Image Analysis Guide
7th Edition
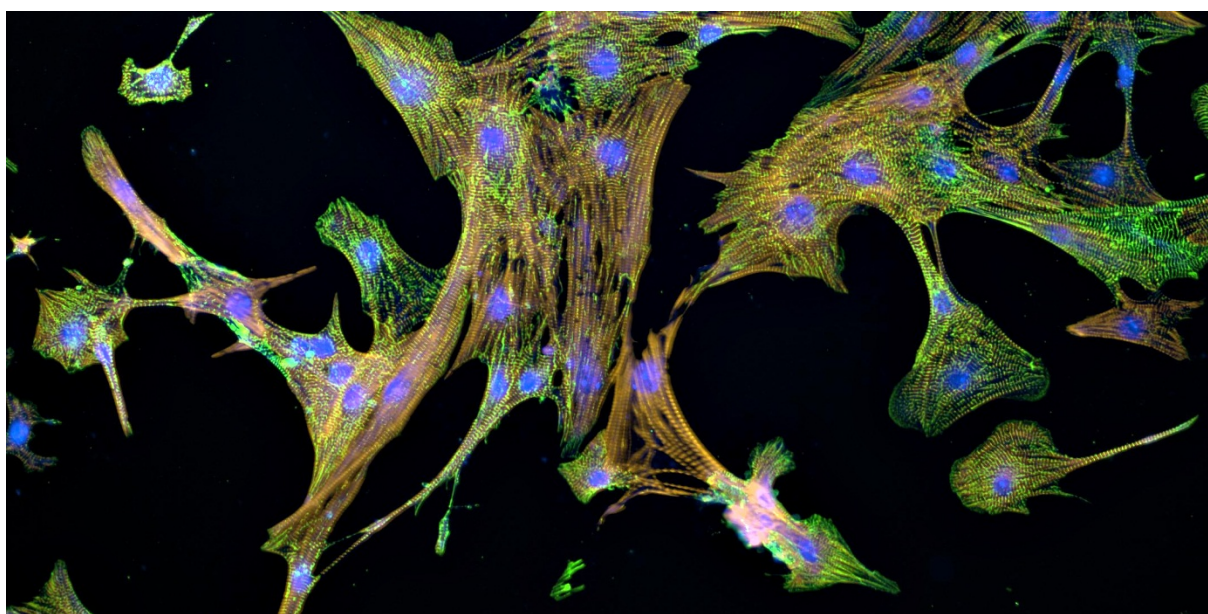
A Guide for Automated Image Analysis
for Cell-based Assays

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For the Better

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Preface – How to use this document

The full High Content Screening workflow is covered by two guides:

Part I – Application Guide

➤ Preparing the samples (cells, stains, plates, slides)

➤ Measuring the samples (objectives, filters, confocal planes, stacks, time series)

➤ Managing measured data

Part II – Image Analysis Guide

➤ Setting up automated image analysis (find and classify objects, measure features)

➤ Statistical evaluation (quality, hits, dose responses, phenotypes)

The guide is directed to:

➤ Users new to imaging assays: Learn basic concepts.

➤ Users migrating from other software to Harmony: Get an overview.

➤ Advanced users: Comprehensive reference to look up details on demand.

➤ Expert users: Find advanced background information.

We assume that the biological background to the cellular process under investigation is well known. We also assume that the reader has knowledge of how to set up cellular assays, from assay design and choice of appropriate controls through to assay evaluation.

In **Part II** which covers **image analysis**, we assume that you have completed image acquisition and want to analyze the data. Alternatively, you can use one of the example datasets included to get working hands-on immediately (see Appendix for a list of datasets).

Depending on your aims, there are four different routes to get your results. From top to down, they get more complex. Start with the simplest and progress as needed.

A – Find a Ready-Made Solution and use it → Go to Chapter 2 → RESULTS

B – Modify a Ready-Made Solution to perfectly meet your needs → Pick a Ready-Made Solution from Chapter 2, pick extensions from Chapter 1 → RESULTS

C – Build a new analysis sequence from scratch using basic building blocks → Start with sections 1.1 to 1.5, study other sections of Chapter 1 as needed → RESULTS

D – Build advanced image analysis algorithms not covered by **A** to **C** → Use Chapters 1 and 2, and have a look at the sections marked with a star * (EXPERTS ONLY)

You will find that virtually all applications can be done with **A** and **B** . As you get more experienced, it may be convenient to quickly build your own tailored analysis sequences on a case by case basis (Route **C**). Route **D** gives access to lower level image and object manipulation features. Use with care, there are few safety fences on this route! Advanced sections are marked with a * symbol. Standard users do not need to know these parts.

A good way to get started is to navigate step by step through some of the ready-made solutions in Chapter 2 using the example data set and analysis sequence provided.

We hope you enjoy working with our instruments and software!

What's new in this Edition?

For a quick overview of the main changes in this edition of the Image Analysis Guide please see the following list:

Changes in the 7th Edition:

➤ Page 106: New section 1.12 "Multiscale Analysis / Global Image"

➤ Page 153: Updated section 1.18 "Various sized Spots – Spot Co-localization"

➤ Page 165: Updated section 1.21 "Considerations Regarding Image Analysis Speed"

Changes in the 6th Edition:

➤ Page 40: Updated section 1.3.6 "Step 6 – How to Calculate Collated Readout Values" – Changes in building block "Define Results"

➤ Page 54: Updated section 1.5.4 "Storing Single Cell Results" – Changes in building block "Define Results"

➤ Page 146: Updated section 1.17 "3D Analysis" – New XYZ view for stack measurements (building block "Input Image")

➤ Page 158: New section 1.20 "Analyzing FRET using Harmony and Tibco Spotfire"

Changes in the 5th Edition (new for Operetta users):

➤ Page 9: Updated "Chapter 1 – High Content Analysis: Getting Started" – Harmony 4.0 building block changes and additions. See release notes in the Harmony Help System for a detailed list what is new or updated.

➤ Page 18: New section 1.2.2 "Pseudocolor Images"

➤ Page 93: New section 1.10 "Regions of Interest" – Restrict the analysis to a set of Regions of Interest

➤ Page 146: Updated section 1.17 "3D Analysis" – Using the maximum projection "Plane Map" for 3D analysis

➤ Page 153: New sub-section "Spot Co-localization" in section 1.18 using the "Restrict by Mask" method

➤ Page 165: Updated section 1.21 "Considerations Regarding Image Analysis Speed" – reflecting Harmony 4.0 speedup of the analysis and new hints on computer and network configuration

➤ Page 302: New section 2.31 "Microtissue Analysis" – New Ready Made Solution

➤ Page 323: New Example Dataset for Microtissue Analysis

➤ Page 324: New section "Converting Acapella Scripts to Building Block Sequences"

Chapter 1 – High Content Analysis: Getting Started

This chapter describes how to develop and optimize High Content Analysis for screening. It is designed to guide you through the creation of a new image analysis sequence. We describe how to work with image analysis modules in general, how to optimize parameters and how to identify subpopulations.

Here you will find:

In addition, a selection of sections on special topics is included:

1.1   The Harmony Software

Figure 1–1 shows the Harmony graphical user interface (GUI) screen during image visualization and interactive image analysis. This screen allows the following steps of the workflow:

➤ Dataset selection (panel 1): Allows a plate and a measurement to be selected from the Harmony database. The same plate may have been measured multiple times.

➤ Navigation in the dataset (panel 2): Allows a well, image field and stack plane to be selected.

➤ Image view (panel 3): Allows multi channel images to be viewed as a color overlay. Analysis results (masks) can be overlaid onto the images.

➤ Image analysis sequence (panel 4): Allows an image analysis algorithm to be set up by combining a series of pre-made building blocks.

➤ Object list view (panel 5): Provides a list of objects, e.g. whole cells, and their associated numerical properties, as returned by the image analysis.
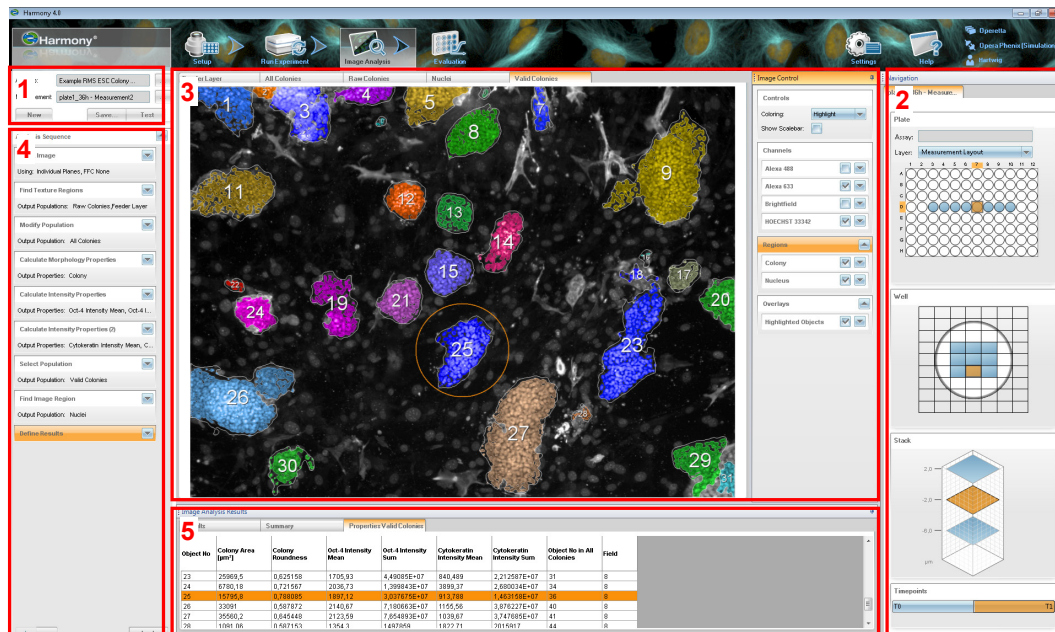
Figure 1–1: Harmony GUI as seen at the "Image Analysis" step. Panel 1 – Plate and measurement selection. Panel 2 – Navigation in the plate dataset: Well selection, field selection, stack plane and time point selection. Panel 3 – Multi channel image viewer. Panel 4 – Image analysis sequence. Panel 5 – Object list display.

Information on how to start the software, browse the database for measurements, load a measurement, view the images and corresponding metadata, select an analysis sequence, run the analysis and view and save the results in the database is described in detail in the "Harmony Help Function" (which can be accessed using the F1-key or the "Help" icon in the menu bar) and the manuals. See the section "References" (on page 331) for a full list of all the user manuals.

1.2　Image Visualization and Quality Control

➤ Image visualization and quality control is the first important step of every image analysis.

➤ Make sure you can see the biological effect of interest in the image. You can't perform automated image analysis on cells or cell properties that you haven't imaged.

➤ For a checklist of aspects to consider before analyzing your images, see *Table 1-1*.

➤ Use zoom and scroll tools, multi color overlays, gamma correction, intensity correction, fixed intensity thresholds and many more, for image inspection.

➤ Use the navigation panel (*Figure 1–1*, panel 2) to navigate through the dataset and get a throrough overview.

➤ To set up an image analysis algorithm, select a representative positive and negative control image.
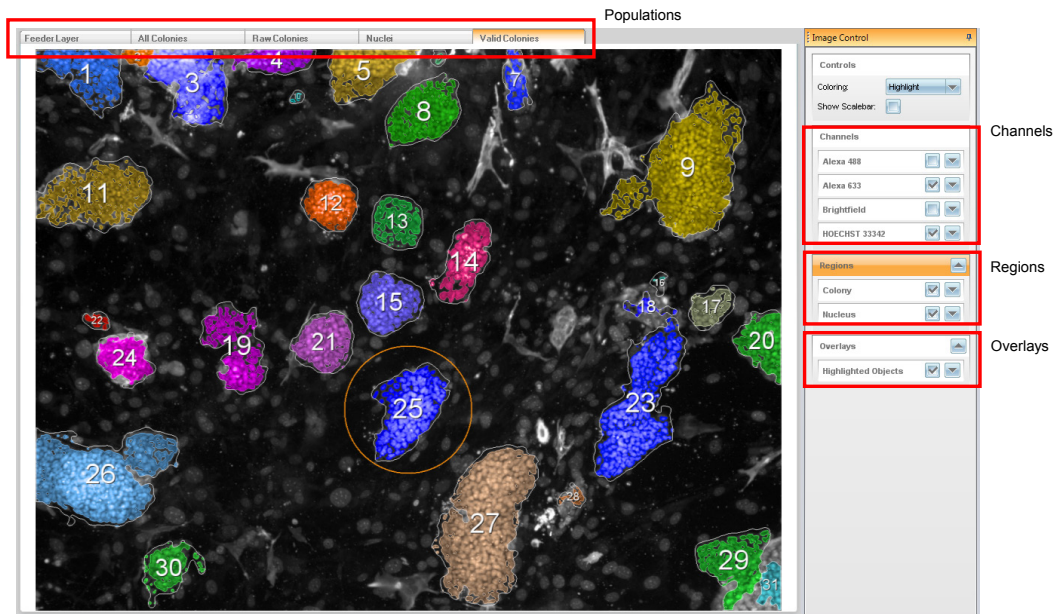
Figure 1–2: Harmony image view in more detail.

The image view allows measured channels (digital camera images) and image analysis results to be combined in one interactive illustration. Displayed objects in the illustration can be highlighted and selected by clicking on them.

The image view also serves as a data browser:

It shows which channels are available in the loaded measurement ("Channels" control box) and which results have been generated by the current image analysis sequence ("Populations" tabs). For the population selected, the list of regions in the population is shown in the "Regions" control box.

The "Overlays" box lists additional elements that can be overlaid on the image, e.g. currently selected objects.

Each element can be selected for display by a checkbox. A number of options are available to configure the display style for each item, including color adjustments and overlay style (border, body, number, circle, etc.). More information can be found in the following sections.

Checklist for the images to be analyzed:

- ✓ Do you see the effect that you expect to see?

- ✓ Is the effect unambiguous?

- ✓ Are there artifacts such as variations in cell density, dead cells, debris or bright agglomerates of dye?

- ✓ Is there crosstalk between image channels?

- ✓ How big is the dynamic range in your channels? Is there clipping of a signal?

- ✓ Is the background constant across the image?

- ✓ Are there significant differences between individual cells in one image (cell to cell variability)?

- ✓ Are there differences between image fields in one well (field-to-field variability)?

- ✓ Are there differences between wells with the same conditions (well-to-well variability)?

Table 1-1: Image analysis checklist.

1.2.1   Image Display Hints

The instrument camera has a much wider dynamic range than a computer screen. A 14-bit camera can resolve 16384 (= 2^{14}) different intensity levels and a 16-bit camera can resolve 65536 (= 2^{16}) levels, whereas even the best computer screen currently available can only resolve < 256 intensity levels (three colors, 256 levels each, gives 16.7 million combinations). The same applies to the color channels: whilst the Operetta system can acquire any number of channels, the computer screen only has three basic colors (red, green and blue). This means that the complete information contained in the acquired images cannot be correctly displayed on the computer screen for visual inspection. The information in the image needs to be compressed which is inevitably connected with the loss of some information and over exaggeration of other information.

When interpreting images, be aware that you never see the "real" camera images on the display. The **intensity** of the dye **measured** inside the cell is evaluated by the automated analysis. The **brightness** and color **displayed** on the computer screen can be adjusted in order to enhance the visibility of some features. The display settings do not influence either the analysis results or the images and their metadata.

The Harmony software controls that can be used to adjust the **displayed image** are shown in *Figure 1–3*.
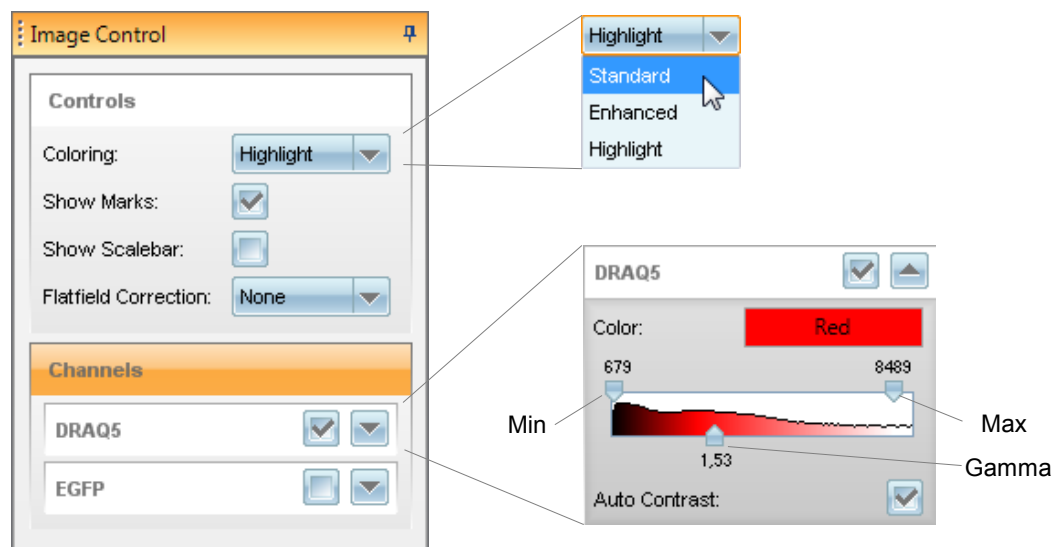
Figure 1–3: Controls available to adjust the image displayed in the Harmony image view. An example is shown for an image with two channels, DRAQ5™ and Alexa Fluor® 488. For each channel, a separate color setting panel (shown on the right) is available.

To display the acquired images on the computer screen, the Harmony software uses the following steps:

➤ The intensity range of each individual channel is mapped to the dynamic range of the display. This is done using the "auto contrast" function which adjusts the intensity settings to try to make both bright and dark regions visible, without losing any detail (see *Figure 1–6* for examples of different intensity settings).

➤ Different channels can be selected to create a joint color image on the computer screen. The color of each channel can be freely defined. Three different color mixing modes (Standard, Enhanced and Highlight) with different characteristics are available to allow the user to adapt the visualization for different purposes (see *Figure 1–8* for an example of a color overlay of three channels).

Each step comes with default values, but also offers a range of adjustment options. These steps are explained in the following sections. For your convenience, you will find keyword labels next to the text.

Dynamic Range Mapping

The brightness and contrast adjustments for image visualization are done automatically by the "auto contrast" function. Nevertheless, it is useful to know some of the basic features in order to customize the settings (optional) and to avoid common pitfalls in image interpretation.

Min / Max

Figure 1–4 schematically shows how the image intensities are mapped to the dynamic range of the display. The "min" and "max" intensity values are auto-adjusted for each individual image and can also be manually changed on the user interface (see *Figure 1–3*).
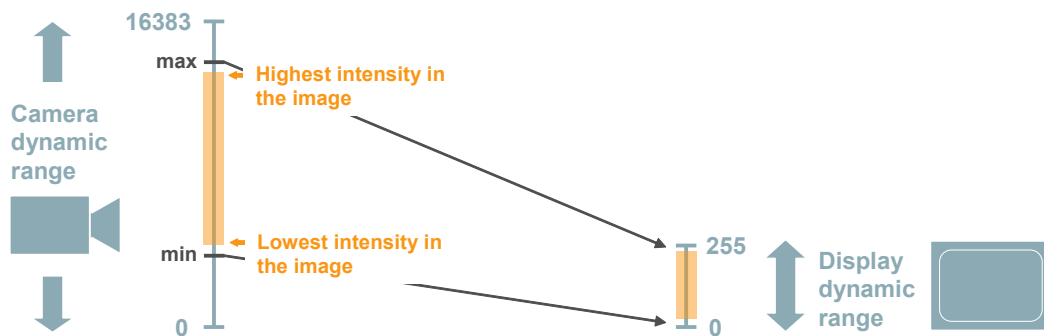
Figure 1–4: Diagram showing how a 14-bit camera image is mapped to the computer display. The blue scale bars indicate the minimum and maximum intensity values of the camera and the display. "0" corresponds to black (no intensity), and "16383" (camera) and "255" (display) correspond to the maximum intensity. The orange bars indicate the intensity range of an example image. The potential intensity mapping is shown by the black lines. Due to the compression, small intensity differences in the image are lost when viewed on a computer screen.

Clipping

The intensity range of different images may vary greatly depending on the image content, staining and the exposure settings used to acquire the image. All intensities above a defined maximum intensity value are scaled down to the maximum value. As a result, the information in the respective image areas is lost. This effect is called "clipping". Clipping may occur during image acquisition (if the sample intensity exceeds the dynamic range of the camera, i.e. "16383") or during visualization (if the image intensity exceeds the "maximum" value of the display intensity). The effects of clipping can be seen as unstructured white areas in the image, or as maximum intensity values of 16383 in the image. When important information is lost due to clipping, the image acquisition settings and the visualization settings respectively have to be re-adjusted.
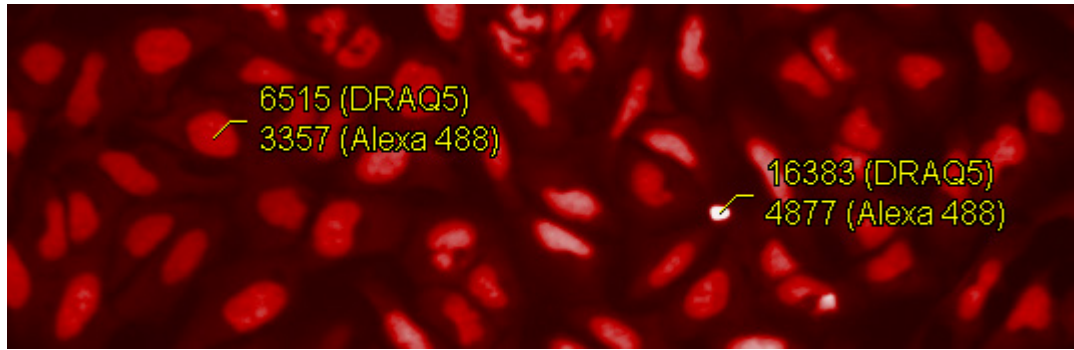
Figure 1–5: Example of "clipping". The image shows a DRAQ5™ channel image. The yellow labels indicate the intensity values of the DRAQ5™ channel and Alexa Fluor® 488 channel (not shown in the image). The intensity of the small bright white spot exceeds the dynamic range of the 14-bit camera and is therefore "clipped" to the constant maximum value of 16383. Clipping can be tolerated in this example since the important parts of the image are inside the dynamic range of the camera.

Gamma correction

In addition to the mapping of the minimum and maximum intensity values, the information in dark image areas can be enhanced by stretching of the image intensities during mapping. This is called "gamma correction" and an example is shown in **Figure 1–6**. A "best guess" gamma value is automatically set by the "auto contrast" feature and can be manually re-adjusted as necessary. In particular, when using combined nuclear / cytoplasmic stains like DRAQ5™, the cytoplasm only becomes visible on the display when gamma correction is used.

Original image (Gamma = 1.0)   Gamma = 2.5   Gamma = 5.0

Original image   Intensity + 50 %   Intensity + 100 %
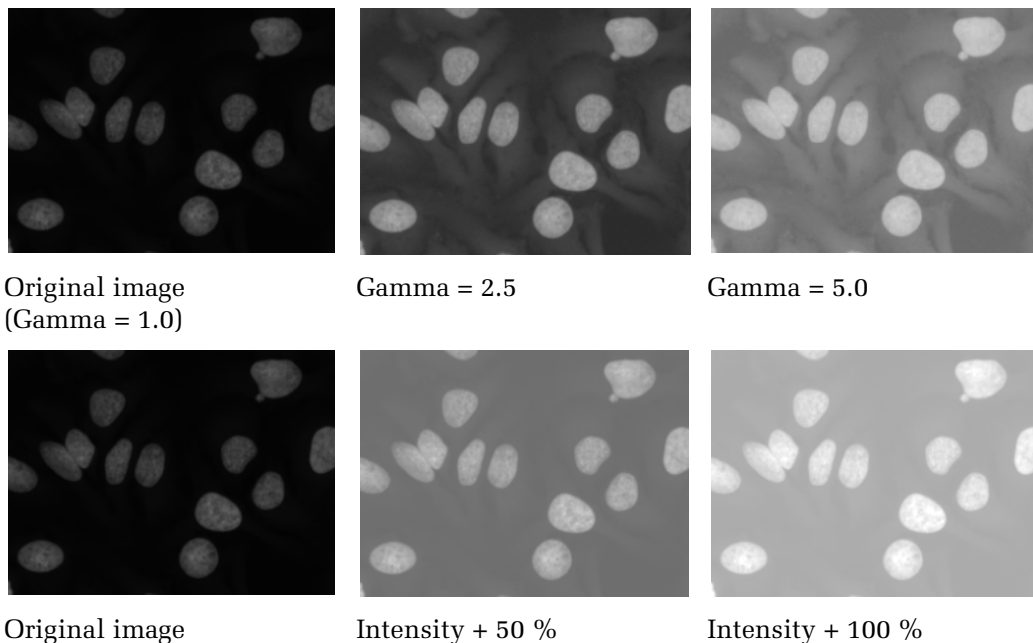
Figure 1–6: Example of gamma correction. Increasing the gamma values will brighten up dark areas while preserving bright areas, so as in this example the cytoplasm becomes visible (top row). In contrast, simply increasing the image intensity will overexpose the existing bright areas (bottom row).

Image intensity comparison

The "auto contrast" feature makes browsing a set of images very easy without having to adapt any settings. However, caution is needed when comparing image intensities. For comparison of the absolute image intensities, fixed settings have to be used (by unchecking the "auto contrast" checkbox). The settings have to be determined using the brightest image to avoid clipping effects. If needed, manually select the "min", "max" and "gamma" values which will allow you to accommodate all images.

For an example of how the automatic adaptation of image intensities can lead to misleading visual impressions, see *Figure 1–7*.

With auto-contrast:

Low concentration   Medium concentration   High concentration

Fixed Settings:

Low concentration   Medium concentration   High concentration
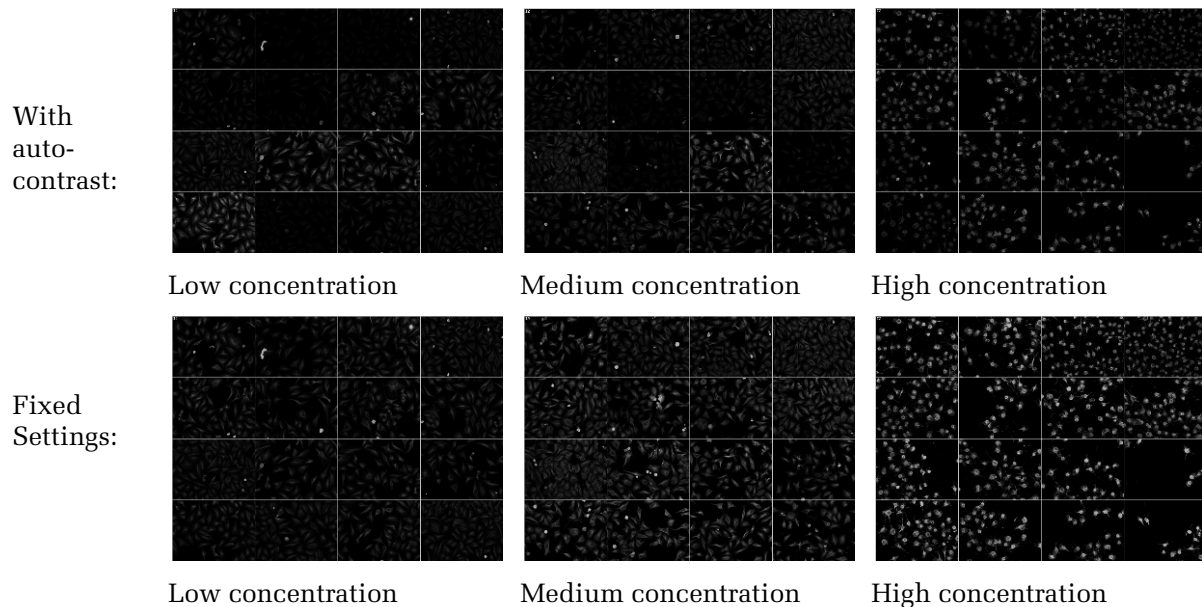
Figure 1–7: Example of a dose-response titration (left to right) with 16 individual grayscale images for each dose (arranged to a matrix). Top row: the automatic intensity adjustment is enabled. The intensity level of each image is adjusted differently depending on individual bright objects and the mean level. Bottom row: same images, but using fixed intensity settings adjusted to best match the dynamic range of all the images. A dose-dependent increase in the dye intensity is now much more apparent. Image field intensities at each concentration also appear at a similar intensity level. For comparison of the absolute image intensities, the auto contrast option must be disabled and fixed intensity settings must be used instead.

Color Images

In a color image the grayscale contents of different channels are combined for visualization on the same display. A distinct color is assigned to each grayscale image prior to overlaying (see *Figure 1–8* for an example). Color images enable:

➤ Comparison of the spatial information from multiple image channels, e.g. the location of a marker dye relative to nuclear or cytoplasmic stains in a second channel.

➤ Combination of a large amount of information in one image for quick interpretation.

Red channel   Green channel   Blue channel   Color overlay
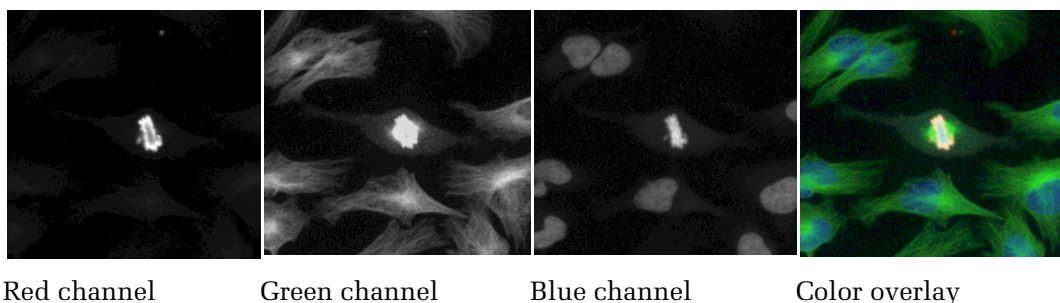
Figure 1–8: Example color overlay of three channels. The relative location of the fluorescence in different channels becomes visible, especially if one color is more dominant. In the bright mitotic cell at the center of the image, the channels cannot be visually distinguished and therefore a comparison of the individual color images is needed.

A very efficient way of identifying all the places where a specific marker signal is located in a color overlay image is to switch the signal of interest on and off periodically ("blinking"). In many cases this works better than comparing two images, or examining a static color overlay image.

Coloring Mode

Due to limitations in the dynamic range and color space of computer monitors, a perfect image without loss of color cannot be created. Harmony provides three modes:

Highlight

➤ The default mode.

➤ Shows a specially enhanced image disclosing every detail that can be used in the automated image analysis.

➤ Best for quickly browsing lots of images of a high content screen for visual assessment (including quality control).

➤ Drawback: all extremely bright image regions will look white independent of the channel color.

➤ Comparison of absolute image intensities of different image regions not recommended (high gamma correction).

Enhanced

➤ Like Highlight mode, but extremely bright regions will show their correct color.

➤ Best choice when using bright markers with different colors.

➤ Drawback: Gives a less clear view for the middle intensity range (which mostly is the largest part of the image).

➤ Comparison of absolute image intensities of different image regions not recommended (high gamma correction).

Standard

➤ Best reflects the absolute image intensities within the image, but shows the least detail.

➤ Best choice to set up exposure parameters for image acquisition or study absolute image intensities.

➤ This mode is commonly used by many other programs.

Highlight   Enhanced   Standard
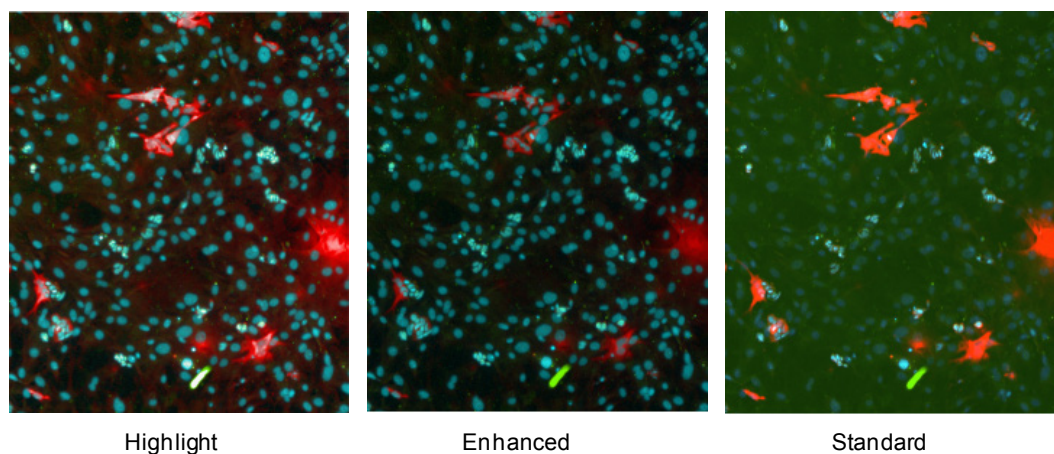
Figure 1–9: Example of the different coloring modes for overlaying three color channels. The images show embryonic stell cells DBA1 (mouse) on a feeder layer of embryonic fibroblasts. Fluorescent labels are Hoechst 33342 (blue), labeled Oct4 antibody (green) and labeled Cytokeratin antibody (red). In the "highlight" mode, the most image details are visible but relative intensities and the color of very bright regions are not correctly reflected. In the enhanced mode, very bright regions have the correct color (instead of white) but the dynamic range is lower. The standard mode has the least adaptations, and intensities within the image can be compared best, but it also shows the least detail. Selection of the most appropriate method will depend on the effect under investigation. Use trial and error to make your choice. Keep in mind that any image coloring may also give a misleading visual impression, so always refer to the grayscale images to confirm conclusions made from color overlay images.

1.2.2   Pseudocolor Images

Pseudocolor images provide a special way of viewing a greyscale image. Each intensity value is mapped to a color according to a predefined table (palette). Depending on the palette, specific details of interest can be highlighted that are difficult to see in the original greyscale image, e.g. small intensity changes in homogeneously looking areas. Here is an example:
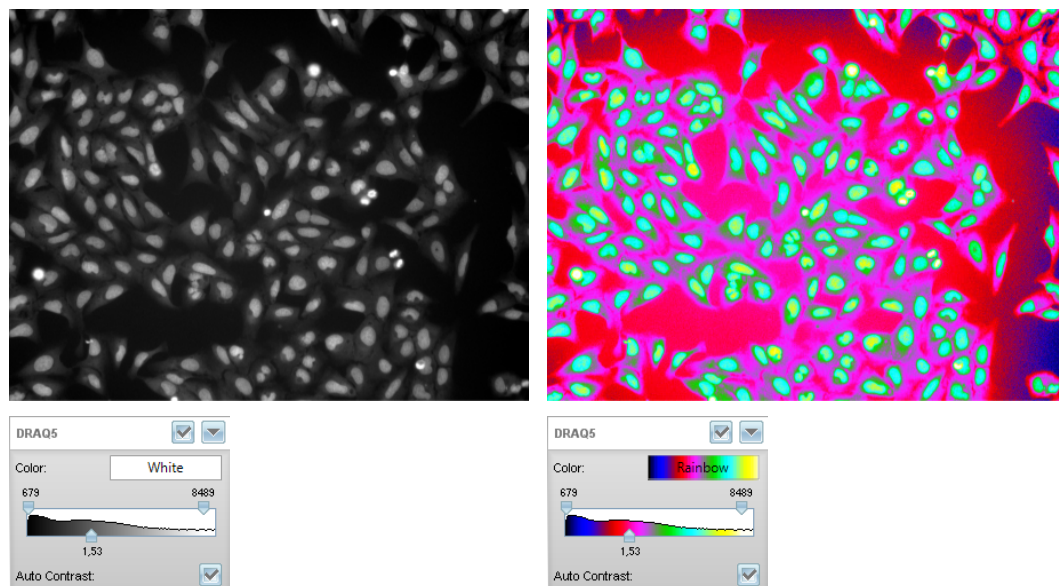
Figure 1–10: Example of using palettes (pseudocolor). Left panel: nuclear dye greyscale image (default display mode). Right panel: After switching to "Rainbow" palette, intensity differences in the regions of the greyscale image that look homogeneous become visible showing: i) there is a drop of background intensity visible towards the top and right borders of the image (can be compensated by enabling flatfield correction), ii) there are intensity variations inside the nuclei (yellow-turquoise). Botton row: The intensity histogram in the image view shows which greyscale intensities are mapped to which color.

Here is an overview of palettes available in Harmony and their characteristics:

Cold-Hot

Emphasises regions with very high ("Hot" = orange & yellow) and very low values ("Cold" = blue). The mid-range is mapped to green. Cold-Hot assumes intensity variations inside the low-mid-high regions are of lower interest.

Black-Cold-Hot

Same as Cold-Hot, but assumes that there is a background intensity which is of no interest (mapped to black). Low and high intensities above that background are emphasiszed.

Geographic

Highlights continuous, bigger regions of the same intensity. It assumes that all intensity levels are equally important. Low levels are mapped to blue ("Sea Level"), followed by green ("Land"), followed by brown ("Mountain") like the height in a geographic map.

Hot Lava

Visually emphasizes high intensity regions. The lighter the color, the higher the value.

Inverse Grey

Reversed standard greyscale. Especially useful in overlays with brightfield images.

Rainbow

Highlights intensity differences. Even subtle intensity differences which are hardly visible in the greyscale image are mapped to different colors and become clearly visible.

➤ To select the best palette, browse through all and find the one that emphasizes the features of interest best.

➤ Consider switching to "Standard" coloring mode to fix the gamma correction to a value of one if you are interested in absolute intensities.

➤ Overlaying multiple pseudocolor images from different channels is possible, but not recommended. Only select a single pseudocolor channel.

1.2.3   Comparing Images at Different Locations

Before the automated image analysis sequence can be set up, the variations between images at different locations inside a well (image fields) and between different wells within a microplate need to be checked. The automated analysis has to be able to cope with these variations without manual intervention. Being aware of the variations will facilitate the set-up and validation of the analysis sequence. Use the image analysis checklist in *Table 1-1* before you start.

Plate
Montage

Typical examples of field-to-field variations are shown in *Figure 1–11*. For an example of typical well-to-well variations, see *Figure 1–12*. A plate montage can easily be created and visualized in Harmony by selecting multiple wells in the plate navigation panel (*Figure 1–1*, panel 2) then clicking the right mouse button on the selected wells and choosing the plate montage from the context menu.
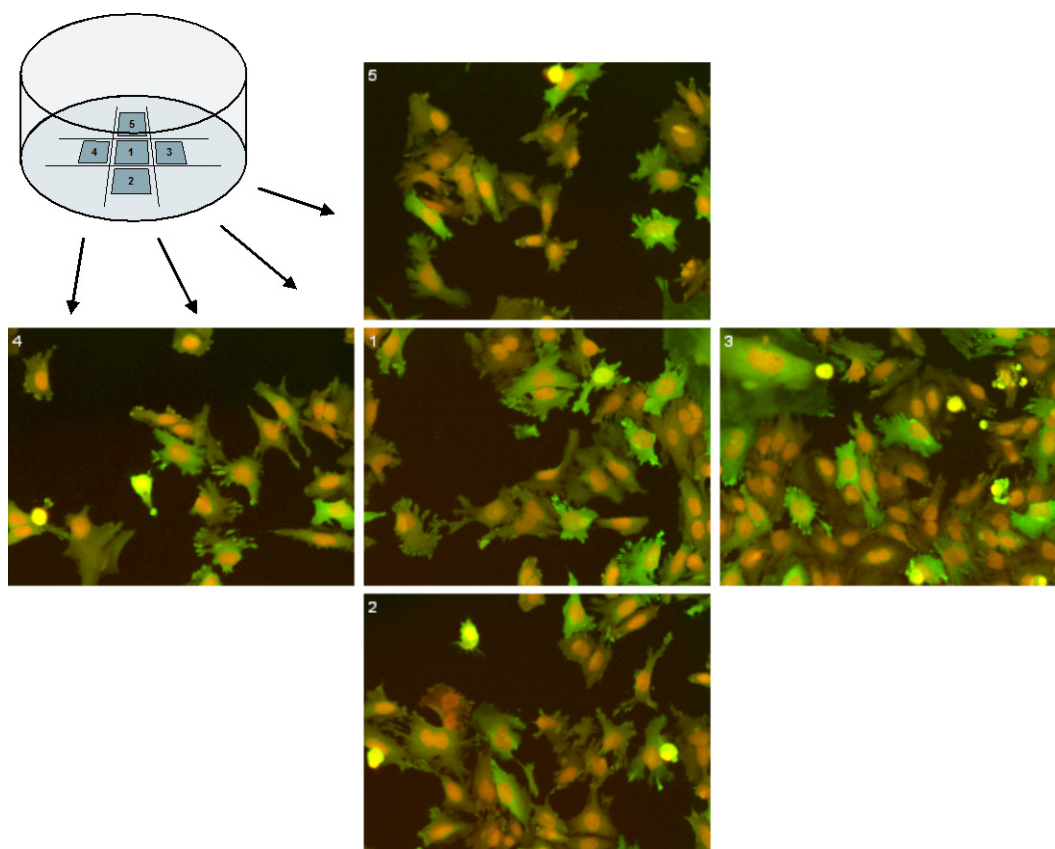
Figure 1–11: Example of multiple image fields taken at different positions in one well. Strong variations in cell number and the degree of confluency can be seen in the different image fields. An automated image analysis must work correctly across all image fields. Field 3 would be a good image for testing how the image analysis works on "crowded" images.
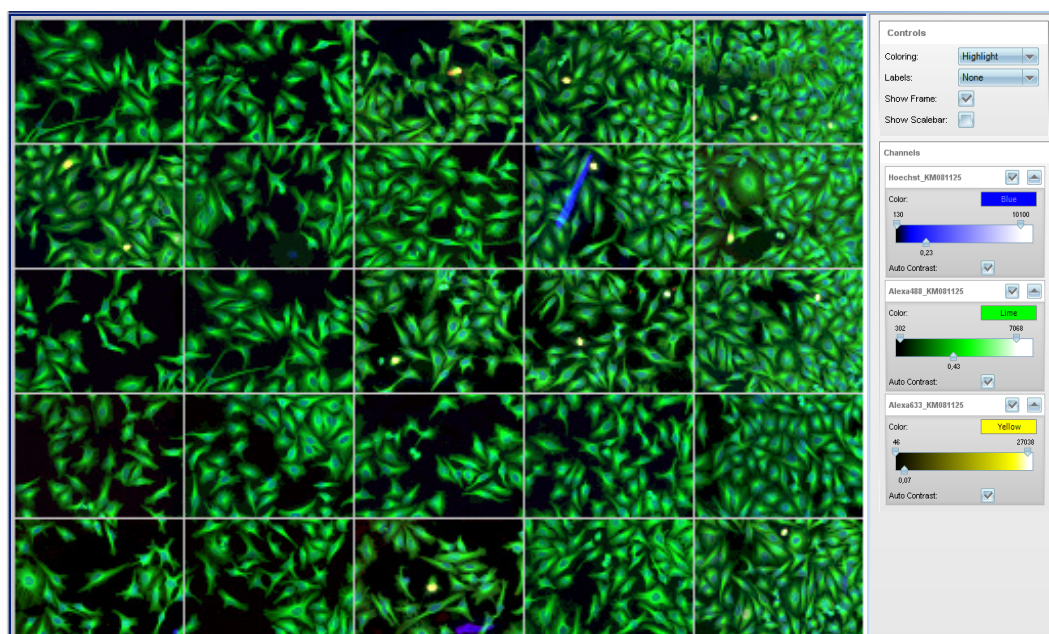
Figure 1–12: Example of a montage of multiple wells within a plate, illustrating some of the common phenomena that are only seen when comparing multiple wells: 1. An increase in the cell density (across the plate from left to right) can be observed. 2. Some wells contain fluorescence artifacts (e.g. dye agglomerations in the blue channel). 3. Some wells exhibit rare events (e.g. yellow spots).

1.2.4 Selecting Images for Set-up of the Automated Analysis

To set up the image analysis you need a set of typical "positive" and "negative" control images (at least one of each), as shown in **Figure 1–13**.

For further validation and optimization of the analysis sequence, you need to have example images showing the extreme cases you want to analyze, e.g. images showing a very high cell density, staining artifacts or untransfected cells.
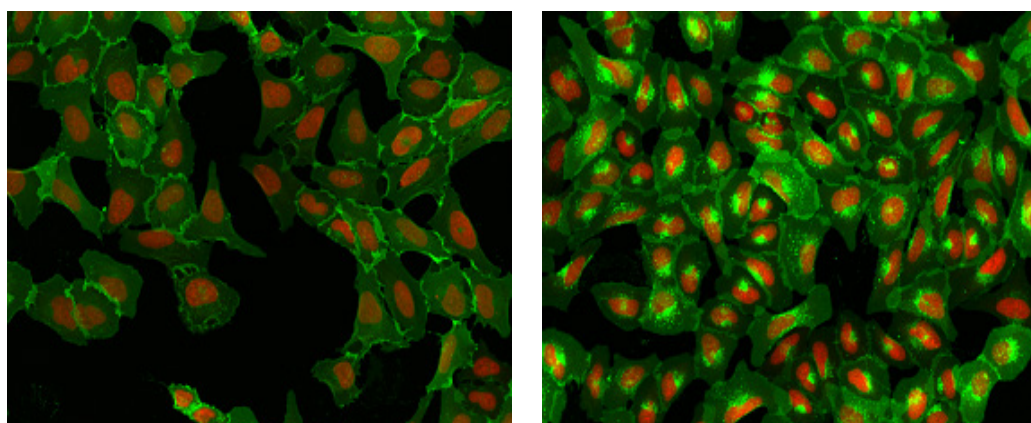
Negative control image – no compound added

Positive control image – internalization triggered by a known compound

Figure 1–13: Positive and negative control images to be used to set up an analysis sequence for an example assay. The images show a color overlay of two channels for a nuclear stain (red) and fluorescently labeled GPCR (green).

1.2.5 Finding an Image Analysis Strategy

During the visual inspection of the images, you should get an idea of which visible features you want to quantify and how you want to implement this measurement in an automated image analysis. One of the following options can be selected:

➤ Option 1 – Load a pre-configured ready-made solution from the database. The ready-made solutions available are described in more detail in chapter 4.

➤ Option 2 – Use a ready-made solution as a starting point, but modify it as appropriate for your application.

➤ Option 3 – Create a new analysis sequence from scratch by combining pre-made building blocks from a set list.

In many cases there are multiple options available to quantify the same effect. Even experts rely on trial and error to find the most robust way to measure an effect. This approach is supported by a fully integrated software environment for fast progression from analysis set-up through to acquisition of results. It is also supported by configurable image analysis sequences (no "black box").

See *Figure 1–14* for an example of three different options for measuring the GPCR internalization in the images shown previously in *Figure 1–13*. All three methods have been tested and, for this example, the best results were achieved using an intensity measurement inside a region around the nucleus (a variation of strategy 2 in *Figure 1–14*), see the RMS "Receptor Internalization" in section 2.11 for more information.

The challenge in setting up the image analysis is to select an algorithm that works for all individual cell shapes (see *Figure 1–15*) and is robust against artifacts such as cell density variations between wells, or dye agglomerates in images (see *Figure 1–12*).

For many more examples see "Chapter 2 – Ready-Made Solutions".
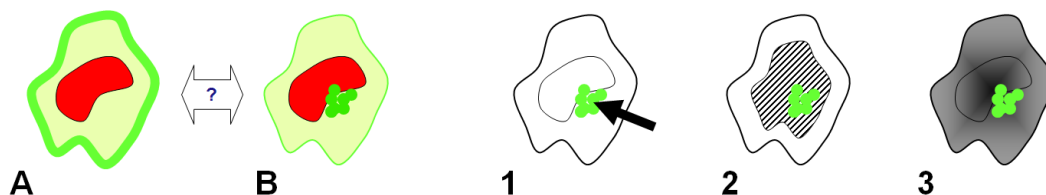
Figure 1–14: Parts A and B show a sketch of a cell, illustrating the effect to be quantified. Parts 1 – 3 show three strategies for measuring the effect. A – The labeled receptor (green) is located on the cell membrane. B – The receptor is mostly internalized to one compact (spot-like) compartment. 1 – The first strategy is to search for a bright spot inside the cell. 2 – The second strategy is to measure the fluorescence intensity in the marked region of the cell (the diameter of the region can be adjusted). 3 – The third strategy is to weight the fluorescence intensity depending on the distance to the nucleus when calculating an average intensity.
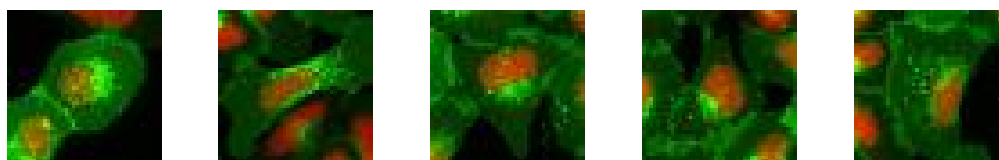
Figure 1–15: The panels show different cells from the positive control image in *Figure 1–13*, and illustrate the variability of internalization. The image analysis should be designed to robustly detect all cells with internalized receptors.

1.3   Setting up an Image Analysis Sequence

The aim of this section is to introduce the generic concepts and terms of the Harmony High Content Analysis strategy. The reader will then be in a position to start using and customizing the ready-made examples and to create analysis sequences from scratch.

This section also provides the background to our Harmony ready-made solutions and describes how to modify them, although they can be easily applied without in-depth knowledge. Our collection of ready-made solutions is discussed in chapter 2. These describe a number of common analysis tasks that are based on the generic scheme introduced here.

Nearly all image analysis algorithms include the same generic sequence of steps. Depending on the individual analysis task, each step may contain a plethora of specialized actions. To allow you to easily create customized analysis sequences, the Harmony image analysis system contains a set of building blocks for each step, which can be arranged into a full analysis sequence (***Figure 1–1***, panel 4). See ***Figure 1–16*** for an example of a building block.
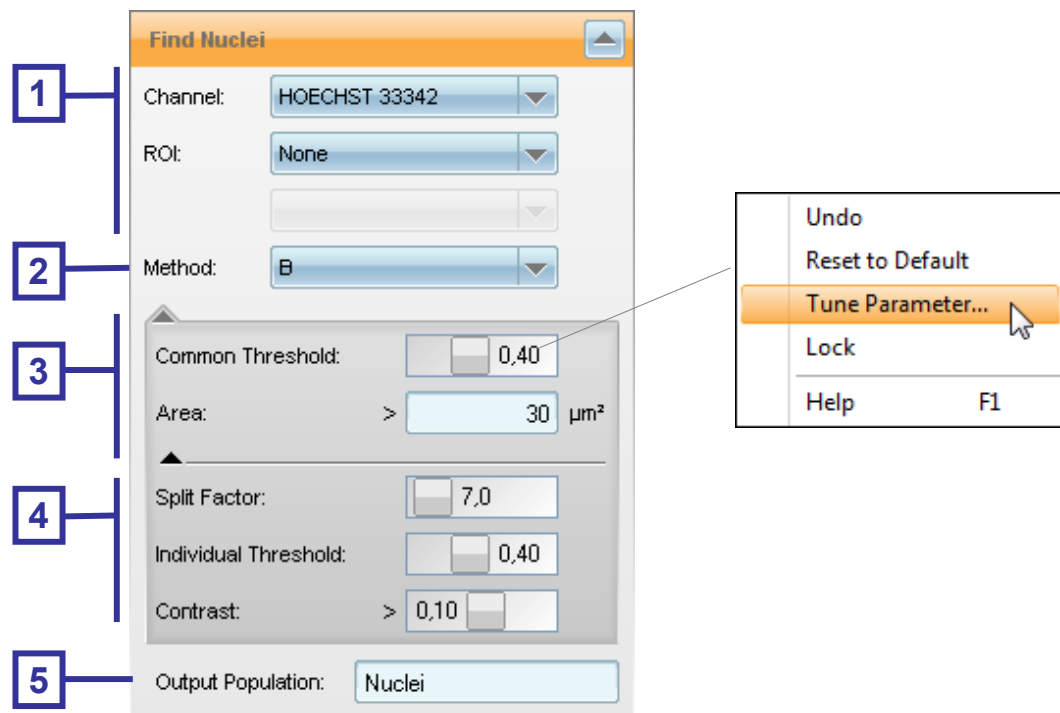
Figure 1–16: Example of a building block (all option panels are opened). 1 – First level parameters. These need to be configured to specify mandatory parameters, e.g. the input channel. 2 – Choice of mathematical models that can be applied to solve the task. These can be selected from a list of different models, allowing you to choose the most appropriate for your experimental conditions (e.g. stains, cells). 3 – Second level parameters. The default values can be adjusted for optimization. Note that the second and third level input parameters may depend on the selected method. 4 – Third level parameters: These offer advanced options. 5 – Name of the resulting output. The right part of the figure shows a context menu containing functions available for each parameter, e.g. an automatic tuning function or a link to the description of the parameter in the "Harmony Help Function". This context menu can be found by right-clicking with the mouse in the appropriate box.

All of the available building blocks are introduced in this section to allow you to pick the most appropriate building blocks for your task. Examples of how the building blocks are used for a wide range of different applications can be found in chapter 2.

A complete description of the building blocks, including all their adjustable parameters, is contained in the "Harmony Help Function" (which can be found in the context menu, see the right box in ***Figure 1–16***).

Information on how to handle the software, e.g. how to load and save data, how to open and run an analysis sequence and how to add a building block to a sequence, is described in detail in the "Harmony Help Function" and the Operation Manual (see page 331 for a full list of all user manuals).

A generic flowchart of an image analysis sequence is shown in the left column of *Figure 1–17*. The middle and right columns show a schematic example of a specific analysis sequence. The individual steps are explained in more detail in the following sections (1.3.1 – 1.3.6). The steps described here outline a straightforward analysis which covers the most common analysis tasks. Using the same basic concept, analysis sequences can be customized to virtually any analysis tasks, including much more complex analysis tasks.
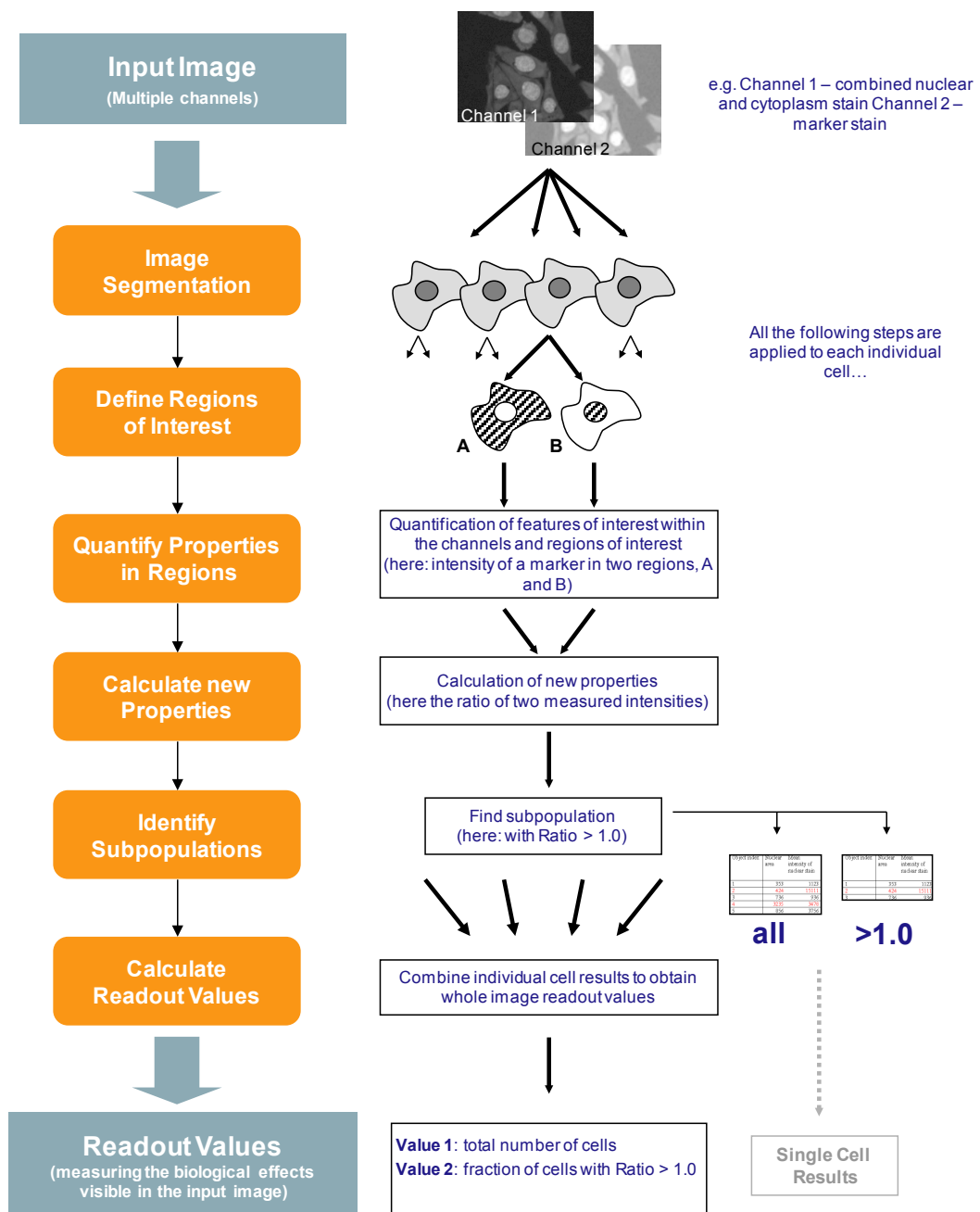
Figure 1–17: Generic flowchart of an image analysis sequence (left column). The middle and right columns show a schematic example of the operations performed on each individual cell during the analysis sequence, and give a specific example. The individual single cell results tables can be stored as an additional well result.

1.3.1　Step 1 – How to find Objects (Image Segmentation)

The first step of an image analysis sequence is the identification of the primary objects of interest, e.g. whole cells, nuclei, or cell groups. These objects will be further analyzed in the subsequent analysis steps. Most cellular high content assays use fluorescent dyes for nuclear and / or cytoplasmic staining to allow for the detection of these cellular areas.
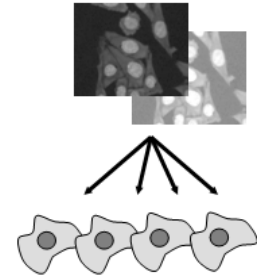
The building blocks for image segmentation are:

> **Find Nuclei**

> **Find Cytoplasm** (around previously detected nuclei)

> **Find Spots** (in previously defined regions)

> **Find Micronuclei**

> **Find Cells (using whole cell stain or brightfield image)**

> **Find Image Region**

> **Find Texture Region**

> Find Surrounding Region (around any type of objects)

Segmentation involves breaking down the image into discrete **objects**, e.g. individual cells or individual nuclei. Each object has an associated set of **regions**, e.g. nucleus, cytoplasm, membrane, and a set of **numerical properties**, e.g. area or intensity.

The set of regions is listed in the regions list in the image view and can be shown as an overlay on the image by activating the corresponding checkbox (see *Figure 1–1*, right part of panel 3, "Image Control"). The numerical properties are listed as an entry in an object list (numerical data based information).

Every building block returns a set of pre-defined results which can be used by the following building blocks. In addition, a set of illustrations and object lists are created which can be used for parameter adjustment purposes when the building block is opened on the user interface.

An example is shown in *Figure 1–18*. The colored lines mark the border of the object regions "nucleus" and "whole cell" (as determined by identifying "nuclei" and "cytoplasm"). The overlays can be displayed on any image for visualization, e.g. on the input image to monitor the correctness of the segmentation or on a marker image to see in which regions the marker is visible.
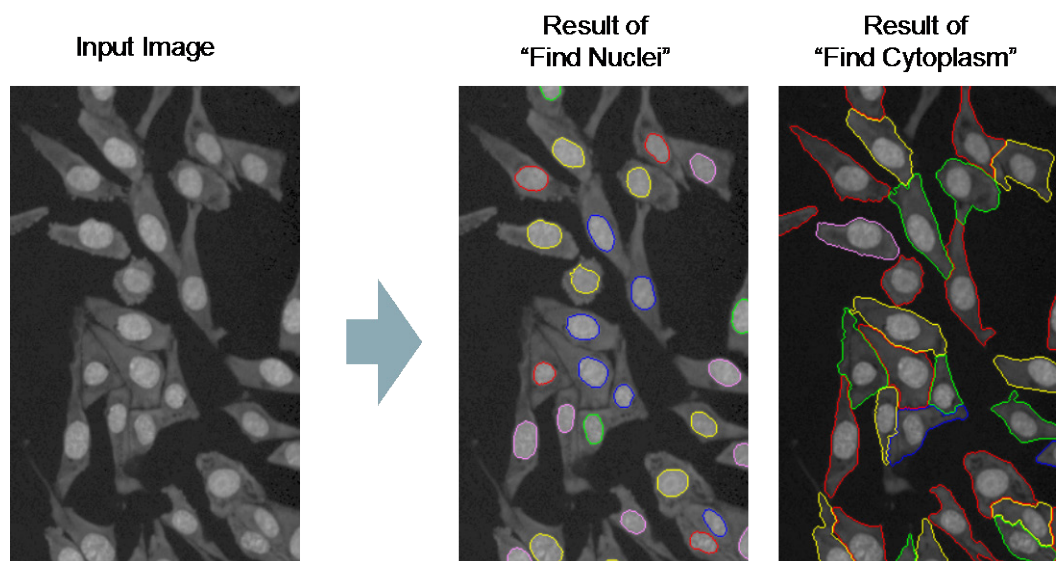
Figure 1–18: Example of image segmentation. Left panel: typical input image showing cells labeled with a nuclear dye (such as DRAQ5™), staining the nucleus (brighter areas) and cytoplasm (darker areas). Center and right panels: results of the nuclear and cytoplasmic detection (borders of "nucleus" and "whole cell" regions) are overlaid onto the input image for visual inspection.

The object list is a table of raw data which lists all the objects found during segmentation (one object per row). Each row contains the object's number and any associated specific properties (numerical values), e.g. the area (size) of the object or its fluorescence intensity (see **Table 1-2**). Any additional properties which are quantified in subsequent analysis steps will also be added to the object list, creating a new column in the list (see **Table 1-3** on page 32). Object lists are displayed in the object list display of the Harmony GUI (**Figure 1–1**, panel 5).

Different segmentations can be applied sequentially in order to identify different types of objects. Nearly all High Content Analysis sequences start with the detection of nuclei and cytoplasm in order to create a complete "map" of the cell during analysis.

Table 1-2: Example of an object list showing the results of a nuclear detection. The **Find Nuclei** building block identifies individual nuclei, assigns an object index number to all of them and calculates the area and mean intensity of the nuclear stain for each individual nucleus.

The illustrations shown in **Figure 1–19** to **1-21** give an overview of all of the available building blocks used for segmentation tasks.

Each building block has a "**Channel**" input which allows you to select the image to be used for segmentation, and a "**Method**" input which allows you to choose from a set of methods for adapting the segmentation to various image types, e.g. high contrast

nuclear staining vs. low contrast. Choose the appropriate method by trial and error using the visual feedback display.

The optional tuning parameters shown in the boxes on the right of *Figure 1–19* to *1-21* can be used for further adaption to specific assay conditions. For more details, please refer to the "Harmony Help Function" which is available from the context menu (see *Figure 1–16*) and section 1.4.

The resulting object list can be renamed using the "**Output Population**" box in the building block.
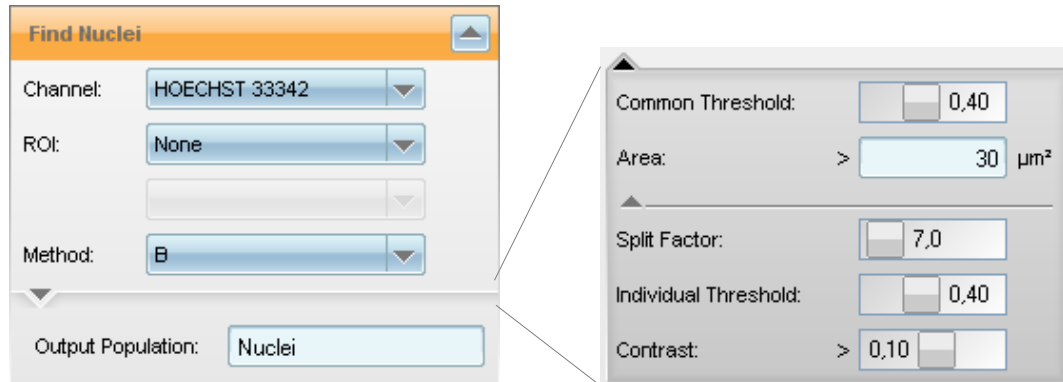
Figure 1–19: **Find Nuclei** building block. Left part: standard parameters. Right part: all of the available input parameters are displayed.
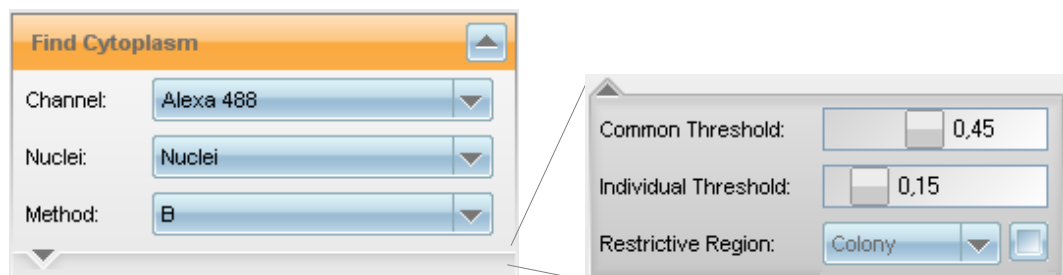
Figure 1–20: **Find Cytoplasm** building block. Left part: standard parameters. Right part: all of the available parameters are displayed.

Please note that you must detect the nuclei before you can detect the cytoplasm. This is because the location of the cytoplasm is determined relative to the position of the previously identified nuclei.
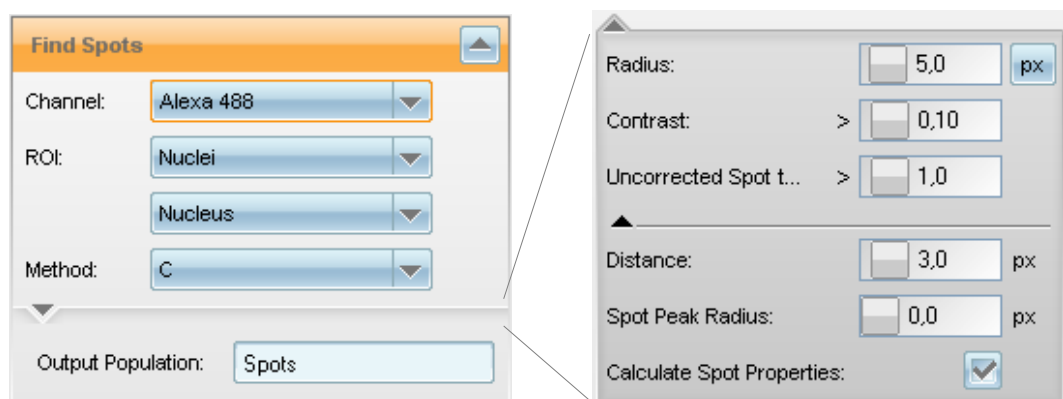
Figure 1–21: **Find Spots** building block. Left part: standard parameters. Right part: all of the available parameters are displayed.

The **Find Spots** building block needs a set of regions of interest (ROIs) in which it can search for the spots. To specify the ROI, select a population of objects (e.g. Nuclei) and a region in that objects (e.g. Nucleus).

The region of interest needs to be created by the preceding building blocks, e.g. **Find Nuclei**, **Find Cytoplasm** or **Find Image Region**.

To search for spots in the whole image, use the **Find Image Region** building block's method "Whole Image Region".

Figure 1–22: **Find Cells** building block. Left part: standard parameters. Right part: all of the available parameters are displayed.

Use the **Find Cells** building block for the identification of cells in digital phase contrast images (label free) or cells stained with a whole cell stain. Most methods of the **Find Cells** are identical to the **Find Nuclei** building block methods but with different default values, adapted for whole cells, e.g. assuming bigger objects.

Figure 1–23: **Find Image Region** building block. Left part: standard parameters. Right part: all of the available parameters are displayed.

The **Find Image Region** building block is a generic building block used to segment an image based on an intensity threshold, e.g. to find bright image regions. For cell analysis, always use the building blocks **Find Nuclei**, **Find Cytoplasm** or **Find Cells**.

To create a region covering the whole image, the **Find Image Region** building block provides the method "Whole Image Region".

1.3.2 Step 2 – How to Define Regions Inside the Objects

The segmentation building blocks create default regions, e.g. the nuclear region. Based on these regions, customized regions can then be created using the following building blocks:

➤ **Select Cell Region** (choose from predefined set of most common cell regions)

➤ **Select Region** (create arbitrary regions in arbitrary objects)

The following regions are available:

➤ The full nucleus or parts of the nucleus

➤ The full cytoplasm or parts of the cytoplasm

➤ The cell membrane or regions around the membrane

➤ Donut shaped areas around the nucleus

➤ Customized zones around any detectable objects, e.g. spot like features

See *Figure 1–24* for some examples.

For each object, multiple regions can be defined, e.g. the nucleus as the first region and a ring inside the cytoplasm as the second region.
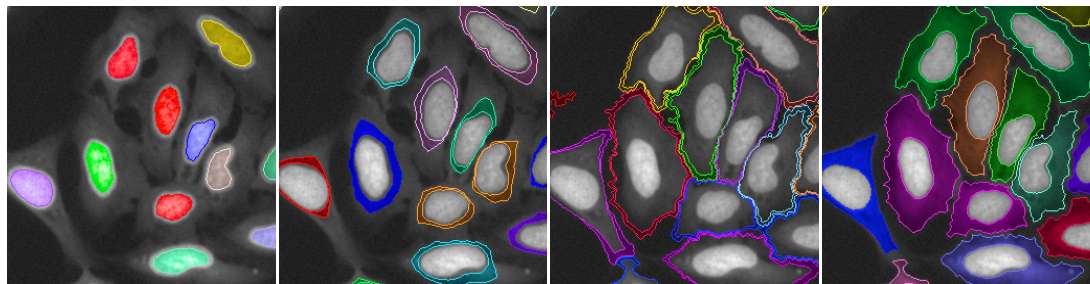
| A "Nucleus Region" is set up to measure marker intensity inside the nucleus. | A "Ring Region" allows the creation of cytoplasmic regions with variable areas around the nucleus. | A "Membrane Region" is set up to reliably identify the plasma membrane area of each cell. | A "Cytoplasm Region" is set up to capture the entire cytoplasmic area for any cell shape. |

Figure 1–24: Examples of region types.

The illustrations in *Figure 1–25* and *Figure 1–26* show the user interface of the **Select Cell Region** and **Select Region** building blocks:

The main input parameter to adjust is the region type. Depending on the region type selection, more options are made available to modify the region shape, e.g. shifting the inner or outer borders.

The region adjustment parameters can be easily explored and adjusted by trial and error using the interactive Harmony user interface.

Detailed information is available in the "Harmony Help Function". More information can also be found in the RMS "Quantification of Cytoplasmic Marker" in section 2.6.

The created regions can be renamed using the "Output Region" field at the bottom of the building block.
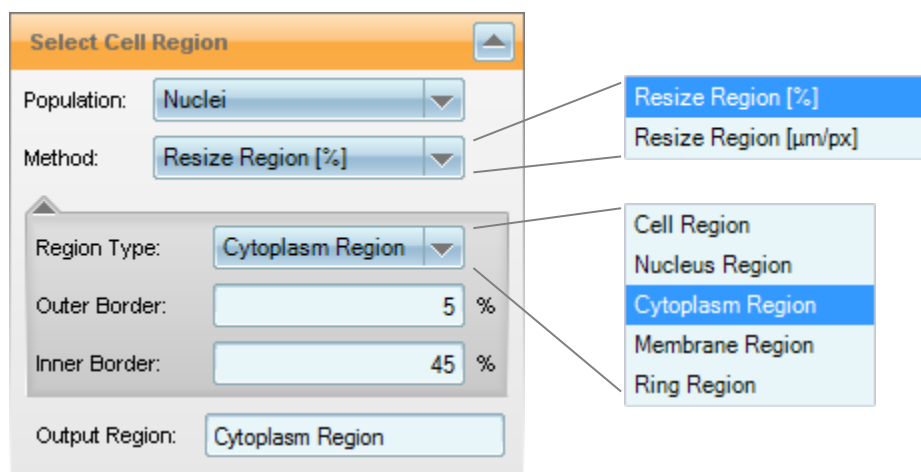
Figure 1–25: **Select Cell Region** building block.

The **Select Cell Region** building block offers the most commonly used regions inside a cell with only a select number of adjustable parameters.

The **Select Cell Region** building block needs to be preceded by the **Find Nuclei** and **Find Cytoplasm** building blocks since it functions by modifying these standard regions to create additional new regions.

In contrast, the **Select Region** building block offers a large set of functions and options to create new regions starting from an arbitrary region. Available regions include, for example, the skeleton or mass center points of existing regions. See *Figure 1–27* for an example. Please refer to the "Harmony Help Function" for a more detailed description.
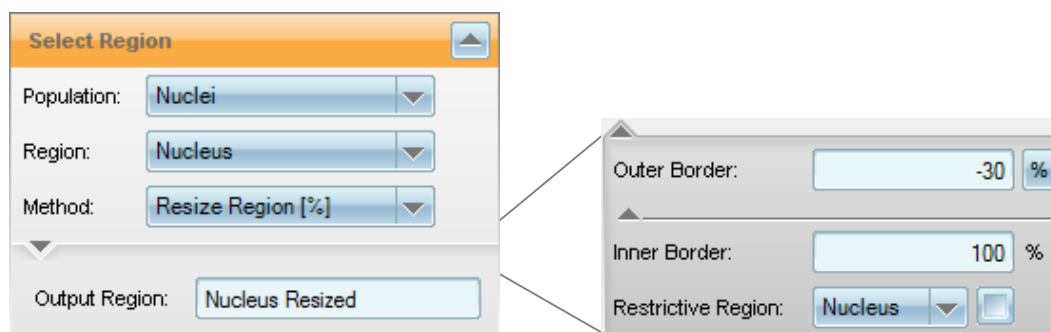
Figure 1–26: **Select Region** building block. Left: the 'Resize Region [%]' method is selected. Right: all of the available parameters are displayed.

Use the **Select Cell Region** building block if you want to modify the default regions (Nucleus, Cytoplasm and Whole Cell) provided by the **Find Nuclei** and **Find Cytoplasm** building blocks.

First try using the **Select Cell Region** building block and only consider the **Select Region** building block if the **Select Cell Region** building block does not provide the desired region.
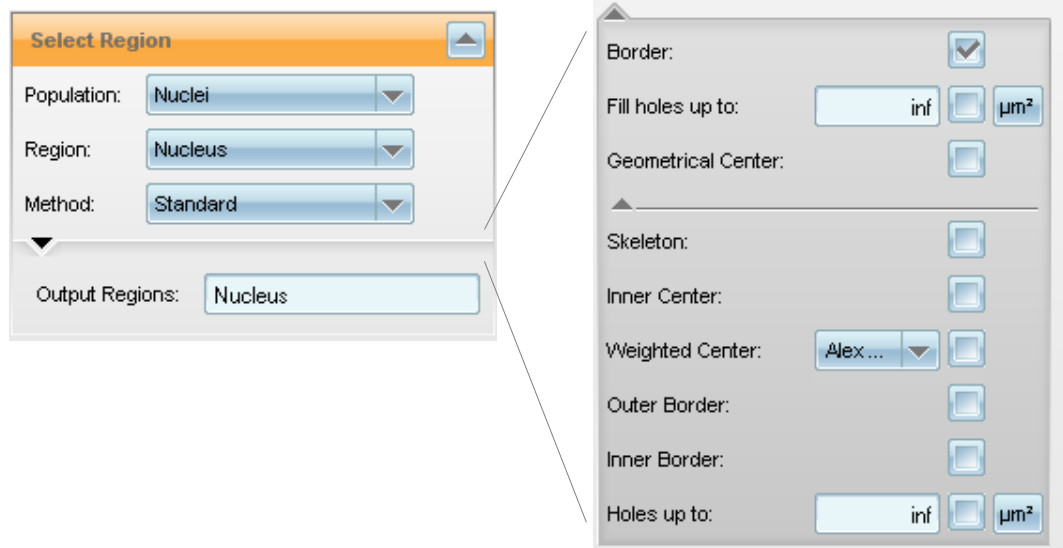
Figure 1–27: **Select Region** building block with all of the available parameters of the "Standard" method displayed. The different regions are described in detail in the "Harmony Help Function" which is accessed by a right mouse click on any of the parameters. Each of the selectable options creates a separate new region. E.g. the "Border" checkbox creates a region "Nucleus Border". The name specified in the "Output Regions" textbox at the bottom of the building block is used as a prefix for the new region's names.

As a third option, **Select Region** allows existing regions to be modified based on other regions, even if they are located in objects of a different population (Restrict by Mask method, see ***Figure 1–28***).
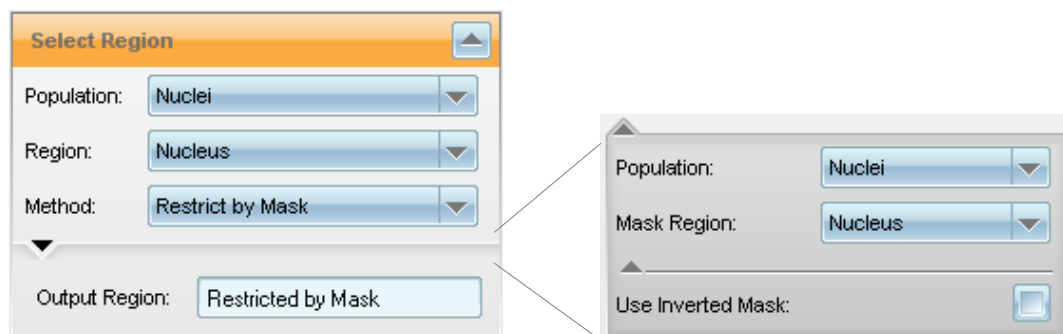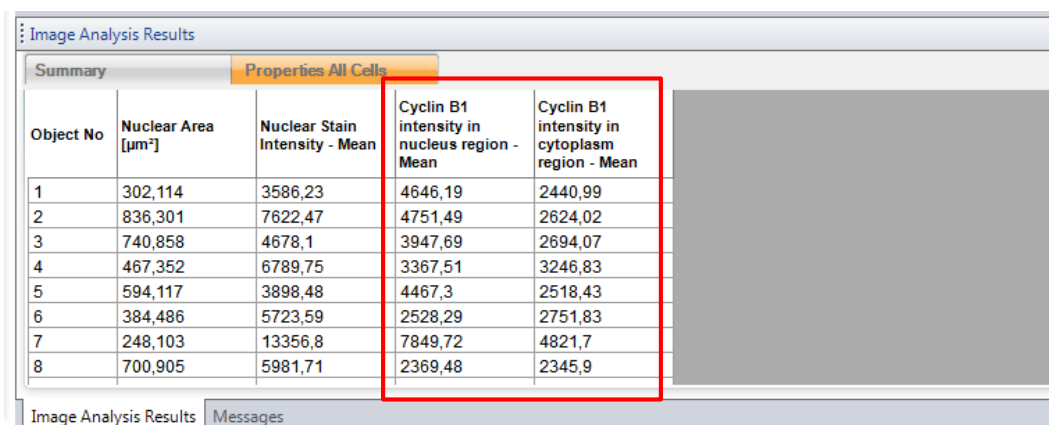
Figure 1–28: **Select Region** building block with the Restrict by Mask method selected. Right part: all of the available parameters are displayed.

1.3.3 Step 3 – How to Measure Properties of the Regions of Interest

After defining regions of interest, the properties of these regions or the properties of features within these regions can be quantified. Common measurements include:

➤ Fluorescence intensities in multiple marker channels inside the different regions.

➤ Shape of the regions ("morphology"), e.g.

- Area

- Length, width

- Roundness

- Set of STAR morphology properties (e.g. Symmetry)

➤ Intensity structure or pattern inside the regions ("texture"), e.g.

- Homogeneity of the intensity (standard deviation)

- Set of SER texture properties (e.g. SER Ridges)

➤ Detection of spot-like features inside a region of interest, e.g.

- Number of spots

- Intensity of spots

The properties are quantified for each individual object and are added to the object list. An example is shown in *Table 1-3*. Here, the measurement of the intensity of the Cyclin B1 marker in two different regions of interest ("Nucleus region" and "Cytoplasm region") is added to the list. Cyclin B1 is a cell cycle marker and was measured in one of the image channels by using a fluorescently labeled antibody.

Object No	Nuclear Area [µm²]	Nuclear Stain Intensity - Mean	Cyclin B1 intensity in nucleus region - Mean	Cyclin B1 intensity in cytoplasm region - Mean
1	302,114	3586,23	4646,19	2440,99
2	836,301	7622,47	4751,49	2624,02
3	740,858	4678,1	3947,69	2694,07
4	467,352	6789,75	3367,51	3246,83
5	594,117	3898,48	4467,3	2518,43
6	384,486	5723,59	2528,29	2751,83
7	248,103	13356,8	7849,72	4821,7
8	700,905	5981,71	2369,48	2345,9

Table 1-3: Example of an object list showing the initial nuclei detection result, with the Cyclin B1 intensities in the nuclear and cytoplasmic regions added (red border).

Building blocks for property calculations are:

➤ **Calculate Intensity Properties**

➤ **Calculate Morphology Properties**

➤ **Calculate Texture Properties**

➤ **Find Spots**

The building block user interfaces are shown in Figures 1-29 to 1-31 and *Figure 1–21* (**Find Spots**). The main input parameters to adjust are the image **Channel** to be measured and the **Region** to be used for the measurement. Please refer to the "Harmony Help Function" (by right-clicking on the mouse) for details. For spot detection, you can also refer to the RMS "Spot Analysis" (section 2.8). For texture analysis and morphology analysis, there are dedicated sections with comprehensive information (sections 1.7. and 1.8)

Multiple building blocks can be used (also repeatedly) to calculate all the properties of interest in various combinations of regions and channels.
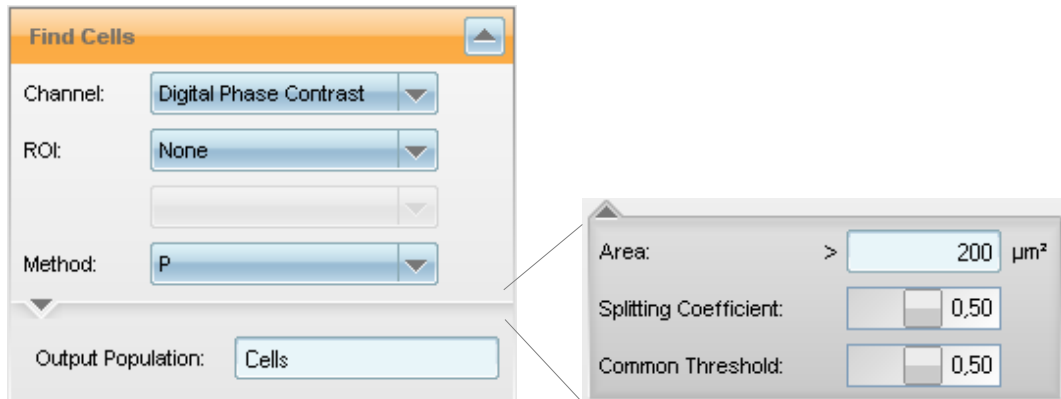

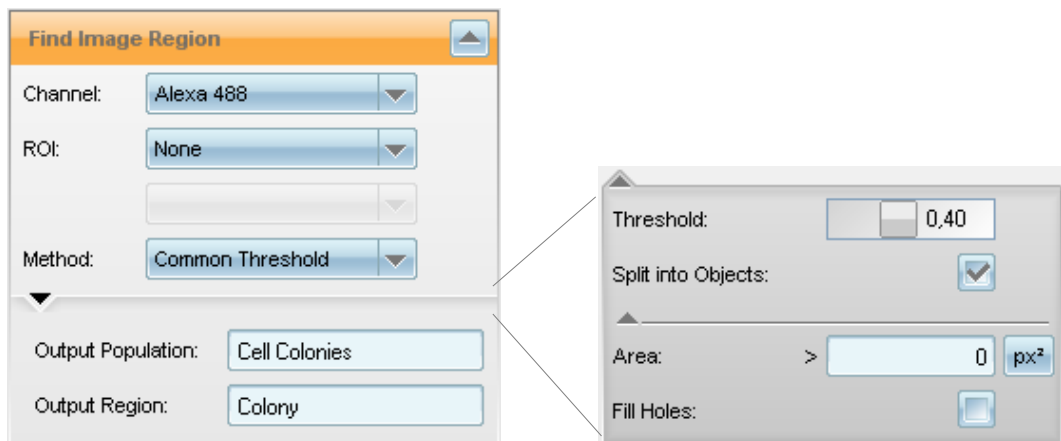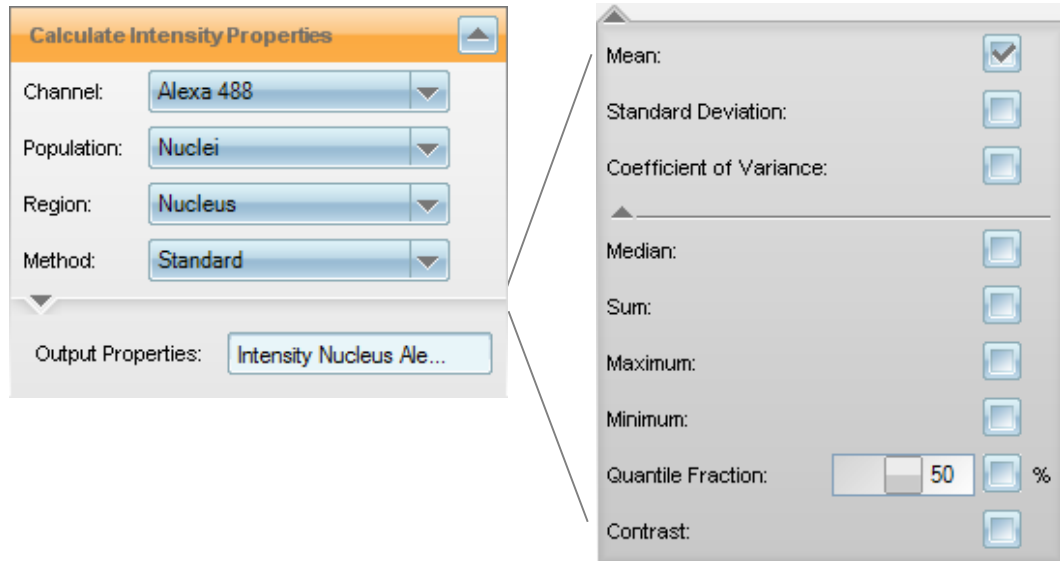*Figure 1–29:* **Calculate Intensity Properties** building block. Left part: standard parameters. Right part: all of the available parameters are displayed.
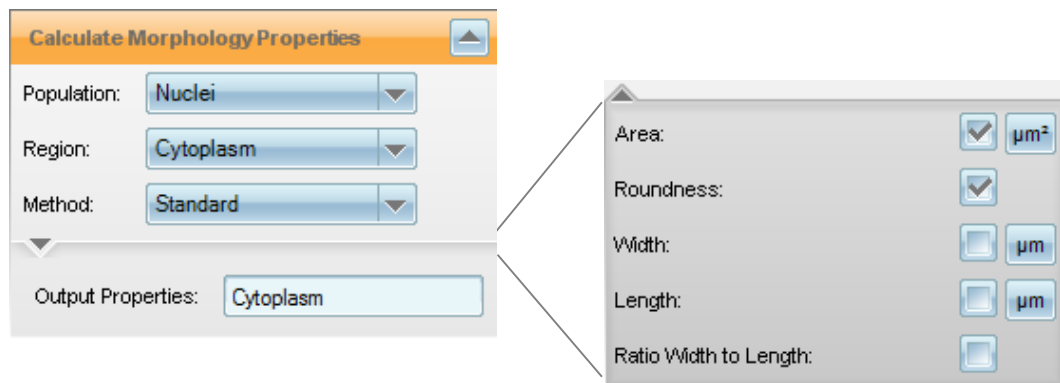
Figure 1–30: **Calculate Morphology Properties** building block, "Standard" method. Left part: standard parameters. Right part: all of the available parameters are displayed.
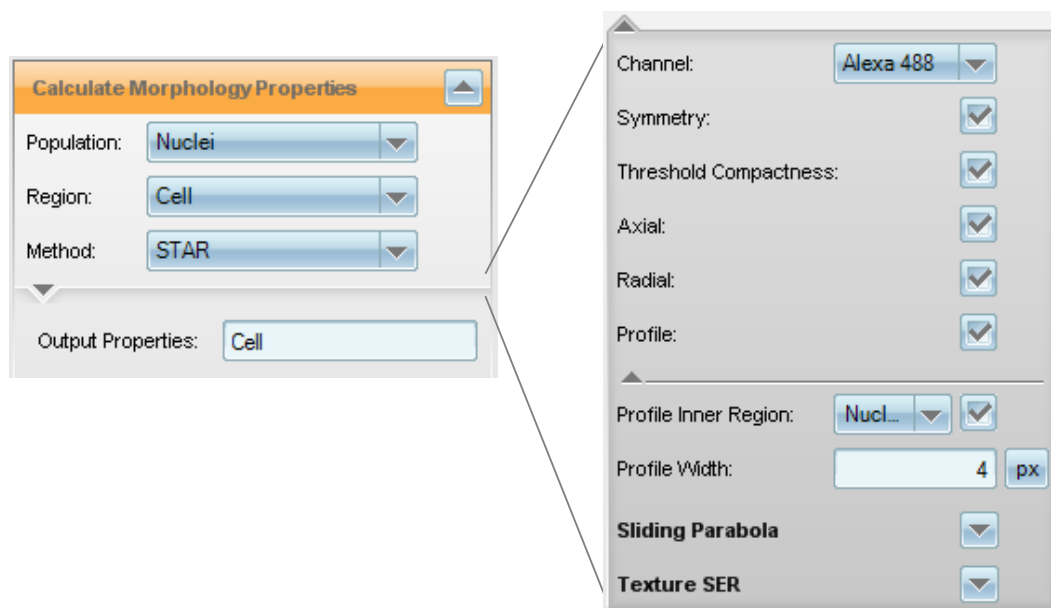
Figure 1–31: **Calculate Morphology Properties** building block, "STAR" method. Left part: standard parameters. Right part: all of the available parameters are displayed.

1.3.4   Step 4 – How to Calculate New Combined Properties

After a list of properties is calculated for each object, these properties can be combined by arithmetic operations (e.g. "New Value = Value A / Value B * 100.0") in order to generate new properties. These operations are applied to the respective columns in the object list table, and new columns are created with the individual results for each object following the operation. Frequently used operations are:
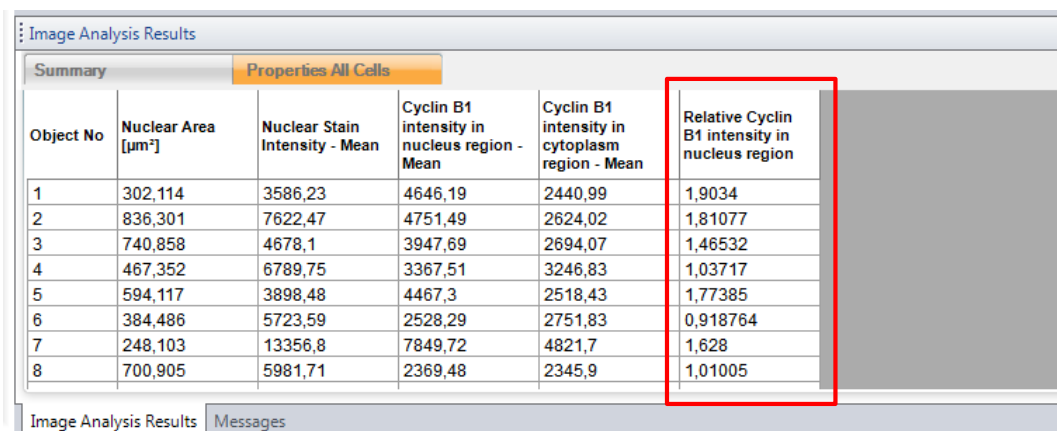
➤ Measuring the translocation / movement of a fluorescent marker from one cellular compartment to another by calculating the intensity ratio between both cellular compartments (identified as "Region1" and "Region2" in the preceding steps).

➤ Calculating the cellular intensity ratio in two different channels ("ratiometric imaging").

See *Table 1-4* for an example.

The building block for calculation of new properties based on existing properties is named **Calculate Properties**, see *Figure 1–32*.

Various calculations can be performed using multiple Calculate Properties building blocks. Any properties calculated previously can be used and the resulting properties are available in all of the subsequent analysis steps.

Multiple analysis tasks can be combined in one analysis sequence to generate multiplexed readouts, e.g. marker quantification combined with cell viability. The most time consuming segmentation steps (**Find Nuclei**, etc.) only have to be done once for both analyses, speeding up the evaluation.

Image Analysis Results

Object No	Nuclear Area [µm²]	Nuclear Stain Intensity - Mean	Cyclin B1 intensity in nucleus region - Mean	Cyclin B1 intensity in cytoplasm region - Mean	Relative Cyclin B1 intensity in nucleus region
1	302,114	3586,23	4646,19	2440,99	1,9034
2	836,301	7622,47	4751,49	2624,02	1,81077
3	740,858	4678,1	3947,69	2694,07	1,46532
4	467,352	6789,75	3367,51	3246,83	1,03717
5	594,117	3898,48	4467,3	2518,43	1,77385
6	384,486	5723,59	2528,29	2751,83	0,918764
7	248,103	13356,8	7849,72	4821,7	1,628
8	700,905	5981,71	2369,48	2345,9	1,01005

Table 1-4: Example of an object list showing the results of the initial nuclei detection and Cyclin B1 intensity calculations in different regions. The Relative Cyclin B1 intensity in the nuclear region is added as an additional property (red border). The formula used for the calculation is "Relative Cyclin B1 intensity in the nucleus region = Mean Cyclin B1 intensity in nucleus region / Mean Cyclin B1 intensity in cytoplasm region".
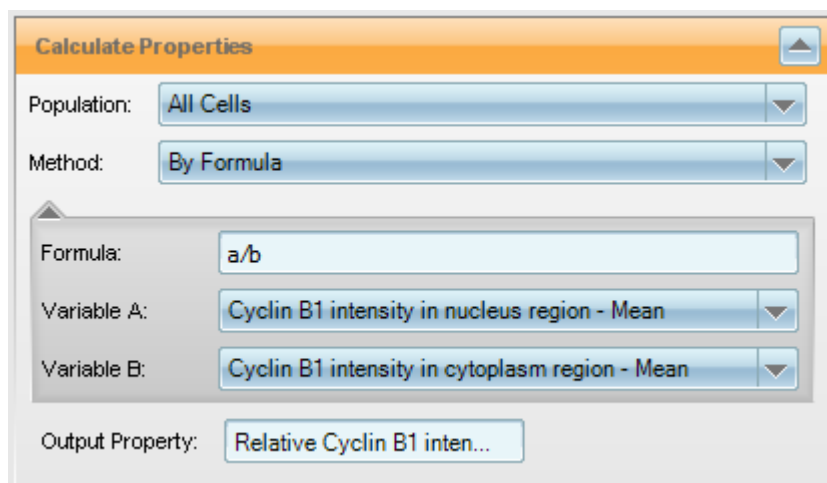
Figure 1–32: **Calculate Properties** building block.

1.3.5 Step 5 – How to Identify Subpopulations

After the measurement of object properties, the next important quantification step is the classification of objects. Classification allows the determination of subpopulations of cells based on their properties (as calculated in the preceding steps). Typical examples explained in the Ready-Made Solution chapter (chapter 2) are:

➤ Finding cells that show a functional marker, e.g. a live stain → population of live cells (see section 2.3: Live / Dead Cell Counting).

➤ Finding cells with condensed nuclei → population of apoptotic or mitotic cells (see section 2.4: Nuclear Analysis – Nuclear Shrinking).

➤ Counting cells with internalized GPCR → population of cells responding to an agonist (see section 2.11: Receptor Internalization).

➤ Identification of cells with spots in the nucleus → population of cells that are positive for a DNA damage marker (see section 2.18: Nuclear Spots).

➤ Identification of cells that show a translocation of a marker → population of cells that respond in an experiment to identify a pathway (see section 2.9: Cytosol to Nucleus Translocation and section 2.10: Cytosol to Membrane Translocation).

➤ Identification of cells with very low GFP fluorescence → population of untransfected cells to be excluded from further analysis steps and reported back for quality control (see section 2.6: Quantification of Cytoplasmic Marker).

➤ Cells with condensed nuclei and pHH3 marker fluorescence → population of early M-phase cells (see section 2.14: Cell Cycle Classification).

➤ Cells with highly irregular nuclear fluorescence → population of early apoptotic cells (see section 2.13: Nuclear Fragmentation).

➤ Cells showing a certain combination of markers → population of cells in a certain cell cycle phase (see section 2.14: Cell Cycle Classification).

➤ Finding round cells → population of cells with re-arranged cytoskeleton, mitotic cells, or apoptotic cells (needs an additional criterion to further differentiate) (see section 2.15: Cell Shape – Cell Rounding).

Each population of cells is represented by an object list (see *Table 1-4* for an example). For each subpopulation, a new property column is added to the object list, flagging each cell which is included in the identified subpopulation (see *Table 1-5*).

Table 1-5: Example of an object list where cells marked in red match the criterion "Relative Cyclin B1 intensity in nucleus region > 1.5". The subpopulation "high nuclear fluorescence" is defined as cells that have a higher Cyclin B1 intensity in the nucleus than in the whole cell region. This could be an indicator that the cell is in the early M-phase of the cell cycle (see Application Guide, section 3.3 "Ready-Made Application: Cell Cycle-1"). This population is flagged in a new cell property column that is added to the table.

Table 1-6: Example of an alternative representation of the data for the identified subpopulation "High nuclear fluorescence". The list contains only those cells in the subpopulation. The red arrows indicate the objects corresponding to the marked objects in **Table 1-5**.

Additionally, a new list is available containing only the identified subpopulation objects (see **Table 1-6**). The new list can be used in further analysis steps.

Each available (sub-) population is represented by a new tab in the image view (**Figure 1–2**, label "Population"). To choose a population for display, select the corresponding tab. The object list table is displayed in the object list view of the Harmony GUI (**Figure 1–1**, panel 5).This table can be used for visualization, further analysis steps in the next building blocks or can be copied and pasted to other data analysis programs, e.g. Excel® or TIBCO® Spotfire.

The detailed single cell level results (object lists tables) can be stored in the database during an automated batch analysis for later export and secondary level analysis with third party software if desired (see **Figure 1–17**, right column, and section 1.5.7).

Even if the tables have not been stored, they can be re-calculated at any time for quality control purposes by opening the corresponding evaluation on the "Evaluation" screen and navigating to the desired well and field on the "Image Analysis" screen.
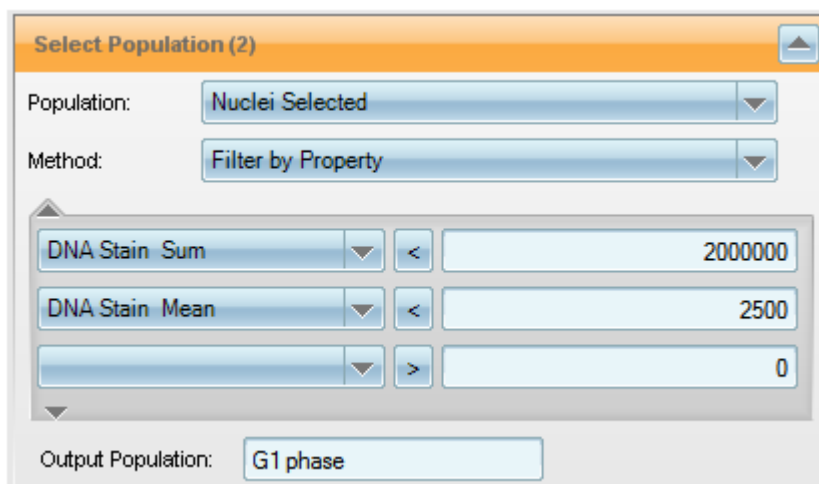
Figure 1–33: Example of a **Select Population** building block. If both of the conditions shown apply to a cell then it is added to the new population "G1 phase" (all conditions are linked by an AND operation by default). Other operations, e.g. OR, can be specified in the advanced parameter section if needed. Please refer to the "Harmony Help function" for more details.

The building block used for creating a new subpopulation based on the object properties is **Select Population**, see *Figure 1–33*.

Two basic methods are available:

➤ Filter by Property to manually create a set of selection conditions.

➤ Linear Classifier based on PhenoLOGIC machine learning (see section 1.13).

In addition to the new object list, two illustrations of the identified subpopulation are also provided to allow you to visually check the results.

➤ A configurable image view to overlay the filtered population outlines, the segmentation results, and the original images. An example is shown in *Figure 1–34*.

➤ A panel of scatter plots to visualize the numerical properties, see *Figure 1–35*.

When using the method "Filter by Property", the **Select Population** building block user interface allows multiple conditions to be specified in order to select objects based on previously calculated numerical properties (see *Figure 1–33* for an example). In addition, a special method is available to remove cells that are not completely visible in the image in order to avoid artifacts, e.g. when monitoring the cell area (Method: "Common Filters – Remove Border Objects").
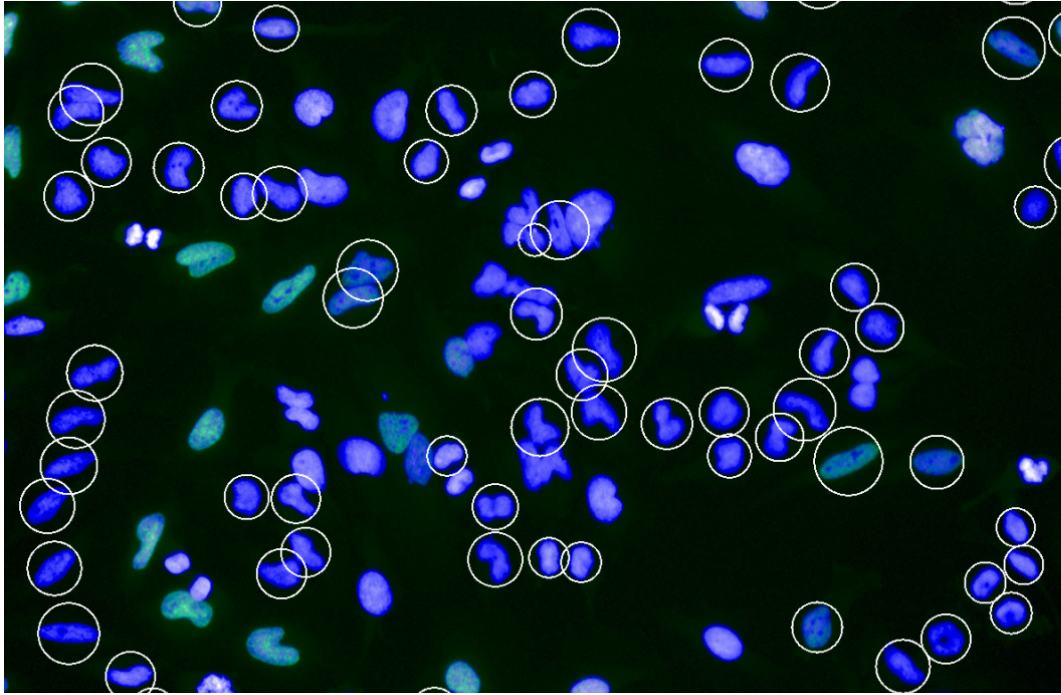
Figure 1–34: Example of an illustration created by the **Select Population** building block. The image allows you to evaluate the results of the subpopulation identification at a glance. The cells that are classified as being in "G2 phase" (according to the filter shown in *Figure 1–33*) are marked by white circles. The green channel shows an EdU stain image, indicating the S phase, and the blue channel corresponds to a Hoechst 33342 nuclear stain signal.
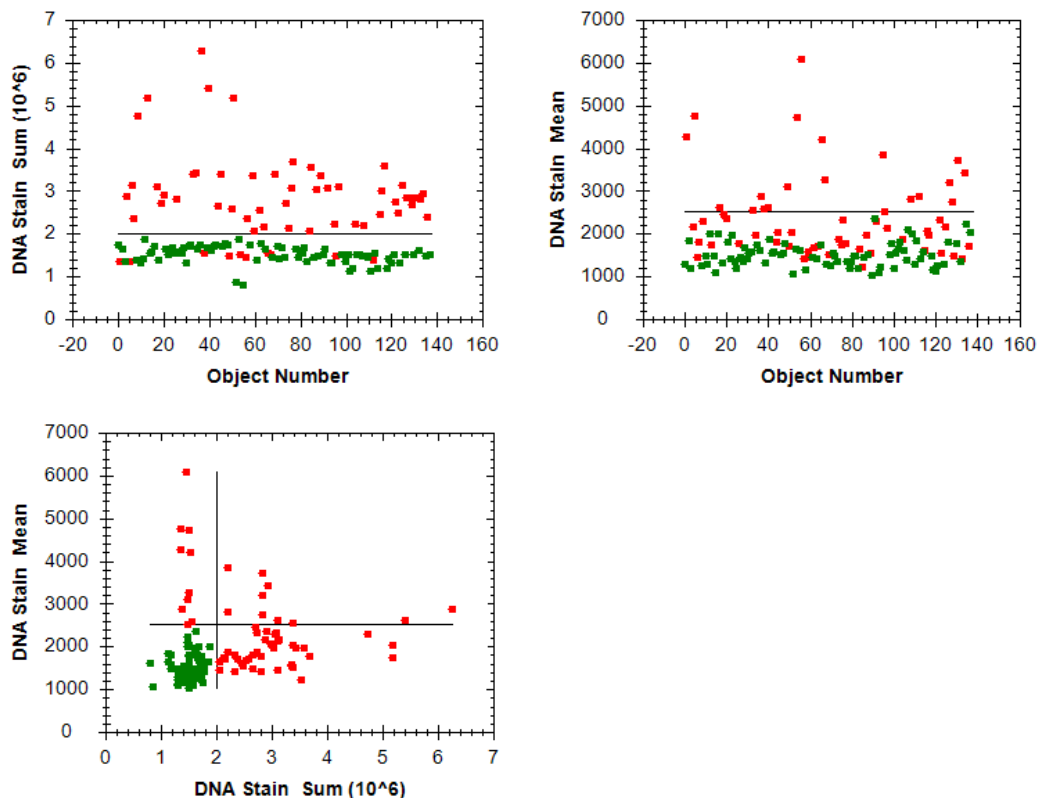
Figure 1–35: Examples of scatter plot illustrations created by the **Select Population** building block (which is shown in *Figure 1–33*). Each data point corresponds to one individual cell. A green color indicates the selected cells which pass both criteria of the filter and the red color indicates the discarded cells. The black lines show the specified thresholds. A separate scatter plot is provided for each filter criterion (top row). In addition, one scatter plot for each pair of properties is shown; here it is the "DNA Stain Mean" versus "DNA Stain Sum" plot in the bottom row. In this example, this plot provides the best overview: Cells with low DNA content AND low intensity per area (mean intensity) in the lower left quadrant are selected. For more information, please also see *Figure 2–33* in the RMS "Cell Cycle Classification" (section 2.14).

1.3.6 Step 6 – How to Calculate Collated Readout Values

The last step of the analysis is to calculate meaningful readout values for each well of the microtiter plate from the individual object (or cell) properties and subpopulations. Due to the high variability within biological samples, averages over 100 – 10,000 events would need to be created in order to obtain statistically relevant readout values for the quantification of a biological effect. Examples of readout values are shown below (see the Ready-Made Solutions in chapter 2 for more details):

➤ "Number of cells" in the full cell population, e.g. for proliferation assays (see section 2.2: Cell Counting).

➤ Number or fraction of cells in a subpopulation, e.g. "Fraction of dead cells" in a live / dead cell assay (see section 2.3: Live / Dead Cell Counting).

➤ Average marker intensity in the full cell population, e.g. for a ligand binding assay (see section 2.7: Quantification of Membrane Marker).

➤ "Fraction of cells with internalized receptor", e.g. for a GPCR internalization assay (see section 2.11: Receptor Internalization).

➤ "Average nuclear size" for a nuclear shrinking quantification application (see section 2.4: Nuclear Analysis – Nuclear Shrinking).

➤ Fraction of a marker that is located in a specific compartment of the cell, e.g. for translocation assays (see section 2.9: Cytosol to Nucleus Translocation or section 2.10: Cytosol to Membrane Translocation).

➤ "Number of cells with spots in the nucleus" in a DNA damage assay (see section 2.18: Nuclear Spots).

➤ Fraction of cells in the M-phase ("mitotic index") for a cell cycle application (see section 2.17: Mitotic Index).

The building block used for performing the statistical analysis of arbitrary populations is **Define Results** (see *Figure 1–36*).

The **Define Results** building block also defines the set of analysis results that are to be reported as the assay readout values for one well of a micro plate (results per well). All properties calculated by the preceding building blocks can be included in this readout, as well as any combinations (by formula).

Figure 1–36: Example of a **Define Results** building block which has been configured to return two final result values per well, as calculated from the image analysis sequence. 1 – To generate the first value ("Number of Cells") the standard output method is selected. 2 – To generate the second value ("Fraction with High Nuclear Fluorescence") the formula output method is used. 3 – Additional output values can be added here by selecting a method from the drop down menu. Another empty method selection drop down menu is always added at the end of the **Define Results** building block.

An appropriate set of values (for the results per well) for the example described previously (*Table 1-3* to *1-6*) is:

➤ Assay readout value 1: Total number of cells.

➤ Assay readout value 2: Fraction of cells with the fluorescent marker located mostly in the nucleus.

The corresponding building block configuration is shown in *Figure 1–36*. For each readout value to be reported, the building block contains one method section to specify how to generate this value. The following methods are available:

➤ **Standard Output**: Choose one previously calculated property to report and select a statistical value to report: Object Count, Mean, Standard deviation, CV %, Sum, Min, Max, Median. Define an appropriate name for the readout value. See *Figure 1–36* for an example.

➤ **Formula Output**: Enter a formula using an arbitrary number of properties. The arithmetic operations "+", "-", "*", "/" and brackets can be used. See *Figure 1–36* and *Figure 1–37* for two different examples.

➤ **List of Outputs**: Offers a list of all previously calculated properties. Values to be reported can be individually selected and the statistical method (if applicable) can be chosen. Alternatively, all properties of a population can be reported (option "Apply to All"). See *Figure 1–38* for an example.
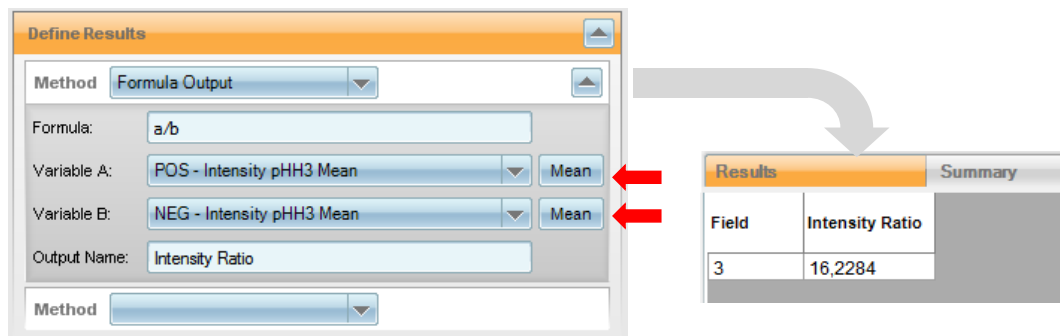
Figure 1–37: Example of a Formula Output in the **Define Results** building block. When using numerical properties in the calculation, e.g. intensity values or sizes, a statistical operation has to be selected to choose how best to convert the single cell values into a single value for the whole population. Here, "Mean" is selected (indicated by the red arrows) to return the ratio of the mean intensities of the "POS" and "NEG" populations. Other options are "StdDev", "CV %", "Sum", "Min", "Max" and "Median".
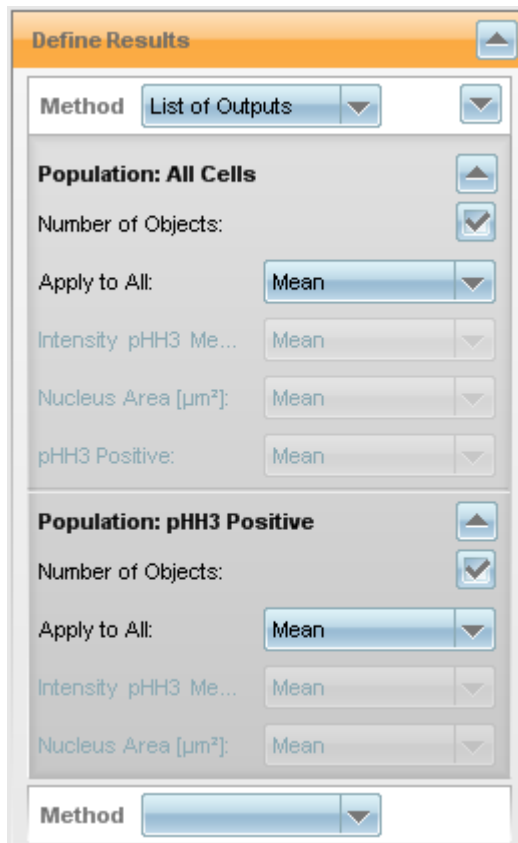
Figure 1–38: Example of how to define the results per well from a list of all the properties and populations created by the analysis sequence. The results are selected by selecting a statistical operation (e.g. "Mean") from a drop down menu (either individually for each property or for all properties at once). This method ("List of Outputs") allows you to quickly select a set of output values; however it does not offer the full flexibility of using a sequence of "Standard" and "Formula" methods, e.g. using custom names, specifying the order of values and performing calculations.

Use the "List of Outputs" method to quickly set up a series of readout values to test whether they provide meaningful information for your assay (see sections 1.5.1 and 1.5.2 for more information on how to judge the quality of a readout value). Once you have identified the best set of readout values for your assay, replace the "List of Outputs" method with a sequence of "Standard" and "Formula" methods with custom names and a specified order, for maximum clarity of the results.

The **Define Results** building block also allows you to define which Single Cell results you want to be stored in the database, see section 1.5.4.

1.4 Optimizing the Image Analysis on Single Images

After an analysis sequence has been set up using a single image, or a few images, its performance then has to be validated on a larger number of images.

In the **Define Results** building block, you can set up an illustration that allows you to visually judge the accuracy of the analysis results at a glance. For example, you can create a color overlay of image channels, add overlays of detected object outlines, and mark identified populations such as mitotic cells.

Browse through the positive and negative control wells that you selected in the first step (section 1.2.4) to confirm that the analysis visually works well on **different** images. Also test the set of "problematic" images (e.g. images with very high or low cell density, clumped cells, artifacts) identified during the visual inspection of your images.

Do not spend too much time at this point on optimization. If you see the desired effect in the positive and negative control wells, first look at the numerical results and then come back to the optimization later.

In most cases, the default or best guess parameters used during the assembly of the building blocks give very good results. This is because the building blocks are tailored to anticipate most common cellular imaging situations and include automated parameter tuning options.

If you find discrepancies with the analysis, e.g.:

➤ A large number of cells have been missed by the analysis.

➤ A region is not located properly.

➤ A large number of cells which should have been identified as "positive" for a marker are missed out.

Go back to the respective building block(s) and:

➤ Re-adjust parameters using the illustrations provided by the building block.

➤ Use the "Tuning…" dialogue provided for each parameter in the menu that appears by right-clicking on the parameter name. See *Figure 1–39*.

➤ Refer to the detailed instructions included in the "Harmony Help Function" for each building block (also available by right-clicking with the mouse). See *Figure 1–40* and *Figure 1–41*.

Continue with the optimization until you are satisfied with the analysis results on the set of positive and negative control images. If you are not sure about the results then continue, perform the next steps, and come back to the optimization later.
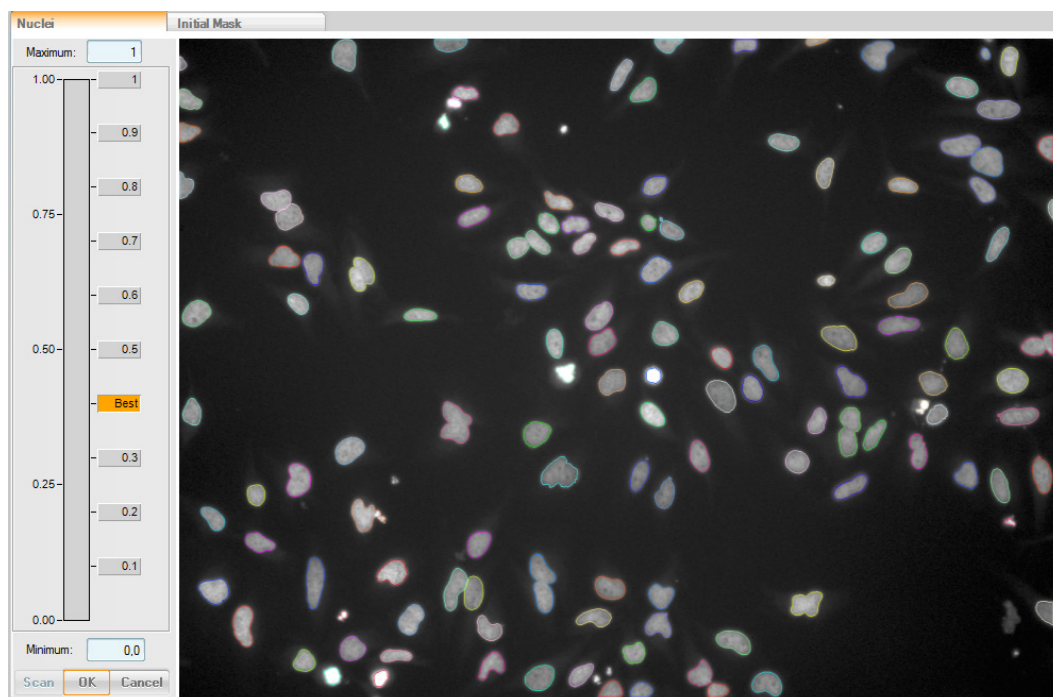
Figure 1–39: Tuning Dialogue for the "Common Threshold" parameter of the **Find Nuclei** building block. The scale bar on the left of the image shows pre-calculated parameter suggestions distributed over the useful parameter range. The value identified by the Harmony software as the best value is marked by "Best" (0.4 in this example). By hovering the cursor over the parameter labels, the image analysis results for that parameter are shown in the image (due to pre-calculation). By switching between different settings whilst viewing the image, the best value can be reliably identified and selected by a mouse click on the best parameter (creating an orange mark). The new selection can be confirmed by clicking the OK button or discarded if no improvement is seen when the parameter is modified by clicking Cancel.
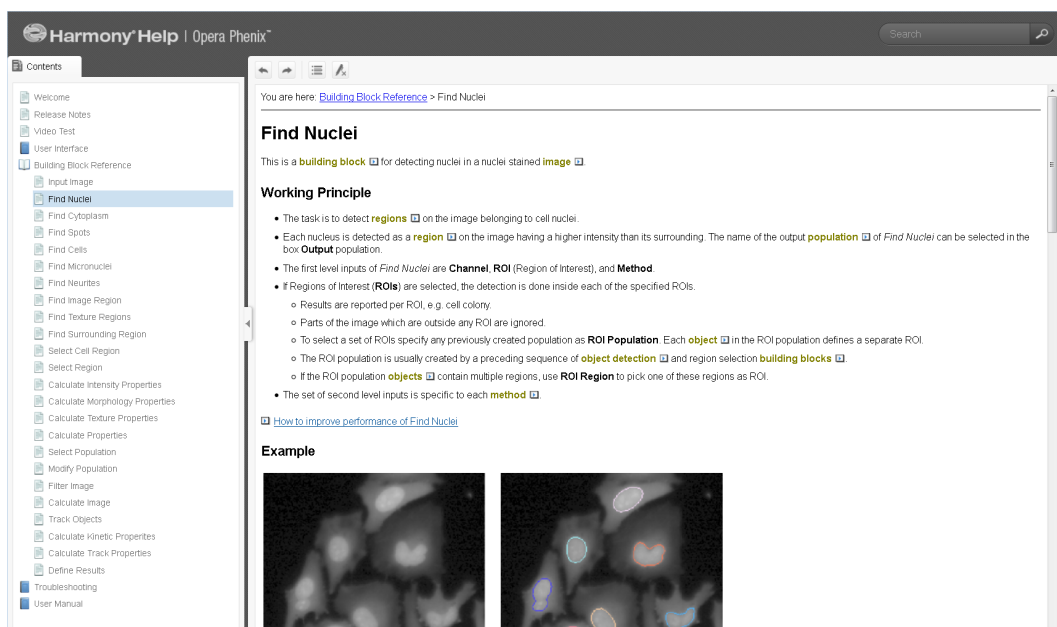
Figure 1–40: "Harmony Help Function" page of the **Find Nuclei** building block. The help page gives detailed information about the function of the building block, step by step instructions on how to improve the performance, and a description of all methods and adjustable parameters in that building block.

Figure 1–41: A page from the "Harmony Help Function" showing an overview of all of the available nuclei detection methods in the **Find Nuclei** building block, and their characteristics. The advantages, disadvantages and tunable parameters for the Method "A" are shown.

1.5 Evaluating Multiple Images and Validation of the Analysis

This is the last step in setting up the image analysis sequence: validating and (if necessary) optimizing the analysis sequence.

The high variability within a cell sample is a challenge for automated image analysis, especially when the goal is to obtain an average result across a cell population and to condense the assay information into a few readout parameters with robust statistics. Each individual cell looks different, for example the shapes of all cell compartments vary, the staining may be non-uniform, cells may touch each other or even overlap, or cell debris may be seen in the images.

Checklist for image analysis:

Quantitative criteria – to examine the quality

- ✓ Reliability, robustness against noise and biological variability
- ✓ Sensitivity
- ✓ Artifacts
- ✓ Ambiguity of the readout, e.g. nuclear shrinking due to apoptosis or mitosis?

Qualitative criteria – how to measure the quality

- ✓ Positive and Negative control well graphs, signal window, noise, outliers
- ✓ Z' value
- ✓ EC_{50} curve of a known control compound

Table 1-7: Criteria used to judge the quality of the image analysis sequence.

It is therefore necessary to look at a representative collection of cell samples and images, rather than single images, when creating or validating a new image analysis sequence.

If the number of cells captured in one image is not sufficient for a good statistical analysis then multiple image fields have to be acquired in one well. See Application Guide, section 2.1.4 "How to set up a suitable well layout" for hints on setting up a multi-field measurement. If multiple image fields are included in the analysis, Harmony will automatically calculate a collated value for all the fields contained in one well.

For the evaluation of multiple images (fields, wells, plates) with your analysis sequence, switch to the Harmony **"Evaluation Screen"**, see *Figure 1–42* for an overview of the hierarchy of data analysis.
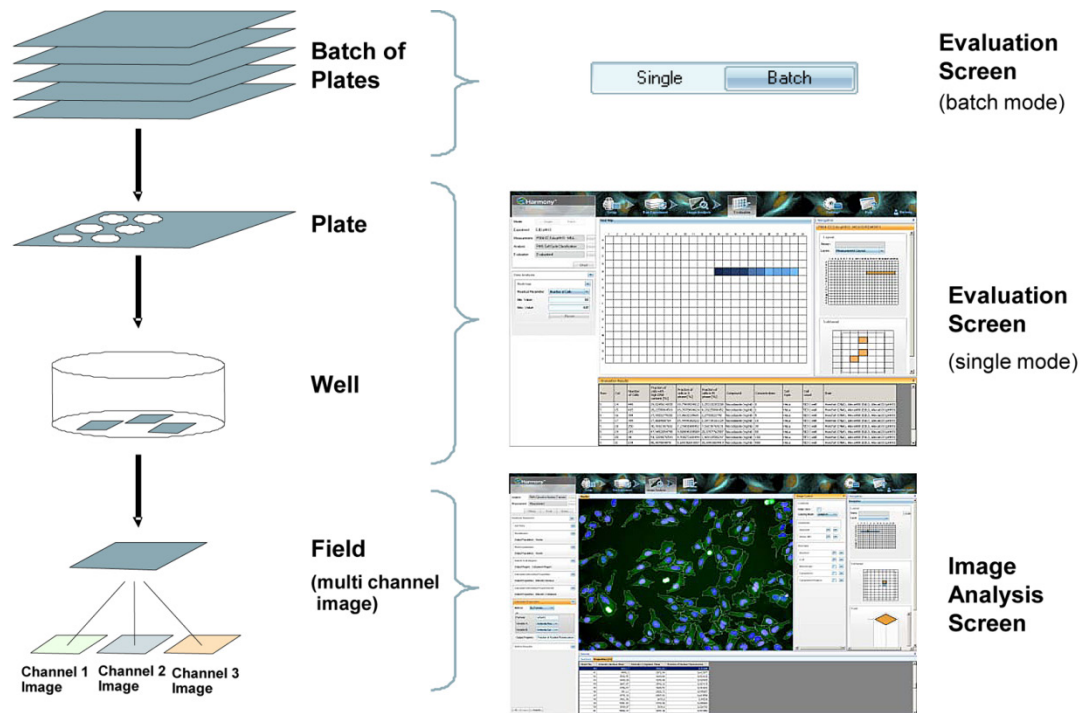
Figure 1–42: Overview of the data analysis hierarchy when analyzing a batch of plates. The left part of the figure illustrates that a batch of plates consists of individual plates, each plate consists of wells, and in each well multiple image fields are acquired. Each image field consists of images from multiple channels (colors / wavelengths). The right part of the figure shows the Harmony screens that are used to set up and control the image analysis at the respective levels.

Figure 1–43 shows a screenshot of the Harmony evaluation screen. You can define a range of wells to be evaluated using the navigation panel of the Harmony GUI (***Figure 1–43***, panel 2). For time series or stack measurements, you can also select the set of time points and / or stack planes to use for the evaluation.

When the analysis is started the selected wells are processed successively. The results per well, which have been defined in the **Define Results** building block, are tabulated (***Figure 1–43***, panel 4), and are visualized graphically in a heat map (***Figure 1–43***, panel 3) or graph plot. For detailed instructions, please refer to the Harmony Operation Manual and the built in "Harmony Help Function".
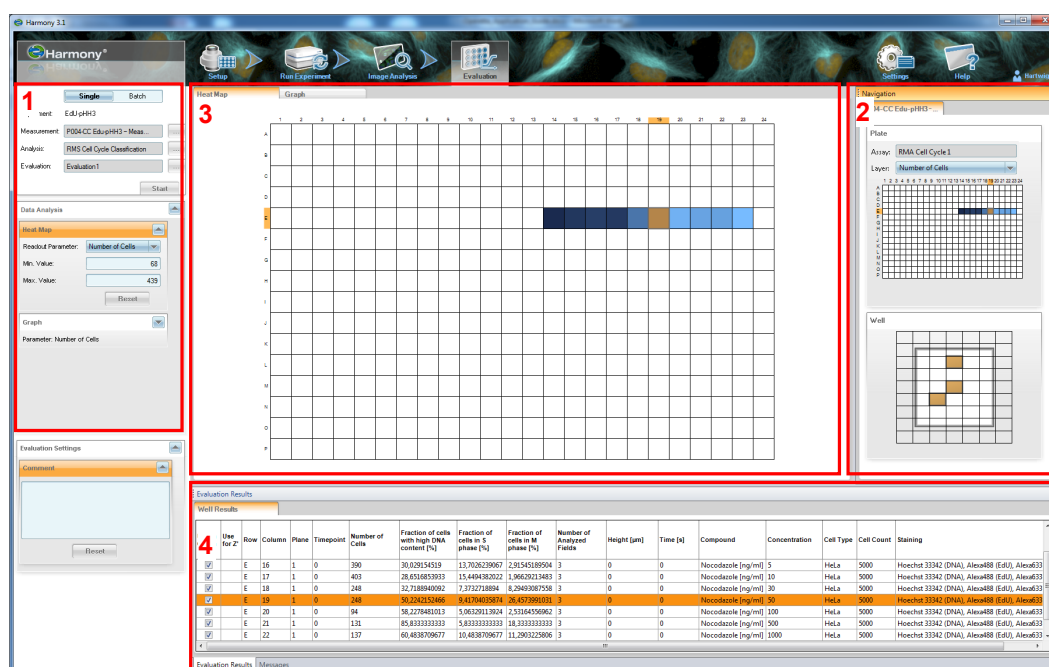
Figure 1–43: Harmony "Evaluation" screen showing a heat map and a tabulated view of the readout values for a range of wells within the plate. 1 – Selection of dataset, analysis sequence and visualization parameters (with the heat map selected). 2 – Navigation in the plate dataset / images (well, field, stack plane, time point). 3 – Heatmap of a selected readout value. 4 – Table of results per well.

1.5.1 Validate the Analysis against a Set of Positive / Negative Control Wells

Select a set of positive and negative control wells from the navigation panel and run the analysis. On the heat map or graph plot display (see *Figure 1–47*), you can immediately check the different readout values for reliability, e.g.

➤ Cell number – constant?

➤ Marker intensities – variations?

➤ Cell area – variations?

➤ Assay readout – do you see the expected effect?

A good way of judging the assay and image analysis quality is shown in *Figure 1–44*. The main readout value of the assay is plotted for a number of positive and negative control wells. This allows you to judge the signal window, noise and Z' value of the assay (refer to the Glossary section in the Appendix for a definition of these terms).

This illustration can be easily created on the Harmony evaluation screen by using the graph plot type: *Z'* (see *Figure 1–45*). The set of positive and negative control wells can be selected interactively from the navigate control or the well result table. Select "Use for Z' positive / negative Control" from the menu available by right-clicking on the selected wells, or lines in the table.

For further statistical calculations or advanced visualizations, copy and paste the table showing the results per well into TIBCO® Spotfire, Microsoft Excel® or other compatible software.

Noise in positive or negative controls

If you see high noise in either of the groups (positive or negative controls), go back to the image analysis screen and inspect the detailed image analysis results of the sample wells from the group. Adjust the parameters if needed.
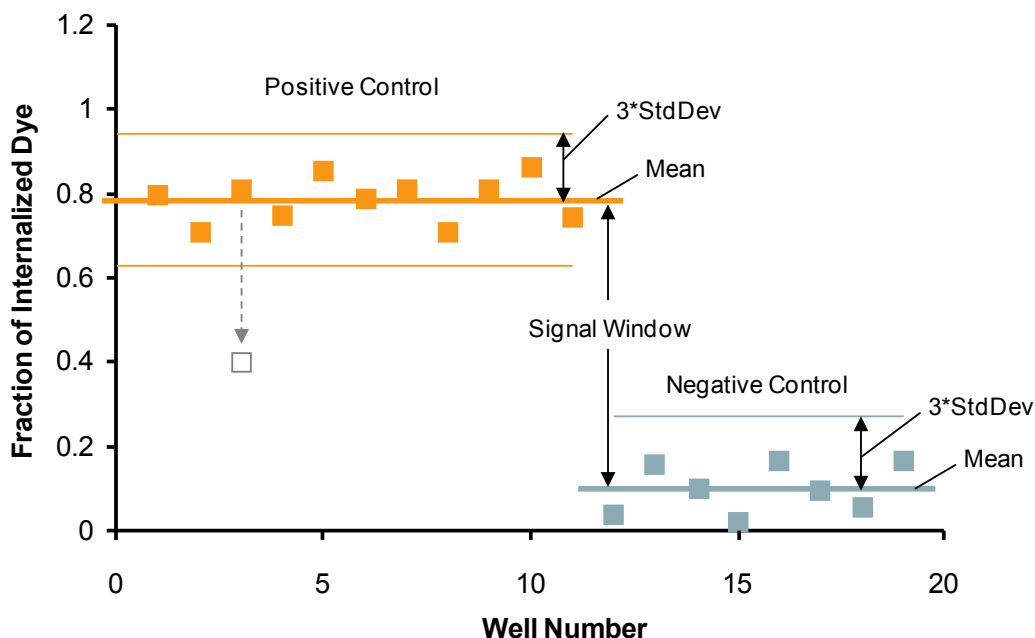
Figure 1–44: Example of a Z' graph showing the results for a set of positive and negative control wells. The readout value ("Fraction of Internalized Dye") is plotted as a square box for each well. Positive control values are marked orange, negative control values are marked blue. The Mean and StdDev values for each group of wells (positive and negative controls) are indicated by their respective colored lines. The Z' value in this example is 0.52 (for formulas please see the Glossary section in the Appendix). If one of the positive control values is shifted to the value indicated in gray (outlier), the Z' value reduces to 0.14. Use the Harmony plot type *Z'* to create the graph in the Harmony Evaluation screen (see *Figure 1–45*).

Z' Value

Z' values of 0.3 or higher are considered significant for cellular screening. The Z' value can be improved by changes to the biological assay setup, the measurement and the image analysis. For hints on potential image analysis improvements, see section 1.5.3.

Signal Window

A wide signal window improves the Z' value (if the noise level does not change). In case of insignificant Z' values, find out if a high noise level is compromising the Z' or if there is a small signal window (i.e. the readout value does not change significantly between positive and negative controls). Depending on the result, either try to lower the variability of your readout value to reduce the noise or try to generate a larger difference between the positive and negative control values.

Outliers

A single outlier in the control wells will compromise the Z' value significantly (see the example in *Figure 1–44*). If you see obvious outliers, go back to the image analysis screen and inspect the detailed results for that respective well to determine the reason. Common reasons include pipetting errors, dry wells, agglomerates of dye, wrong well selection, low cell number in the image, clumped cells, and insufficient staining. Depending on the reason, you may exclude that well from the Z' calculation (singular

events which will be fixed for the next measurement), modify the image analysis to give correct results, or flag the well as an "outlier", e.g. in case of a low cell number or low intensity level (indicating an empty well).

The graph plot is fully interactive: selecting a data point in the plot selects the corresponding wells, stack planes and time points in the well result table and the navigate control, and vice versa.

➤ To see the corresponding images of an outlier in the graph, click on the data point in the graph to select the data point in the navigate control, then create a well montage image (from the right click menu on the navigate control) or navigate back to the Image Analysis screen.

➤ To remove a data point from the Z' graph, click on the data point in the graph to select it in the well results table, then un-check the "Display" checkbox of that data point in the table.

➤ To find out where data points from the plot are located on the plate, select them in the graph (hold down the Control key for multiple selections) and see their location on the navigate control. You might, for example, find that multiple outliers are located in a specific region, e.g. near the border of the plate, giving a hint of what might have gone wrong. See *Figure 1–47* for an example.
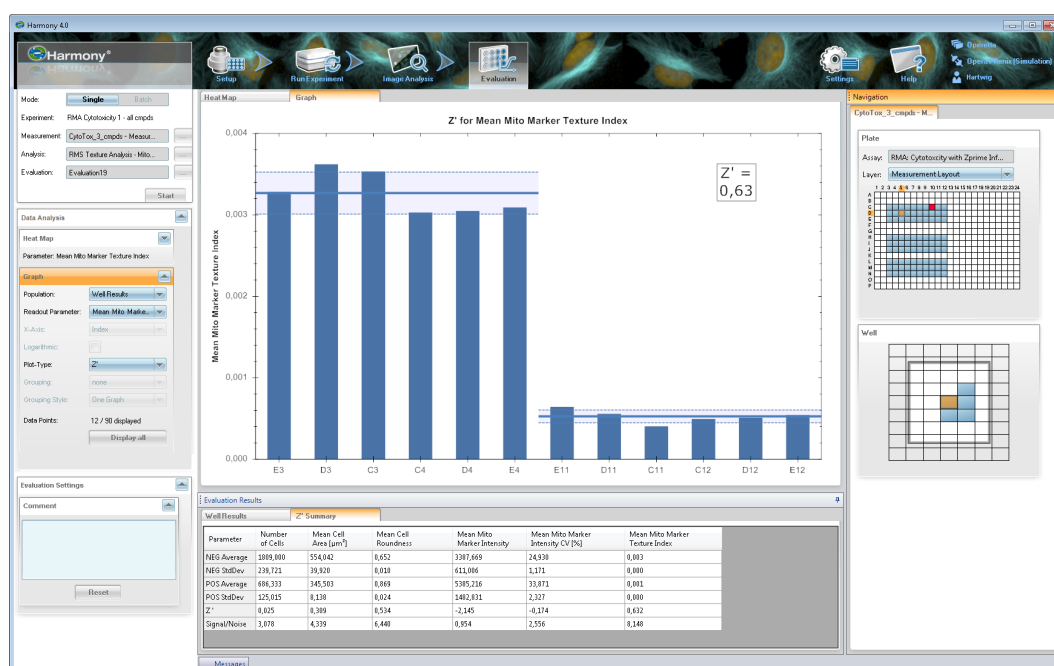
Figure 1–45: Screenshot of the Z' plot type on the Harmony Evaluation screen. Positive and negative control wells can be interactively selected from the plate navigation area on the right hand side of the screen (or the Well Results table in the bottom pane). The mean level and the standard deviation interval are shown for each group. The resulting Z' value is displayed. Details are shown in the "Z' Summary" tab in the bottom pane, e.g. the Z' values for all readout parameters.

1.5.2 Validate the Analysis against a Dose-Response Curve

Only validation of the full dose-response curve provides the final proof that the intermediate values (in addition to the high and low values) have been correctly analyzed.

To create a dose response curve, select the wells on the plate that represent a dose titration of a compound and start the batch analysis.

Make sure an assay layout with a layer "Concentration" has been set up, containing the different compound concentrations in each well of your measurement. See the Harmony Help system for instructions. The concentrations can be used in the graph and in the table, and will be part of your exported results. They greatly facilitate data interpretation.

If you use multiple compounds on the plate, add an assay layout layer "Compound" which contains the compound names for each well (the layer type must be alphanumeric). This allows the creation of multiple dose response curves at once (use the "Grouping" parameter of the graph plot).

To view and judge the results, use the plot type EC_{50} in the graph view, see **Figure 1–46**. The graph is fully interactive, i.e. you can select points in the graph to see the corresponding well(s) on the plate and in the well result table. This may be useful for example to follow up on outliers.

The EC_{50} graph illustration includes the following information to allow you to judge the assay performance (to validate the assay with a known compound) or the compound characteristics (for unknown compounds with a validated assay):

➤ Error bars, if multiple wells are treated with the same compound and concentration (replicates). Hint: To split up the error bars to individual points in order to find an outlier, switch to the plot type "Points".

➤ EC_{50} fit curve (black line, based on a Hill Equation fit with four free parameters).

➤ The "EC_{50} Summary" tab is added to the Evaluation Results pane. It lists all fit result parameters for all compounds, including their standard deviations.

➤ Confidence interval of the fit curve (light grey lines below and above the fit curve). A smaller corridor indicates a higher statistical probability that the fit curve represents the true value.

➤ The EC_{50} concentration value is indicated by a label on the "Concentration" axis of each plot.

For more advanced analysis or customized illustrations, all resulting values can be exported, for example to TIBCO® Spotfire, Microsoft Excel® or GraphPad Prism®. Refer to the biological applications in sections 3.1 to 3.3 of the Application Guide for more examples of dose-response curves that were created by copying and pasting the contents of the results table into the GraphPad Prism® software.
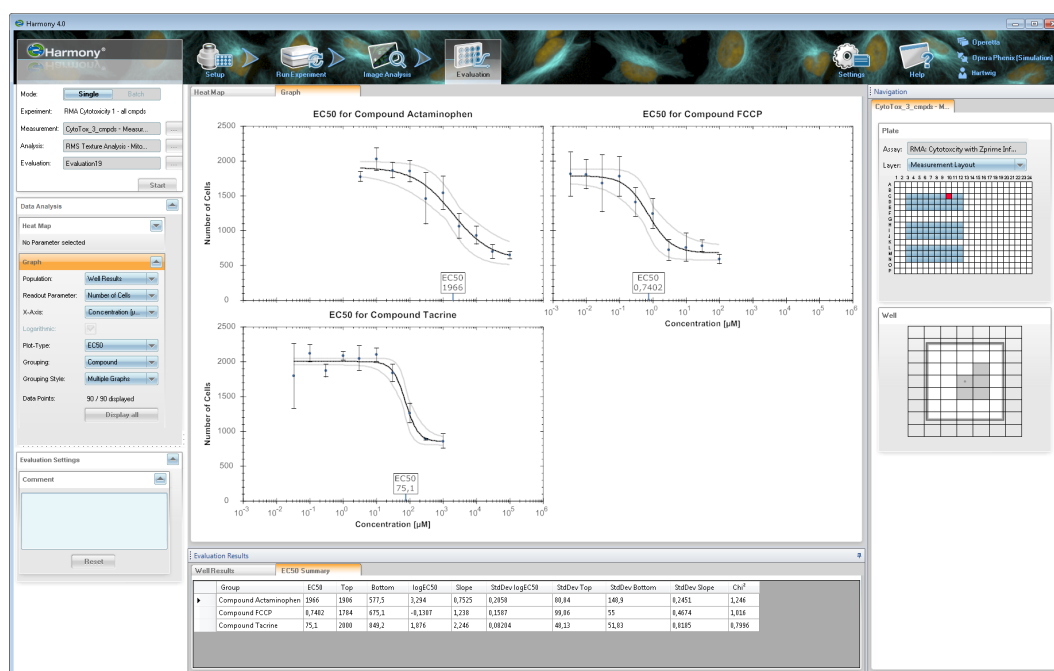
Figure 1–46: Screenshot of an EC$_{50}$ plot on the Harmony GUI. The concentrations are taken from the assay layout layer "Concentration" (see the Harmony Help for details on how to add and modify an assay layout). The graph option "Grouping" is used to group the data by the assay layout layer "Compound", creating a separate plot for each compound. The fit includes error bars, reflecting replicates for a concentration, and an interval of confidence (thin grey lines below and above the solid fit curve). EC$_{50}$ values are indicated by a label on the concentration axis. Numerical parameters are collected in the results table tab "EC$_{50}$ Summary" in the bottom pane.

1.5.3 Optimization of the Numerical Results

In all cases – also in apparently "ideal" cases – the readout values returned by the analysis sequence not only reflect the biological property of your interest but will also be influenced by irrelevant properties of the cell, sample preparation and measurement process. For example, organelles may be out of focus (blurred) in some cells due to the 3D shape of the cell, some cells may not be transfected, or staining intensities may vary from plate to plate. This section lists some measures to reduce these unwanted influences on the readout values and increase the robustness of the analysis.

You can optimize the performance of the analysis sequence by:

➤ Adjusting the tuning parameters of individual building blocks. Two levels of tuning parameters are available.

➤ Using a different readout parameter to measure the biological effect.

➤ Adapting a region of interest to maximize the signal-to-noise ratio.

➤ Including a filtering step to remove artifacts, e.g. untransfected cells (using the **Select Population** building block).

You can also improve the performance of the overall experiment by:

➤ Using more image fields in the same well to increase the number of cells used to generate the result.

➤ Using sample replicates to minimize well-to-well and plate-to-plate variability.

You can monitor the performance of the assay by:

➤ Using control wells as "positive" and "negative" references, e.g. using compounds that induce a known effect.

➤ Using dose-response curves of the known compounds to show a gradual increase in the effect, from low to high.

➤ Using additional readout values for quality control. See *Figure 1–47* for an example. The homogeneity of the nuclear stain (coefficient of variation value) was used here to detect a region in the plate with an increased number of apoptotic cells.
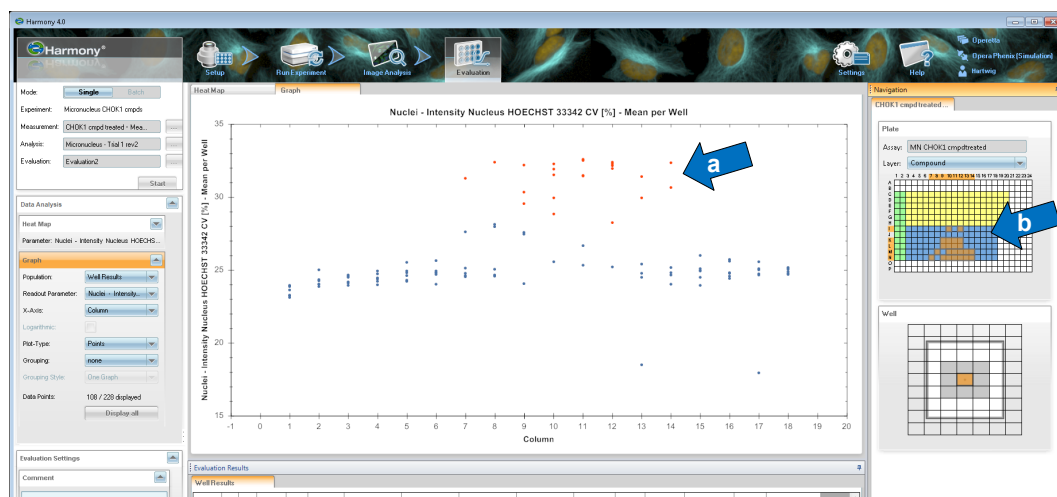
Figure 1–47: Example of tracking artifacts using the interactive graph display on the evaluation screen. The intensity homogeneity of the nuclear stain (CV) of all wells of the microplate is plotted against the column number of the wells in the plate. Each dot represents one well. Some wells in the middle of the plate show increased inhomogeneity. These cells were selected in the graph (blue arrow "a"). The selection is propagated to the corresponding wells on the plate layout (blue arrow "b") indicating the location of those wells. Studying the images in that region revealed an increased rate of apoptotic cells, compromising the results in this area of the plate. The location and shape of the area of apoptotic cells suggests a problem with cell handling rather than a compound effect (compound regions are marked by colors on the navigation control, with doses increases from left to right).

1.5.4   Storing Single Cell Results

When working with single cell results (in addition to or instead of the collated well results), all of the single cell properties can be saved in the database during batch analysis. The results stored can be analyzed within the Harmony Software (see section 1.5.5) or exported for secondary analysis in third party software (see section 1.5.7). The following RMSs provide examples on how to work with single cell data:

➤ RMS "Cell Cycle Classification" (section 2.14)

➤ RMS "Nuclear Classification – DNA content" (section 2.12)

➤ RMS "Colony Formation Analysis" (section 2.25)

➤ RMS "Cell Tracking – Migration Analysis" (section 2.29)

➤ RMS "Cell Tracking – Cell Division" (section 2.30)

To avoid taking up disk space and cluttering the database, the amount of detail stored in the database can be adapted to the needs of the application. Three different options can be selected in the **Define Results** building block (see *Figure 1–48*):

➤ None – Single cell results are not stored in the database to save disk space (default setting).

> Note that single cell results for a particular field of view can be accessed on the Harmony Image Analysis screen. This option is most suitable for assays where you do not need to export the results for further processing with third party software.

➤ All – All object properties which are calculated using a building block (e.g. **Calculate Intensity Properties**, **Calculate Morphology Properties**) are saved in the database, i.e. all columns of the object list tables displayed by the **Define Results** building block are saved.

➤ Selected – Only those cell properties which have corresponding well results are saved in the database, e.g. if the "mean nuclear area" is defined as a well result then the "nuclear area" is also saved for each individual cell.

> Properties which are only used in formula outputs (Method: "By Formula") are not stored as single cell data when method "Selected" is used. To store single cell data for these properties, they have to be selected as an individual output value.
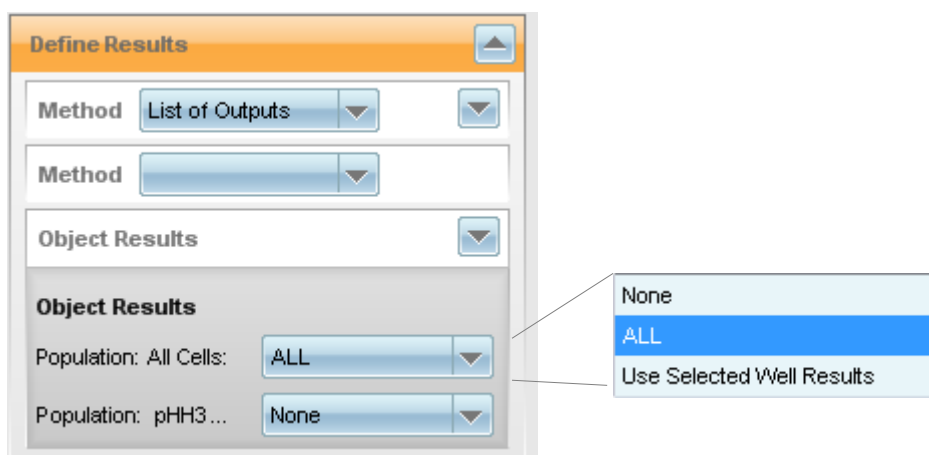
Figure 1–48: Storage options for single cell results provided by the **Define Results** building block. Storage options can be individually selected for each population.

1.5.5   Analyzing Single Cell Results

Single cell results that were stored in the database can be accessed on the Evaluation Screen via the **Population** dropdown list in the **Graph** panel. The list will contain one entry per population that has associated single cell results (see *Figure 1–49*). However, since the data volume of single cell results can be huge compared to the volume of well results, it is not recommended to load the single cell results for an entire microplate. The recommended workflow is as follows:

➤   Identify wells of interest by analyzing the well results. The resulting selection can comprise different wells, planes and time points.

➤   Reduce the data points displayed in the graph, for example by using **Graph – Display Selection** from the context menu of the navigation area or the results table.

➤   Select the single cell results from a particular population using the **Population** dropdown list in the graph panel.

➤   The Harmony software will start to load the numerical properties for all objects from the selected population and the corresponding wells, time points and planes.

Harmony will only load up to 500,000 data points at once. If the selection of wells, planes and time points contains more than 500,000 single object data points a warning will be given once this maximum number is reached and the loading of data will stop. Therefore, it is likely that data from one of the selected wells will not load completely. To avoid this situation try to narrow down the initial selection of wells, planes and time points.

Together with RMS "Cell Tracking – Migration Analysis" (section 2.29) and RMS "Cell Tracking – Cell Division" (section 2.30) evaluations are provided that contain single cell data. The respective chapters contain examples on how the Harmony software's **Evaluation** screen can be used to create plots from single cell data.

Focusing on subsets of data points

In most cases the aim of single cell data analysis is to identify cells or objects that have special properties. Often the properties of these objects are masked in a well-based analysis by the noise created by the majority of objects. In general, the workflow to focus on the objects of interest is as follows:

➤   Select data points of interest. This can be done either by selecting points in the graph (e.g. outliers in a scatter plot) or by selecting lines from the **Evaluation Results** tables (see *Figure 1–49*).

➤   Select **Graph – Display Selection** from the context menu of the data table to reduce the graph to the selected data points. Select an appropriate plot type to visualize the results.

When working with single cell data the evaluation results tables can be very large making the selection of lines difficult. Sorting the table by relevant properties facilitates the selection of the desired subset (e.g. long tracks or bright cells).

The selection of data points is fully synchronized. Selecting data points in the graph will select the corresponding lines in the evaluation results tables and vice versa. This also applies when single cell results from a kinetic population (generated by object

tracking, see section 1.16) are displayed. In this case, the corresponding lines in the additional **Track Properties** table are highlighted when data points are selected in the graph or the single cell results table. In turn, selecting a line in the track properties table will select all lines in the single cell results table that relate to the selected track.
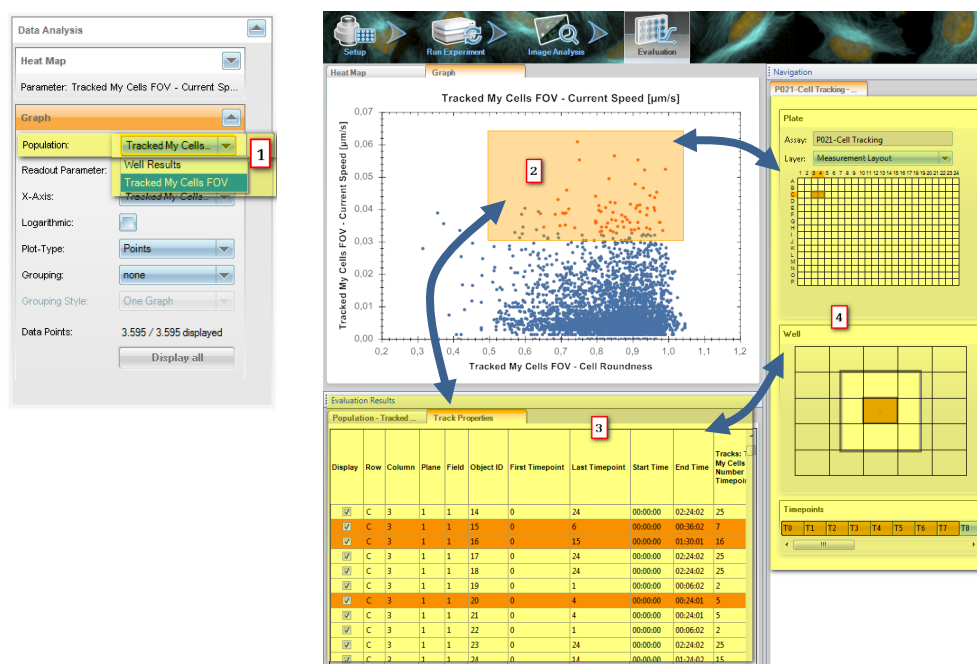
Figure 1–49: Single cell data analysis in the Harmony software. A population with single cell data is selected via the **Population** dropdown list (1). The single cell data displayed can be refined by selecting data points in the graph (2) or by selecting lines in the evaluation results (3). Note that selecting data points in any way will always highlight the corresponding items in the graph (2), the evaluation results (3) and the **Navigation** area(4).

Using the **Display all** button will display the set of single cell data in the graph that was loaded initially. By selecting additional wells, fields, time points or planes further single cell data can be loaded using the context menu **Results table – Add Selection** function. Note that the limit of 500,000 data points still applies.

Linking back to the corresponding images

Once a particular group of objects has been identified, it is desirable to visually inspect these objects. To this end, proceed as follows:

➤ Select the objects either via the graph or the evaluation results tables. The corresponding wells, fields, time points and planes are highlighted in the navigation area (see *Figure 1–49*).

➤ Go back to the image analysis screen. The building block **Input Image** must be active.

➤ If there are objects from multiple images selected, no image will be shown. Select one of the images by refining the selection in the navigation area to one well, one field, one time point and one plane.

➤ The objects that were selected on the evaluation screen will be marked with an orange circle (see *Figure 1–50*).

➤ Select other combinations of well, field, time point and plane to inspect selected objects located in other images.

To follow particular objects over time, select all data points that belong to the objects. This can be easily achieved by selecting the corresponding lines in the track results table. From the image analysis screen, use the selected time points to create a time window (see Harmony Help). In this way, a movie can be generated where particular cells are marked over the entire track length.
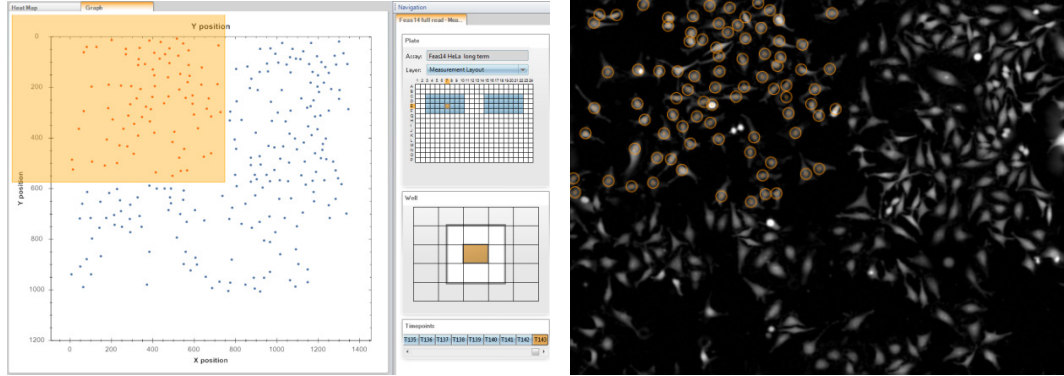
Figure 1–50: Connecting data points to images. The Y versus X coordinates of cells from one field of view were displayed in the graph on the Harmony Software's evaluation screen. Data points in the upper left corner were selected. After switching to the image analysis screen the objects in the upper left corner of the image are marked with an orange circle.

Examples for typical plots

In general, single cell analysis within the Harmony Software can be used to generate a variety of plots of all possible properties. Some of the potential plots are shown in this section:

➤ Scatter plot to identify subpopulations (see *Figure 1–51*, left)

➤ Plot of a property versus time for a subset of cells to analyze cell-to-cell variation (see *Figure 1–51*, right)

➤ Plot of cell tracks for one field of view (see *Figure 1–52*, left)

➤ Plot of the relative cell migration to analyze chemotaxis or chemokinesis (see *Figure 1–52*, left)
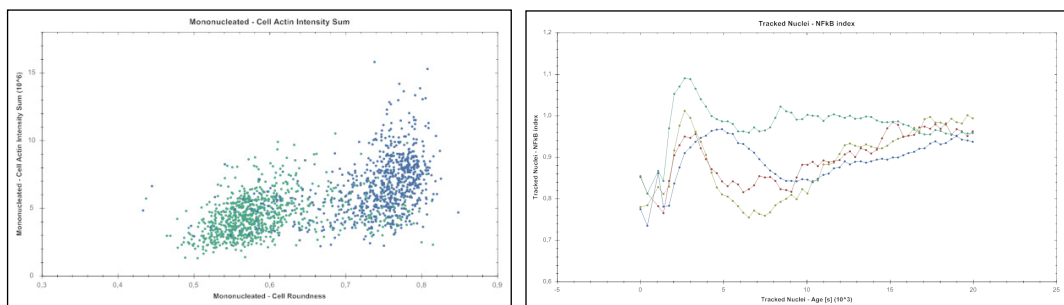
Figure 1–51: Analysis of subpopulations using single cell data. **Left:** Scatter Plot of two cellular properties for two cell populations that were subjected to different treatments. Plot type was set to *Points*, grouping by treatment and grouping style was set to *One Graph.* The two populations (green and blue) can be clearly distinguished in the scatter plot. **Right:** Kinetic of the response of 4 different cells to a compound treatment. A readout parameter reflecting the cellular response was plotted versus the observation time. Plot type was set to *Lines*, grouping by *Object* and grouping style was set to *One Graph.* The variability in the response is clearly visible.
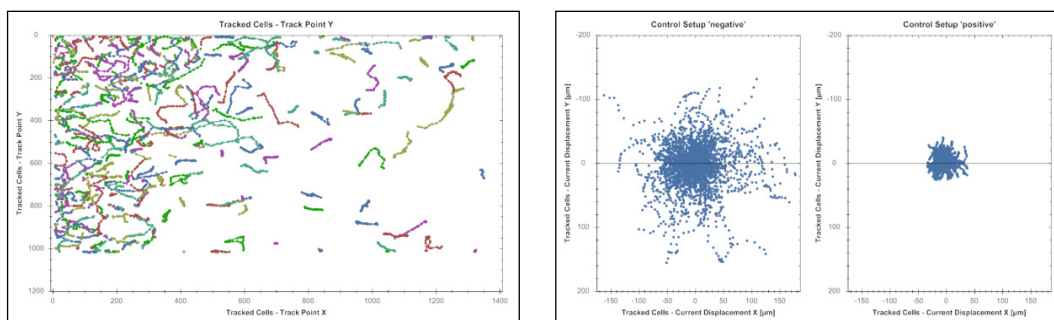
Figure 1–52: Analysis of cell migration using single cell data. The underlying data set is provided together with RMS "Cell Tracking – Migration Analysis" (section 2.29) **Left:** Plotting the Track Point Y versus the Track Point X using single cell data from one field of view and 25 time points. Grouping option was set to *Object,* grouping style was set to One Graph and the plot style was set to *Lines*. The individual cell tracks are clearly visible. **Right:** Plotting Current Displacement Y versus Current Displacement X. In this way all tracks are normalized to a common origin. In the given example single cell data from 2 different wells was used, where one well was treated with an inhibitor of cell migration. Plot style was set to *Points*, grouping was by Control Setup and grouping style was set to *Multiple Graphs*.

1.5.6　Analyzing Batches of Plates

Once an image analysis sequence has been set up and its performance has been validated, it can then be used to automatically evaluate a full plate or a batch of plates.

In a batch analysis, all the wells of all selected plates are sequentially processed in the same way without user intervention. The generated well and single cell results are saved in the Harmony database as an attachment to the corresponding measurement. Multiple evaluations of the same measurement can be done and saved in the database, allowing the user to re-analyze measurements or compare different analysis sequences.

If analysis speed is an issue, see section 1.21 for hints on optimizing the analysis sequence for enhanced speed.

Any Harmony image analysis sequence can also be used to analyze images "online", i.e. in parallel to the image acquisition.

For unattended batch and online analysis of plates it is particularly important to:

➤　Use a robust analysis sequence with respect to the well-to-well variations.

➤　Use additional readout values for quality control, e.g. cell number.

➤　Use control areas on each plate, e.g. "high" and "low" controls.

This will ensure that possible errors in the automated data acquisition or analysis are recognized.

1.5.7 Exporting & Documenting Results

Several interfaces exist for transferring images and numerical data to external programs, e.g. for recording protocols in electronic lab notebooks, for using custom visualization software, or for further analysis e.g. by spreadsheet programs or corporate screening databases. Extended archiving and importing / exporting functions are available from the "Settings – Data Management" menu of the Harmony software (see *Figure 1–53*). For a detailed description, please refer to "Harmony Help" or the instrument's Operation Manual.
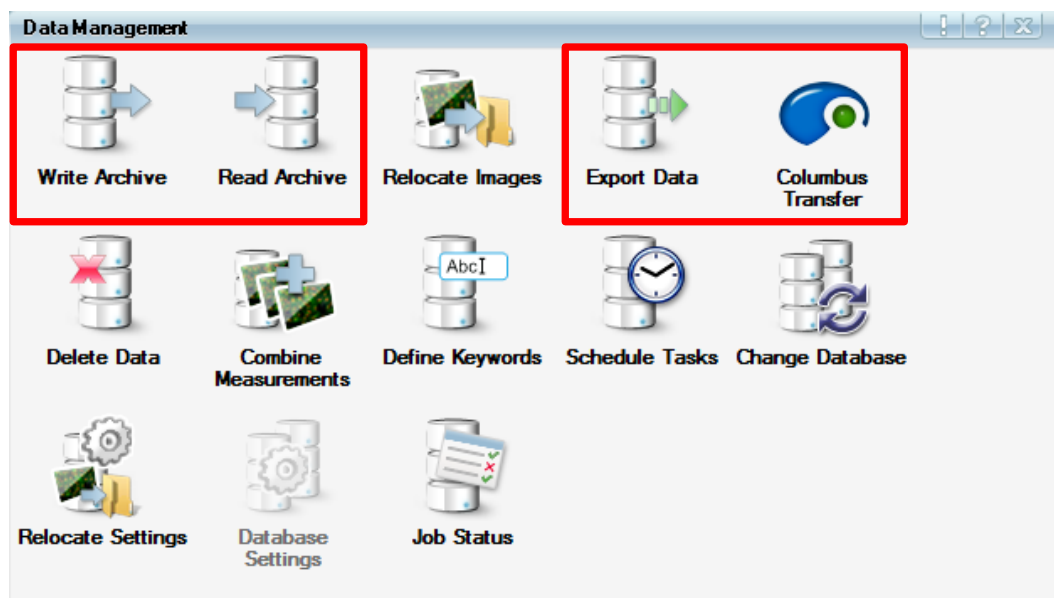
Figure 1–53: Data management options in the Harmony database. Data import and export options are marked by a red box. Archived images, measurements, and analysis sequences can be stored on disk or DVD, shared, sent over a network and / or re-imported (also partly re-imported) into the same, or other, databases.

Different formats for the export are available:

➤ **Copy and Paste** to the Windows clipboard for immediate transfer of images, graphs and tables during interactive work.

➤ **Harmony Archives** for storage and archiving contents from the Harmony database and for transfer between different Harmony systems.

➤ **Export Data** for transfer to Spotfire® or third party software. The images can be included as a copy or as a reference to the Harmony database (avoiding duplicating the images).

➤ **Columbus Transfer** for directly transferring the data to Columbus. If the Columbus Tranfer has been defined as an online job in the experiment setup, the data will be transferred automatically after the measurement has been finished (see Application Guide, section 4.1.10 "Automated data transfer to Columbus" for detailed information).

Please note: Harmony archives (created by "Write Archive") cannot be read by programs other than Harmony. They contain a 1:1 copy of the Harmony database contents to fully restore the saved contents.

In contrast, the exported information (created by "Export Data") can only be used in other software. The exported data cannot be re-imported into a Harmony database. Some Harmony specific information is lost (e.g. used filters for image acquisition).

Exported measurements always contain the associated assay layouts and flatfield correction parameters. If the "incl. associated files" method variant is selected, all evaluations (including analysis sequences) that have been done for the selected measurements are included as well.

Illustrations

Every image or illustration that is visible on the Harmony GUI can be transferred to other Windows programs using the standard Windows copy and paste ("Clipboard") mechanism (available from the right mouse-click menu). This is especially helpful for documenting and communicating results during interactive work with Harmony.

Acquired Images

The images acquired during an automated measurement are stored in the Harmony database and can be exported using the "Write Archive", "Export Data" or "Columbus Transfer" functions (see *Figure 1–53*).

➤ Harmony archives are folders on the hard disk that can be re-imported to the same, or another, Harmony database.

➤ Exported images are packaged in a folder to be read by third party software. The folder may either contain a copy of the images or a reference to the images in the Harmony database (see *Figure 1–54*, "Measurements" repectively "Measurements – referenced images").

Using references to the images is an efficient way of avoiding duplication of large amounts of data, saving disk space and time when transferring the data. The recipient software can directly read the images from the Harmony database using an internet connection ("Web Interface").

Well Results

Well results can be:

➤ Transferred to third party software for further visualization and analysis by copying and pasting the table of well results on the Harmony Evaluation screen (panel 4 of *Figure 1–43*). This is very useful for quick inspection of the results during assay development or for manual quality control, e.g. for calculations in Spotfire®, Excel® or preparation of special plots.

➤ Stored in Harmony archives together with a self-contained package of all the settings and analysis sequences that were used to acquire and analyze the images.

➤ Exported as a text file to be read by third party software. The file format is designed to be easy to import into software such as Spotfire®, Excel® or GraphPad Prism®. The file also includes the assay layout information entered during the assay setup, e.g. the compound concentrations and location of control areas (see *Figure 1–54*, choose method: "Evaluation Results per Well").

➤ Transferred to Columbus. When a measurement is transferred all corresponding well and single-cell results are automatically included.

Single Cell Results

During interactive analysis on the Harmony **Image Analysis** screen, all displayed object list tables (see *Figure 1–1*, panel 5) can be copied to the clipboard and pasted directly into, for example, Spotfire® or Excel® for further calculations (e.g. to create **histograms** or **pivot tables**) or visualization (e.g. **scatter plots**). See the RMS "Nuclear Classification – DNA content" (section 2.12) or "Colony Formation Analysis" (section 2.25) for an example.

Single-cell results that are stored in the Harmony database during batch analysis can be exported for further analysis in third party software in the same way, and in the same file format, as the well results (see *Figure 1–54*, choose method: "Evaluation Results per Well and Object").
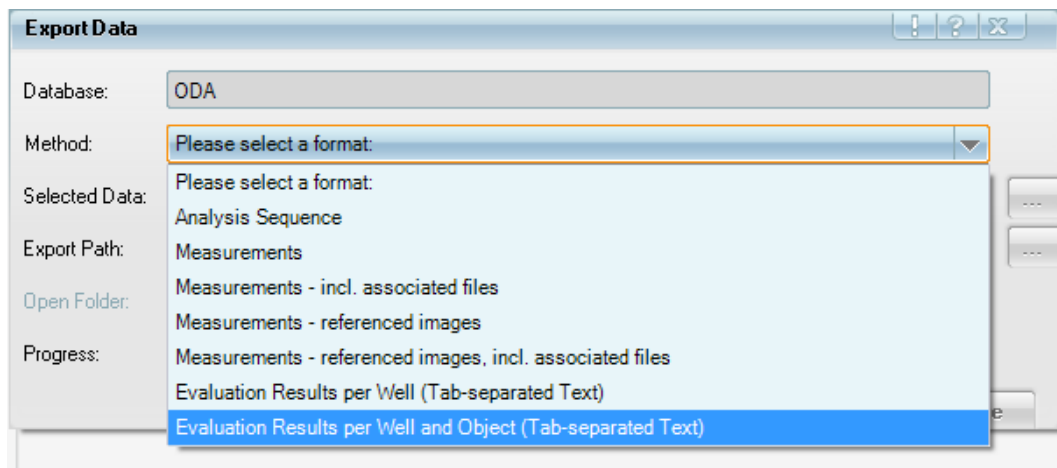
Figure 1–54: Dialogue for exporting images and results from the Harmony database for import into Columbus or third party software for further processing.

1.6 Flatfield Correction

The illumination of optical devices is not perfectly homogeneous throughout the field of view which mostly leads to an intensity drop at the image borders (vignetting). The magnitude of this effect mainly depends on the choice of objective, channel and image acquisition mode (confocal/non-confocal).

The resulting non-flat illumination may affect visualization of the images, segmentation and analysis of absolute intensities. Objects in the corner of the image exhibit lower intensity values even if they have identical expression levels of a fluorescently labeled marker.

Harmony allows you to mathematically correct for this uneven illumination. Based on your sample images, the illumination profiles are automatically derived simultaneously to the measurement. No user interaction is required to start the process and the profiles are tailored to your experiment. Furthermore, it is always the user's choice whether to apply the correction in Harmony.

Applying the flatfield correction may be beneficial for:

➤ Visualization

➤ Segmentation

➤ Measurement of absolute intensities

How does it work?

The flatfield correction profile estimation is automatically done in the background. After acquisition, the images are directly passed to the computing engine to determine the illumination profiles. After completion, the flatfield correction can be applied optionally, but in the Harmony database only uncorrected images are stored. Images that were measured with older Harmony versions (before Harmony 3.5) can be corrected by a flatfield profile as well – simply open the measurement and the profile calculation will be started.

As the profile's shape differs between the channels and for background and objects, two separate profiles need to be determined for each channel (see ***Figure 1–55***). For each profile valid pixels are accumulated until a robust estimate of the illumination profile is possible. To prevent any reduction in the image quality due to correction, there is a quality check for the input images during the estimation process as well as the resulting correction profiles.

The required number of images for profile estimation strongly depends on your sample, especially on the confluency. For background profile estimation at least 7 images are needed, but typically 10–30. For object profile estimation many more images are required due to high intra and inter object intensity variations (typically at least 100). The more objects you have in the images, the fewer images you will need for object profile estimation.

Illumination profiles
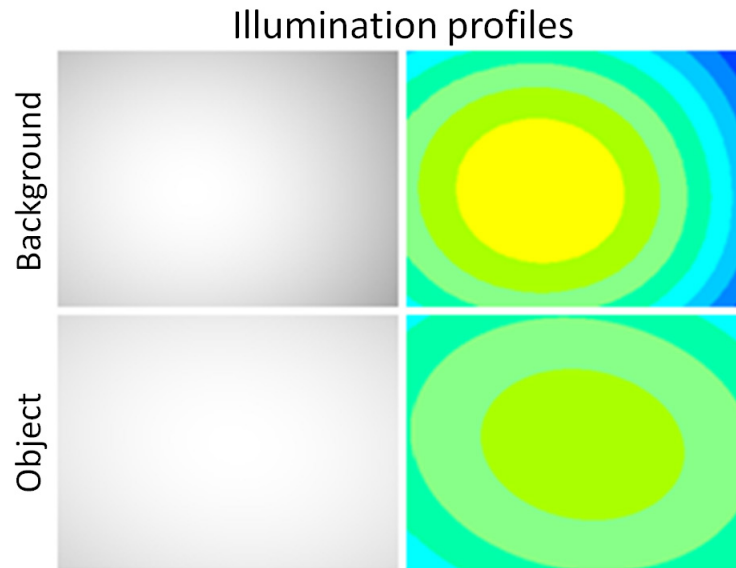
Figure 1–55: Graphical representation of background and object correction profiles. Correction profiles are illustrated as a grayscale image (left) and a color-coded image (right) where each color represents 5 % intensity variation. Correction profiles differ considerably between background and object, therefore it is important to determine and apply both profiles in order to provide a reliable correction of images.

In the image, the background and object illumination profiles are superimposed. Each pixel has a background and an object intensity part, which need to be corrected separately.

Harmony offers two different flatfield correction modes: basic and advanced. When using the basic mode, only the background proportion of each pixel is leveled. In the advanced mode, the basic correction is applied first, followed by a correction using the object profile to level the object intensity proportion of each pixel.

Basic correction is available if the background profiles for all channels were determined successfully. For the advanced correction not only the background profiles, but also the object profiles need to be present for all channels. Availability of profiles for all channels is mandatory to ensure traceability and to allow for ratiometric measurements (e.g. FRET applications).

Flatfield correction does not set the background level to zero. Mean background and mean object intensities of each image remain unchanged after correction, only the global illumination dependent intensity variation is minimized.

In the flatfield corrected images, you may discover background structures which you have not seen in the uncorrected images. After removal of the global background intensity variation, the autocontrast function visually pronounces tiny intensity variations in empty image areas. You can avoid this effect by tuning the contrast parameters on the uncorrected images and switching off the autocontrast before applying flatfield correction.

Please note that for stack measurements, the flatfield correction will yield the best result if you apply it to the mid plane. During the profile estimation only the mid plane from a stack is considered. Applying the flatfield correction to planes far from the focal plane will reduce the quality of the correction.

The current flatfield calculation method is not applicable to brightfield images. Therefore, changing the flatfield methods will not have an effect on brightfield images. For digital phase images no flatfield correction is required as they are inherently flat due to the DPC calculation method.

There is an additional keyword "Available Correction" in the database which allows you to sort measurements by their available correction.

It is not possible to apply the flatfield correction during online analysis as at start of the measurement it is not known which correction will be available.

Recommendations for the use of flatfield correction

Visual image improvement

No matter which correction method you chose, you will always achieve a visual improvement of your images regardless of the assay. *Figure 1–56* demonstrates the effect of the correction in a well montage. The visual effect is mainly achieved by correcting the background and thus basic correction is sufficient. As objects show strong intensity variations, the object illumination profile is hardly visible to the human eye. Therefore it is difficult to observe the differences between basic and advanced flatfield correction, when switching back and forth.
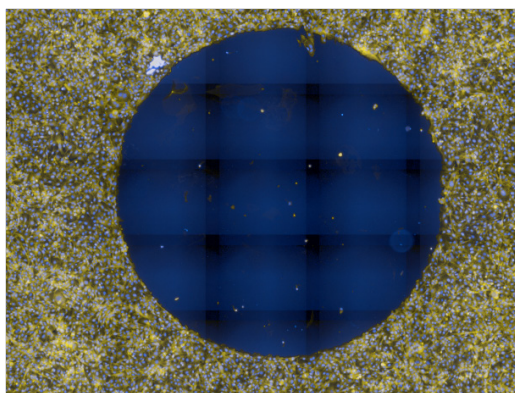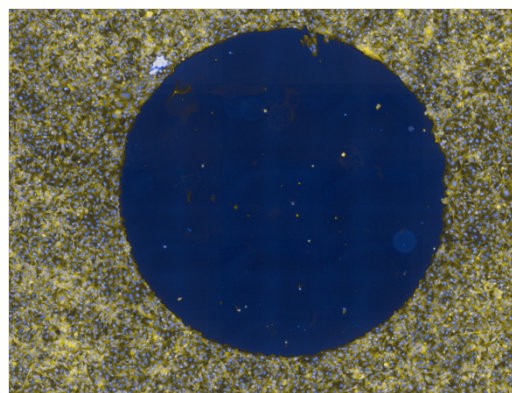
No flatfield correction   Basic flatfield correction

Figure 1–56: Effect of the flatfield correction in a well montage. In the well montage without correction (left), the borders of the individual images are darker compared to the rest of the image thus creating a check pattern in homogeneous image regions. This is especially visible in the center area of the well without the cell layer. In the corrected well montage (right), the basic flatfield correction levels the background in the individual images and the pattern in the center area of the well vanishes.

Improving segmentation

The illumination inhomogeneity also impacts some segmentation algorithms. Very simple thresholding methods like **Find Image Region** are sensitive to a non-flatness in the image, but also more advanced algorithms e.g. in the **Find Cytoplasm** building block work more robustly if the correction is applied. *Figure 1–57* compares the segmentation results of an uncorrected and a corrected image using **Find Cytoplasm**. In the uncorrected image, the detected cell contours sometimes extend into the background. Such segmentation errors are most likely to occur if the intensity difference between background and objects is rather small. Applying the basic correction addresses these segmentation issues.

No flatfield correction   Basic flatfield correction
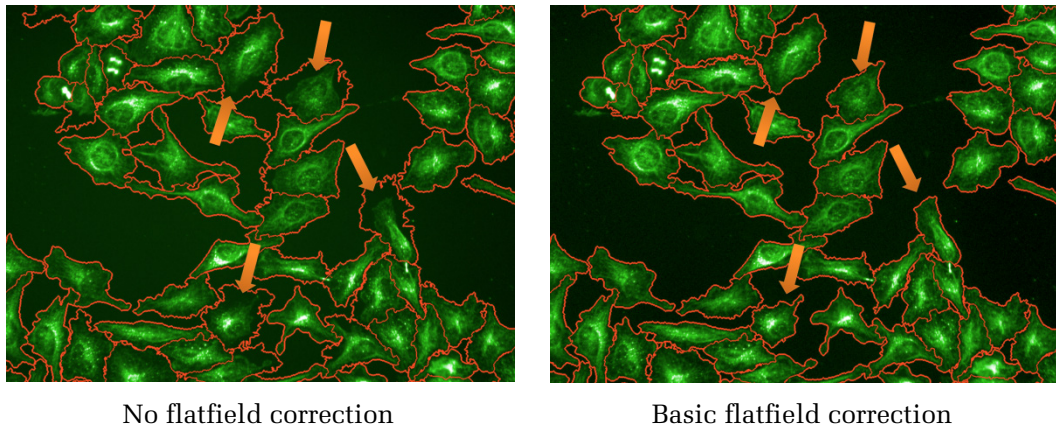
Figure 1–57: Impact of flatfield correction on segmentation results (Find Cytoplasm). Without flatfield correction (left), the detected cytoplasm regions show a tendency to extend into the background in dim samples, as highlighted by the orange arrows. After applying the basic flatfield correction (right), all cytoplasm regions are detected accurately.

Measurement of absolute intensities

For many applications, such as translocation assays, no absolute cell signal readout is required as relative intensities can be calculated. Relative readouts have the advantage of being independent from the transfection rate, staining protocol and the illumination profile of the lamp. However, for some applications absolute intensity readouts are necessary, and for these it is beneficial to correct for any inhomogeneity of the illumination profile:

➤ Quantification of marker intensity in a cell or cell organelle

➤ Determine the DNA amount of cells based on their nuclear staining (cell cycle assays)

➤ Determine the expression level of transfected cells

If your assay requires absolute intensity readouts, we recommend using the advanced flatfield correction, especially if your assay exhibits only subtle effects (small differences between positive and negative control).

No flatfield correction   Basic flatfield correction   Advanced flatfield correction
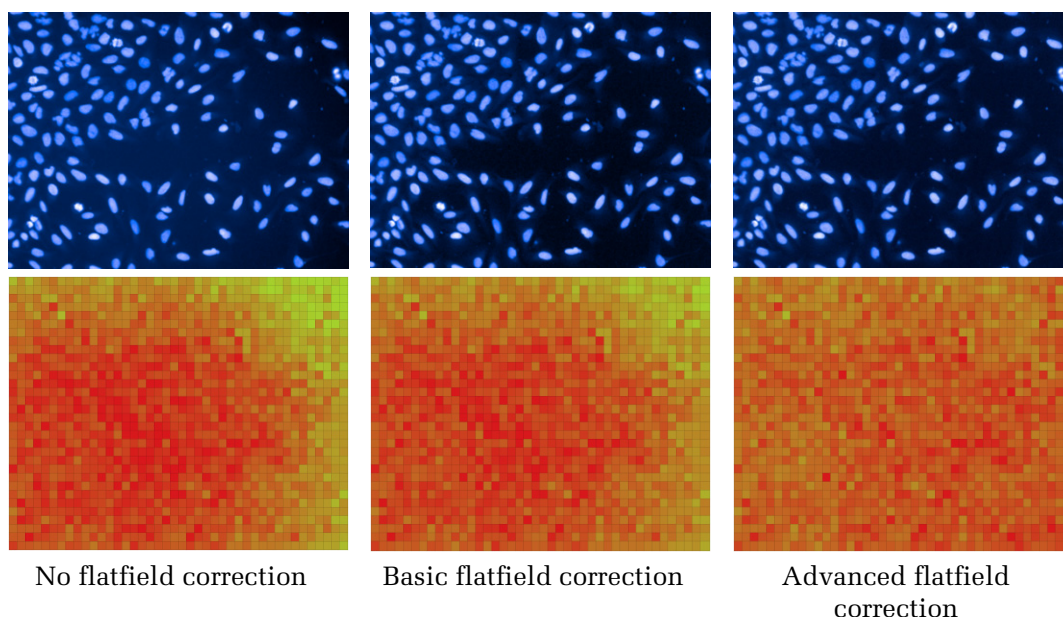
Figure 1–58: Effect of basic and advanced flatfield correction on image visualization and quantification. Hela cells were stained with Hoechst (upper row). The mean Hoechst intensity in the nucleus was determined and a heatmap was generated displaying the mean intensity of all nuclei in the dataset with regard to their x-y coordinates in the field of view (lower row). While the basic correction improves the visualization in this dataset, the advanced correction leads to an even distribution of object intensities across the field of view.

In case there is only a small difference between object and background intensities, even the basic correction (blue curve) will improve quantitative readouts of object intensities (see ***Figure 1–59***). This might be important for datasets where the advanced correction mode is not available.

High signal sample   Low signal sample
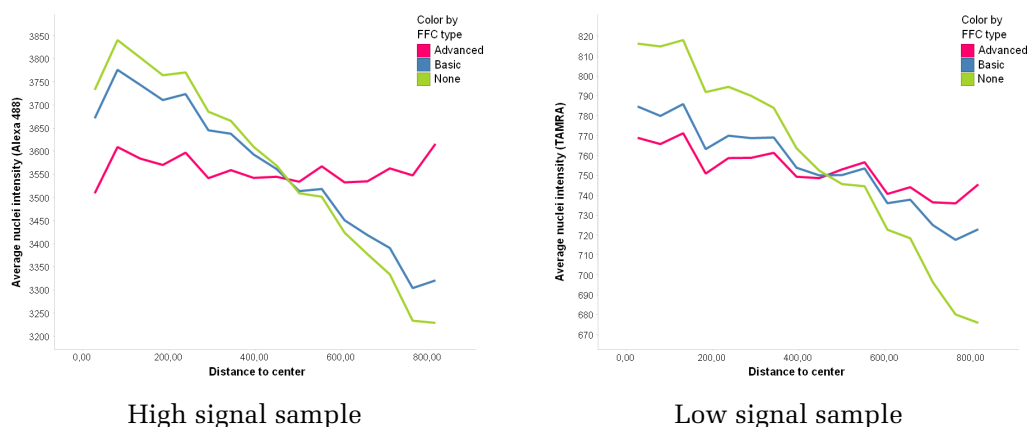
Figure 1–59: Effect of the different flatfield correction modes on a high and a low signal sample. The mean object intensities of a strong and a dim signal were plotted against their distance from the image center. The advanced correction (pink curve) shows the best improvement and leads to a nearly flat signal for both samples. In samples with dim objects, also the basic correction (blue curve) considerably improves image flatness for object intensities.

> The flatfield correction will not have any effect on quantitative analysis if your readouts are based on e.g. relative intensities (e.g. Nucleus to Cytoplasm intensity), texture or morphology parameters.

If one channel shows very rare events, such as transfected cells at low transfection rates, it is likely that the object correction profile cannot be determined and thus the advanced mode will not be available. In this case, you may consider acquiring more wells and/or fields than needed for your experiment to increase the total number of images for profile estimation.

Flatfield correction in Columbus

In Columbus versions higher than 2.4, you have the option to apply flatfield correction which was generated in Harmony. No matter how you import the images into Columbus (using automated Columbus transfer or export if you are not connected to the network) the profiles for flatfield correction will be transferred as well.

1.7   Texture Analysis

Texture analysis is a special technique that can be used to quantify biological effects. It makes use of the "pattern" or structure of the fluorescent region, rather than using the average intensity or shape of the region (see ***Figure 1–60***).

For generic information on how to set-up an image analysis sequence for a specific application, see section 1.3. In the following section only additions that relate to texture analysis are introduced.

For an example of a ready-made analysis sequence using texture analysis, see the RMS "Texture Analysis – Mitochondria Classification" (section 2.22).
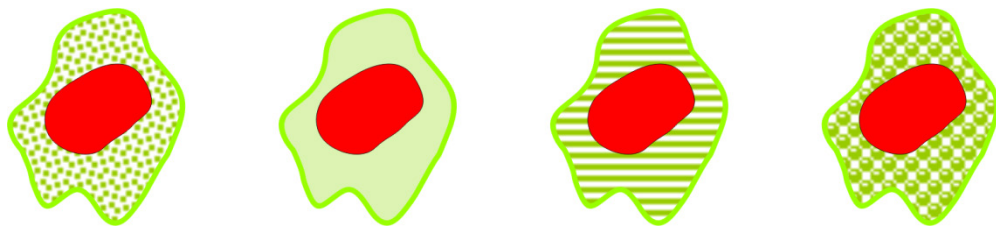
Figure 1–60: Examples of different textures. All cells have the same morphology (shape) and intensities but different textures within the cytoplasmic area.

➤   Texture analysis is performed in step 3 of the generic image analysis sequence (described in section 1.3) when quantifying properties of regions that have been identified in the preceding steps of the analysis sequence.

➤   In general, texture analysis can be used for two purposes:

- **Image segmentation**, i.e. identifying objects or image regions based on their texture. An example might be the identification of areas covered by cell colonies in brightfield images, as the cell colonies have a different texture to the background. This is not covered in this section; please see section 1.14 for more information.

- **Object Characterization**, e.g. for classification of nuclei into "apoptotic" and "healthy" classes. The **Calculate Texture Properties** building block can be used for this purpose.

➤   Potential applications are (see ***Figure 1–61***):

- Evaluation of the cytoskeletal structure, e.g. well organized fibers running in similar directions versus irregular structures.

- Nuclear fragmentation, apoptosis detection.

- Mitochondrial structural analysis.

- Cytosolic marker structural analysis (smoothness, pits, vesicles), e.g. endosomes, clathrin-coated pits, cellular vesicles, protein aggregates or clusters, homogeneous versus inhomogeneous distribution.

- Nuclear marker structural analysis, e.g. cell cycle proteins, histones, homogeneous versus inhomogeneous distribution.

Typical descriptions associated with texture properties are smooth, rough, granular, spotty, homogeneous, inhomogeneous, irregular, regular, fiber structure, line pattern, etc. If you can describe a phenotype by these terms, try using texture parameters for quantification.
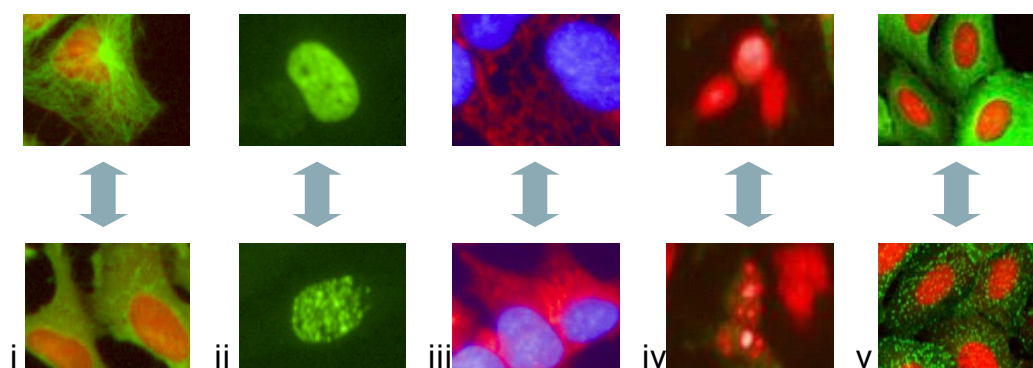
Figure 1–61: Examples of texture changes that can be quantified using the **Calculate Texture Properties** building block. i) Tubulin structure change due to Demecolcine treatment. ii) Different textures of EdU stained nuclei. iii) Cytotoxic effects of FCCP on MitoTracker™ Red-stained mitochondria. iv) Nuclear fragmentation of Hoechst 33342 (red) and Caspase-3 marker (green) -stained cells. v) Example images for negative (low) and positive (high) B2 adrenergic receptor activation. In the negative image (top), the green GFP fluorescence is more homogeneous, whereas a strong granularity is visible in the positive image (bottom).

Background

➤ Because texture recognition is an essential part of our visual perception, it has already been studied for decades from different perspectives such as human vision, object recognition, machine vision, image segmentation, texture simulation, object tracking.

➤ Due to its complexity and the wide scope of applications, there is no generic solution or system for texture analysis. There are still a number of rather distinct fields of research related to texture, each generating many publications every year.

➤ Typically, object classification consists of two steps:

1. Calculating a set of numerical properties which describe the texture of an object. There is a plethora of different sets of properties, based on different mathematical approaches. These are either generic or are model-based and tailored to different application areas.

2. Classifying the objects by their set of texture property values using different approaches such as the Fisher Linear Discriminant Analysis (LDA), Support Vector Machines (SVM), Artificial Neural Networks (ANN). Classification is either supervised (i.e. examples of classes are provided by the user) or unsupervised (i.e. different classes are generated by the computer).

➤ The Harmony **Calculate Texture Properties** building block offers a selection of three different methods for the first step described above (calculating a set of numerical properties to quantify the texture of an object).

➤ These methods have been carefully selected from many potential methods described in the literature, in order to:

• Cover a large variety of different applications for cell image analysis.

• Include diverse mathematical approaches to allow you to select the most suitable.

• Be suitable for an easy-to-use, interactive, visual set-up and tuning (no need for neuronal networks, etc.).

➤ For the second step – classifying objects based on their texture properties – PhenoLOGIC™ classification is an ideal tool, see section 1.15.
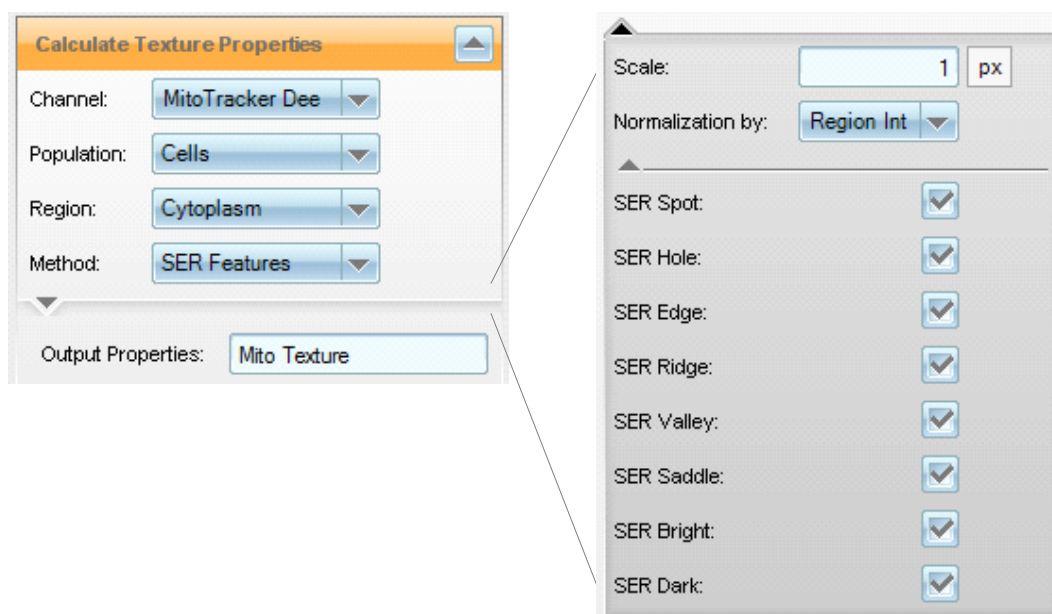
Figure 1–62: A **Calculate Texture Properties** building block with the method "**SER Features**" selected. Left: standard parameters. Right: all of the available input parameters are displayed.

Calculate Texture Properties Building Block

See *Figure 1–62* for a screenshot of the user interface of the **Calculate Texture Properties** building block.

➤ **Calculate Texture Properties** follows the same principles as the **Calculate Intensity Properties** and **Calculate Morphology Properties** building blocks.

➤ For the texture property calculation, e.g. "calculate the **tubulin stain texture** inside the **cytoplasm region** for **all cells**", the required input parameters are a channel, population, and region.

➤ The selection of methods offered are:

- **SER Features**: "Spots, Edges and Ridges", a set of eight properties that are sensitive to different characteristic intensity patterns as described by the property name. These include spots, holes, edges, ridges, valleys, etc. **We recommended this as the first choice for trial and error since this method works well for most applications** (see *Figure 1*–63).

- **Haralick Features**: A set of four features (Correlation, Contrast, Homogeneity and Sum Variance) that are based on the comparison of sets of two pixels in order to identify different textures by their typical intensity co-occurrence patterns. These are a subset of the well known parameters described by Robert Haralick [Haralick *et al.*, 1973] (see *Figure 1–64*).

- **Gabor Features**: Two selected features are generated by Gabor filtering. These properties are based on the analysis of spatial repetitions in the texture pattern. A number of parameters need to be adjusted to specify the characteristic "wavelength" and "scale" of the features to be identified (please refer to the "Harmony Help function" for more details), see *Figure 1–65*.

Image Analysis Guide
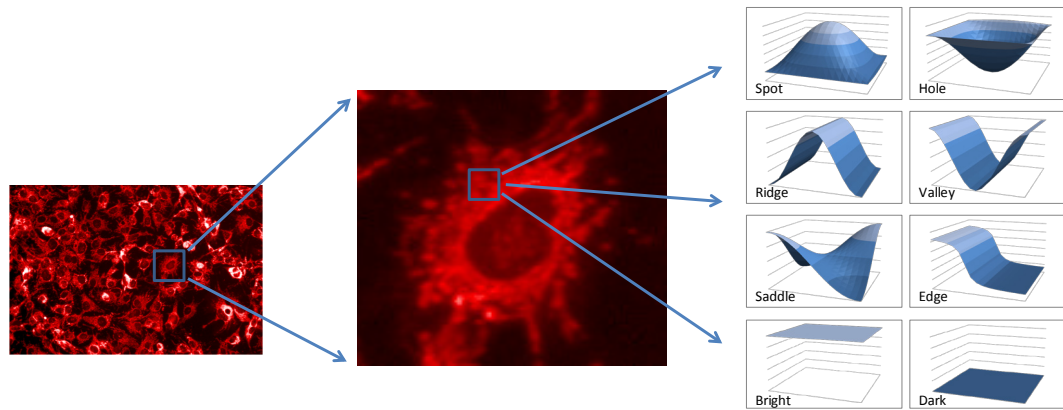
Figure 1–63: The principles of texture analysis using the "SER features" method in the **Calculate Texture Properties** building block. The intensity structure of a region (here: the cytoplasmic region around a nucleus) is analyzed for the occurrence of typical intensity patterns, e.g. "edges", "ridges" or "spots". The biological state is characterized by the frequency of a specific feature or combination of features. Left: Operetta image of HepG2 cells with the mitochondria stained with MitoTracker™ Red. Middle: Enlarged region of the image (marked by a blue box). Right: The set of 8 intensity patterns that can be searched for in the image region.

➤ Depending on the method, one or more optional input parameters are available to fine tune the behavior of the method. See the Harmony Help system for more details.

➤ To choose a suitable texture feature for your application, always start with the default values. Try several features and select the one which best describes the effect that you are measuring. This is the same principle that is used when selecting the best nuclei and cytoplasmic detection methods (by trying different algorithms; A, B, C, etc).

➤ When the best method has been identified, you may want to further tune the optional parameters, e.g. tuning the scale parameter to match the typical length scale of your texture (e.g. the diameter of a spot or hole).

Try using the set of **SER Features** first. If none of these features give sufficient results then consider testing the Haralick or Gabor features (see *Figure 3-51* and *Figure 3.52*).
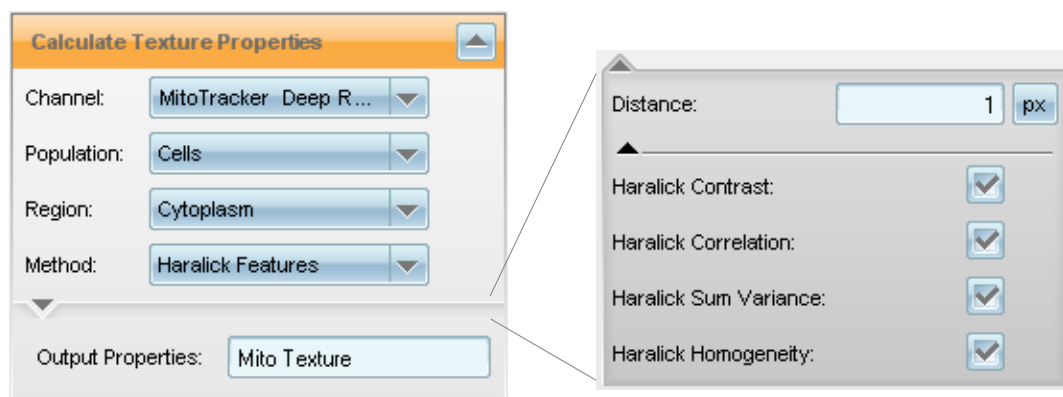
Figure 1–64: **Calculate Texture Properties** building block with the method "**Haralick Feature**" selected. Left: standard parameters. Right: All of the available input parameters are displayed.
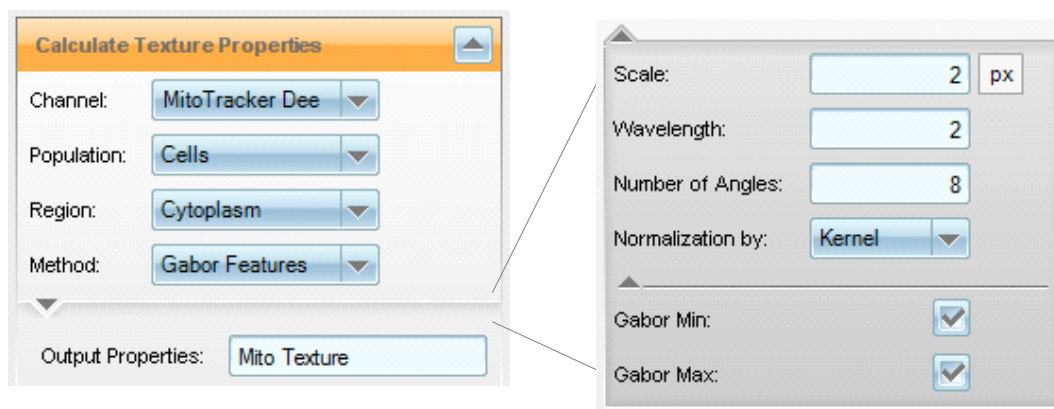
Figure 1–65: **Calculate Texture Properties** building block with the method "**Gabor Features**" selected. Left: Standard parameters. Right: All of the available input parameters are displayed.

Hints and Tips

➤ The texture features are independent of the overall intensity (unless normalization is explicitly disabled). This means that cell-to-cell, plate-to-plate or day-to-day variations in the staining intensity do not influence the results!

➤ Texture features may therefore be more robust than absolute intensity or shape-based features.

➤ Warning: Texture features may be sensitive to image blurring, slightly de-focused cells and / or imaging of thick structures at different heights. In situations prone to these interferences, e.g. due to the assay setup, cell line, etc. carefully check the robustness with respect to the image acquisition height. First, try to select a higher value of "Scale" (in case of SER features) or "Scale" and "Wavelength" (in case of Gabor features) or "Distance" (in case of Haralick features). If that does not work, consider using intensity or shape-based assay readout values instead of, or in addition to, texture features.

➤ For a review of texture analysis from the perspective of a computer scientist, see Tuceryan and Jain, 1998 or Srinivasan and Shobha, 2008 (listed in the references at the end of this section).

1.7.1 SER Feature Details*

This section contains detailed information on the SER features, which is the most frequently used texture parameter set, intended for more advanced, expert users.

Meaning of the Numerical Output Parameters

The SER parameters are calculated by the following steps:

1. The original image is passed through a set of filters, each of which is sensitive to a certain property of the pixel neighborhood. For example, the SER Spot filter is sensitive to spots (see *Figure 1–66* for illustrations).

2. The texture property of a region, e.g. the cytoplasm, is the average intensity of the corresponding filtered image in that region.

The filters are based on gradient and curvature estimates for each pixel (which characterize intensity landscapes in the neighborhood of the pixel). The formulas are undisclosed at the moment.

The SER features are designed to generate robust results for a wide range of different cellular images but the absolute values have no general or easily interpretable meaning. They are meant to be used for relative comparison of values between cells or regions.

It is possible to create artificial images which result in a particular high value of a single SER feature, while the others stay low. On the other hand, there are SER features which never result in a high value on their own. For example, whenever SER Spot is high, SER Bright is also high in the same pixel. In a random pixel of a real image, usually four different SER features are simultaneously increased. For a multi-pixel object in a real image, you will usually get a mixture of all SER features at one time.

Here is an example of an artificial situation resulting in a specific numerical output value (compare this to Figure 1–63, "Ridge" panel): for a pixel on top of a perfect "Ridge" the curvature is equal to zero in one direction ("following the ridge"), while in the perpendicular direction the curvature is negative ("left and right go downhill, when walking along the ridge"). In this perfect case, the SER ridge value corresponds to the negative curvature value.

The absolute SER values have no useful meaning. SER values are meant for relative comparison between cells or regions.

Texture Feature Normalization

If normalization by Kernel is used (default option), the SER values are independent of the image intensity. If normalization is disabled, the SER values scale with the image intensity. See *Figure 1–66* for an example. This option is useful for judging both the presence of a specific intensity pattern (texture) and its intensity. See the Application Note "Image-based Quantification of PKC Activation using Operetta" for an example (listed in the references at the end of this section).
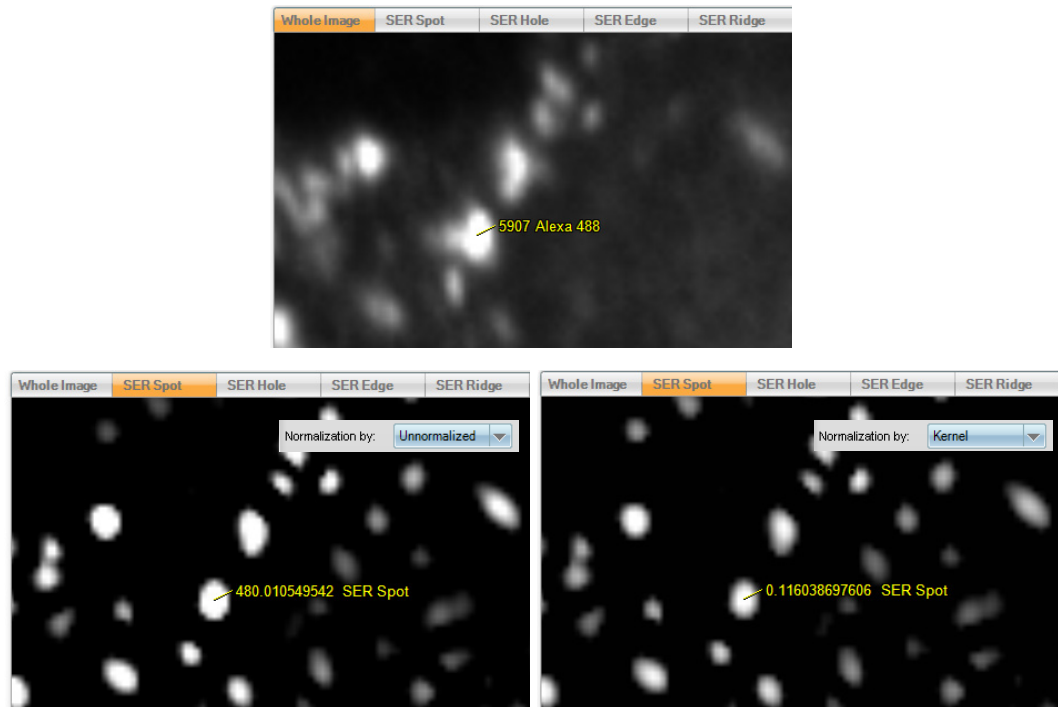
Figure 1–66: Example of SER feature normalization. Top: Original image with the intensity of one pixel shown. Bottom left: SER Spot filtered image (scale = 2). The intensity of the same pixel is shown and normalization is set to "Un-normalized". Bottom Right: The same conditions apply, but normalization is now set to "Kernel".

➤ Normalization is ONLY used to get rid of the intensity dependence of the texture features, NOT to fit the numbers into a fixed range, e.g. 0…1.

➤ The possible range of values is any positive real value but typically much less than one. See ***Figure 1–67*** for an example with a value greater than one.

➤ The average SER values you see in the object properties tables are generally very small because the values are averaged over the full region of interest; many pixels in the area have very low or even zero values for the specific texture feature.
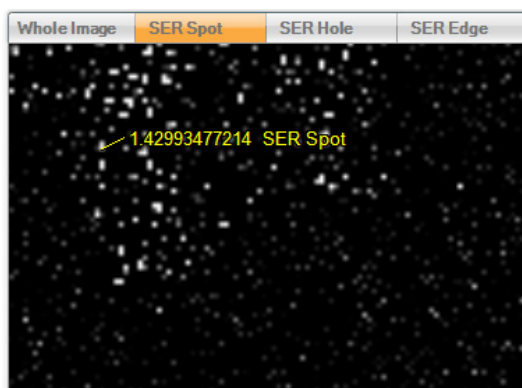
Figure 1–67: Intensity of the SER Spot feature for one image pixel on the **Calculate Texture Properties** building block's illustration "SER Hole". Normalization is set to "Kernel" and the "Scale" parameter is zero. Please note that the texture values are not limited to a range of 0…1, they can be any positive real number although for most applications the values are very low.

➤ Normalized SER values are dimensionless (default setting, recommended).

➤ Un-normalized SER values have the same dimension as the intensity values ("intensity units").

➤ The signal to noise ratio of both values is not affected by re-scaling the image intensity, i.e. multiplying the intensity by a factor e.g. due to a different staining intensity or exposure time.

➤ Normalized texture values are independent of the intensity of the feature being quantified; this is extremely powerful because cell to cell, well to well, batch to batch, etc. intensity variations do NOT influence the texture signal!

Please note that the SER texture features still depend on the image sharpness (because a change in contrast also means changes in intensity gradients and curvature).

➤ Un-normalized texture values are proportional to the intensity of the respective features and can be used to quantify both the presence of a feature and its intensity with one value. This is useful, for example, for cell membrane intensity quantification by quantifying the intensity of ridges: The more ridge-like the structure is (=part of a stained cell membrane) and the brighter the structure is, the higher the readout value is (kind of a "score").

References

Tuceryan, M., Jain, A. K. (1998) "Texture Analysis". In The Handbook of Pattern Recognition and Computer Vision (2nd Edition) by C.H. Chen, L.F. Pau, P.S.P. Wang (eds.), pp. 207-248, World Scientific Publishing Co. (Long review).

Srinivasan, G. N., Shobha, G. (2008) "Statistical Texture Analysis". PWASET 36, pp. 1264-1269. (Short review).

Haralick, R. M., Shanmugam, K., Dinstein, I. (1973) "Texture features for image classification". IEEE Trans. Systems Man Cybern. 3, pp. 610-621. (Original theory, for reference only).

Operetta Application Note "Texture Analysis of the Plasma Membrane", available from the PerkinElmer website: http://www.perkinelmer.com/lab-products-and-services/cellular-imaging/hcs-resources.html.

1.8   Morphology Analysis

The Harmony morphology analysis allows various aspects of the form and structure of objects to be quantified, e.g. elongation, symmetry, smoothness of the border and spatial distribution of intensity or texture features (like spots) inside the object.

The morphology properties can be used for:

➤   Classification of objects (e.g. using the PhenoLOGIC linear classifier)

➤   As a readout value quantifying specific biological changes

Requirements

➤   A population of objects must have been identified, e.g. by Find Nuclei.

➤   Optionally, an inner region inside the objects can be selected, e.g. the nucleus inside a cell.

When to use it?

➤   When intensity measurements of specific fluorescent markers in different regions are not sufficient to measure the biological effect.

➤   If counting objects or measuring the object sizes is not sufficient.

➤   In cases where the form and structure of objects should be used to classify them, see *Figure 1–68* to *Figure 1–73* for examples.

➤   When the location of texture areas inside an object are important to identify an effect.

➤   Note that morphology properties only take individual objects into account. If the relationship of objects to their neighbors (e.g. number of neighbors or properties of the neighborhood) is of interest, morphology properties will not help.
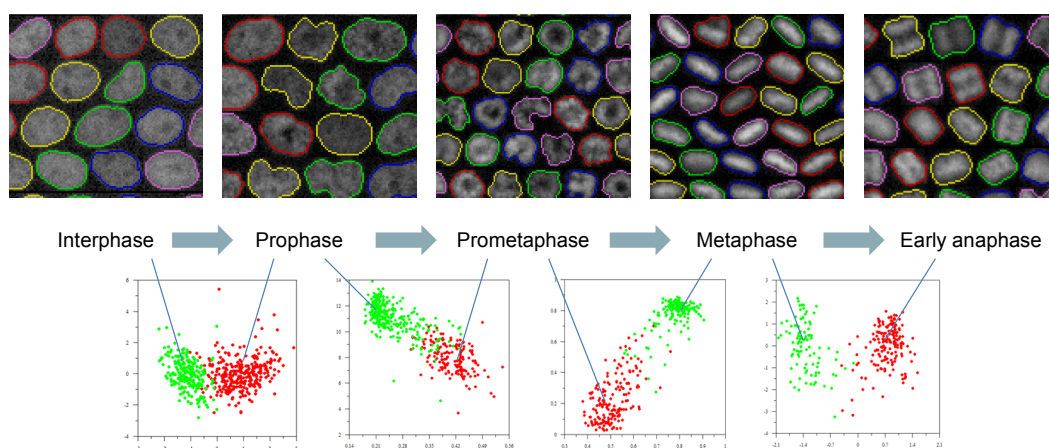
Figure 1–68: Nuclear Morphology: Cell cycle analysis using nuclear morphology properties. Top row: Montage of nuclear stain images of various nuclei in five different consecutive stages of the cell cycle. Image data and staging from Held et al. (Nat Methods. 2010 Sep; 7(9):747-54). The nuclear outlines identified by the **Find Nuclei** building block are marked by colored lines. Bottom row: Example classification results of the separation of a pair of two neighboring stages of the cell cycle. The scatter plot shows the linear combination identified by the PhenoLOGIC linear classifier. Each dot represents one nucleus, the color indicates the class. All stages can be well distinguished. The extended set of STAR morphology properties has been used, see "Hints and Tips" section.
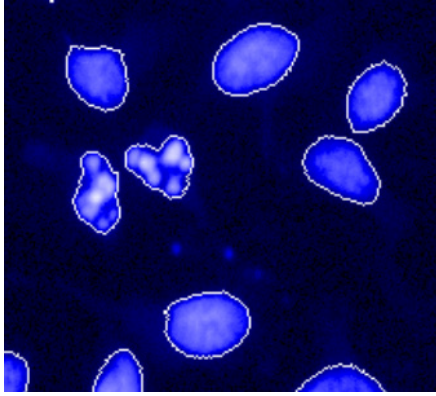
Figure 1–69: Nuclear Morphology: Nuclear stain image with segmentation results of the **Find Nuclei** building block overlaid (white line). Two nuclei are fragmented. This can be very well classified by morphology properties, especially "Threshold Compactness".
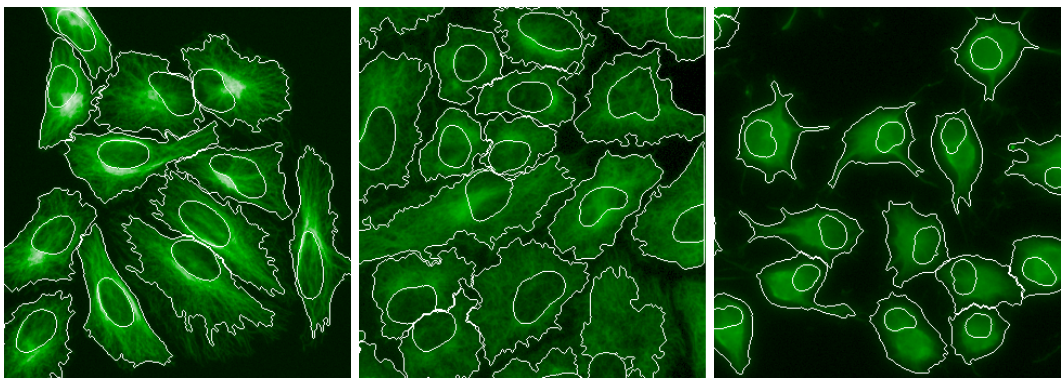
Figure 1–70: Morphology of tubulin distribution in cells. Left: Untreated cells, Middle: Demecolcine treated cells, Right: Cytochalasin treated cells. The segmentation results of the **Find Nuclei** and **Find Cytoplasm** building blocks are marked by a white line. The different phenotypes can be well separated using the STAR morphology properties, preferably the extended set including texture filtered images, see "Hints and Tips".
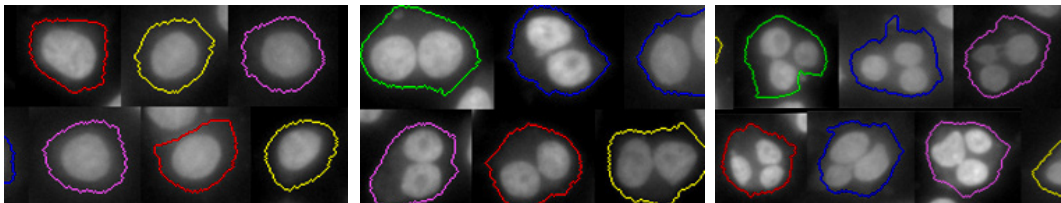
Figure 1–71: Morphology of DNA distribution inside a cell: Segmentation results (colored lines) of the **Find Cytoplasm** building block overlaid on nuclear stain images. The left panel shows mononucleated cells, the middle panel binucleated cells and the right panel cells with three nuclei (potentially due to segmentation errors). The morphology properties ("Symmetry") can be used to reliably discriminate the different classes. Since this approach does not rely on nuclei segmentation and counting, it is robust against nuclei segmentation errors.
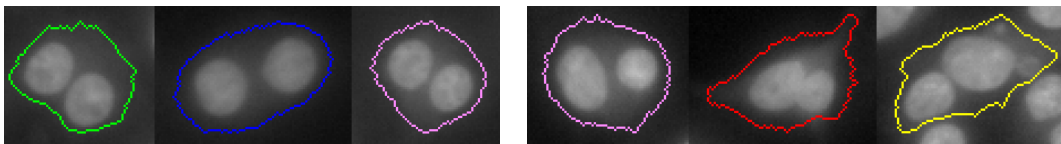
Figure 1–72: Morphology of the DNA intensity in detected regions. Left panel: Symmetric. Right panel: Asymmetric. Morphology properties can be used to easily and reliably discriminate these classes.

| Untreated | Activated cell type 1 | Activated cell type 2 |
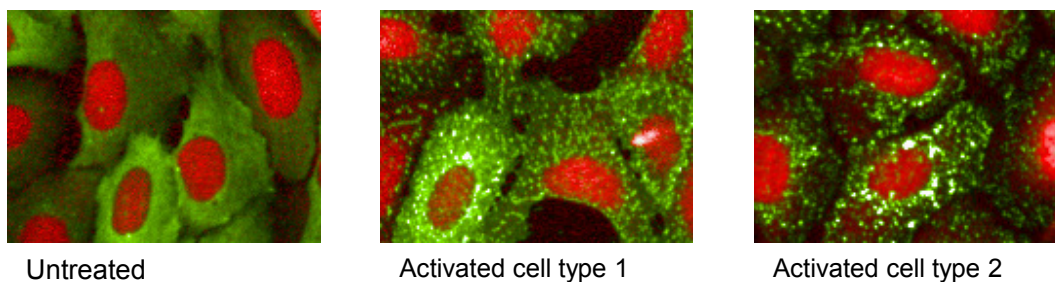
Figure 1–73: Morphology analysis of the redistribution of a fluorescently labeled protein (green channel) from a homogeneous distribution (left panel) to pits and vesicles. Red channel: DRAQ5 ™ nuclear stain. The combined morphology and texture change can be used to very robustly detect the phenotype.

1.8.1   Calculate Morphology Properties Building Block

The morphology properties are calculated by the **Calculate Morphology Properties** building block. Two methods are available:

- ➤ Method "Standard" for area, roundness and width/length.
- ➤ Method "STAR" for a comprehensive set of properties for phenotype classification.

Standard Method

The **standard** properties have an easily understandable meaning, like "length" or "width", and are usually used to directly measure the desired biological change. See e.g. the ready-made solution "Nuclear Analysis – Nuclear Shrinking" (section 2.4), *Figure 2–6* (page 185) gives an overview of the standard properties for different shapes. Another example is the ready-made solution "Cell Shape – Cell Rounding" (section 2.15).

STAR Method

The **STAR** morphology properties comprise of a special set of properties designed to precisely describe as many common cellular phenotypes as possible. Many of the properties are abstract mathematical values and do not have an easily understandable meaning – very much like some of the texture properties described in Section 1.7.

The STAR properties can be used in different ways:

- ➤ Calculate the default STAR property set and use PhenoLOGIC™ to classify the cells or other objects of interest. See section 1.8.2.
- ➤ Calculate STAR properties, report them as well results and select the one with the best Z' as your assay readout on the Harmony **Evaluation Screen**. See section 1.8.3.
- ➤ Calculate STAR properties, report them as **single cell results** and use TIBCO® Spotfire or other third party software to further classify or analyze the individual cells or objects. See section 1.5.4 to see how to access the single cell results.
- ➤ For experts: Interactively identify one or more STAR properties that measure the effect of interest and use it in further calculations or as assay readout. See section 1.8.4.

In the first three scenarios, the choice of the best STAR property (or properties) is made by examining the results. It is not necessary to understand the meaning of the individual properties or adjust tuning parameters.

In most cases, it is sufficient to just select the method STAR in the Calculate Morphology Properties building block (without further changes or tuning) and to add a **Select Population** building block with method "Linear Classifier". See the following example (section 1.8.2).

1.8.2　STAR Morphology with PhenoLOGIC

The simplest way of using the STAR morphology properties is with PhenoLOGIC™ machine learning. For an example, see ***Figure 1–74***. The aim in this example is to find micro patterns on a slide that are populated by exactly one cell.
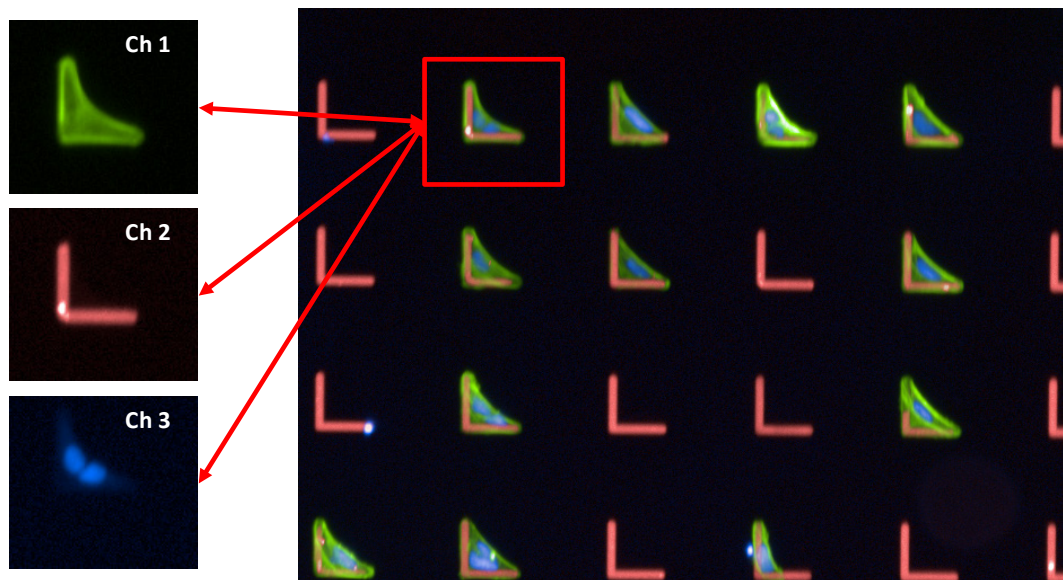
Figure 1–74: Cells growing on a pattern of L-shaped microstructures printed on a slide (Cytoo). Right side: color montage of a 10x fluorescence image. Red channel: microstructures, blue: DNA, green: cytoskeleton. Left side: for one microstructure, the three image channels (red, green, blue) are shown separately. The blue DNA stain shows two cells growing on this structure. Most of the structures only contain one cell.

Step 1

➤ Prepare a population of the objects you want to classify by morphology properties.
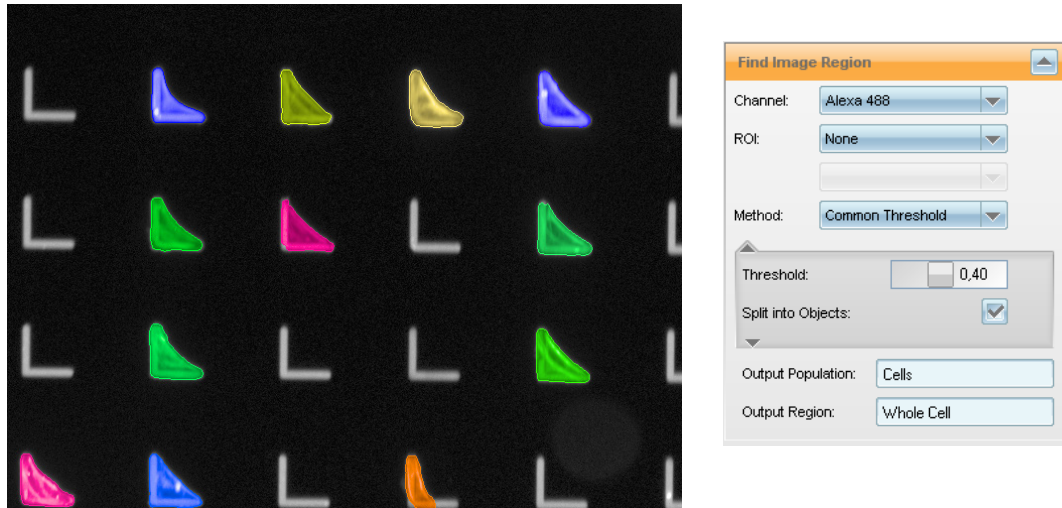
Figure 1–75: The **Find Image Region** building block (right panel) has been used on the Cytoskeleton stain channel to identify the regions covered by cells. The resulting population has been named "Cells". Each object of the population is marked by a different color region overlaid on the cytoskeleton image (left panel).

Step 2

➤ Add a **Calculate Morphology Properties** building block and select method STAR.

➤ Specify the population and the region that defines the shape of the objects.

➤ Select a channel that specifies the intensity information for weighting the shape, e.g. when determining the symmetry.
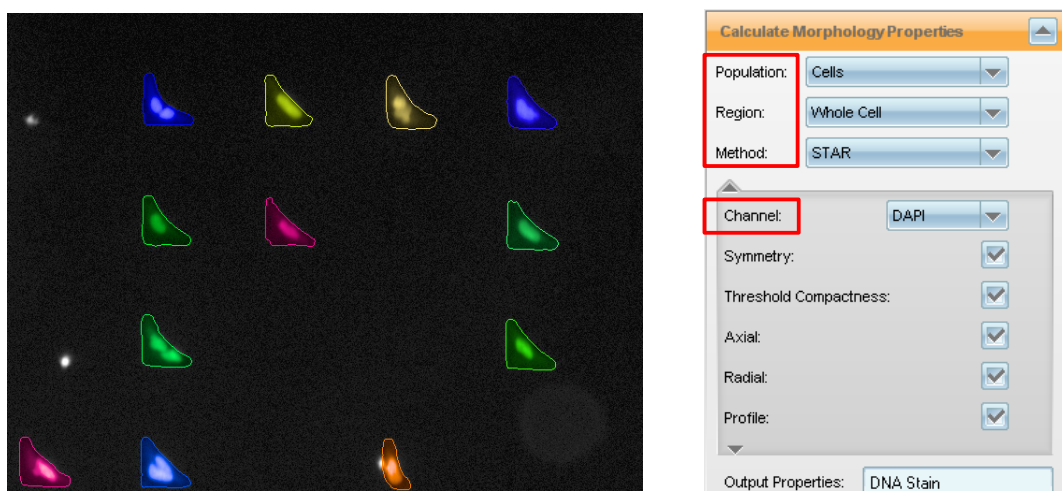
Figure 1–76: Calculation of STAR properties for use in following building blocks in order to identify microstructures containing exactly one cell. Right panel: Calculate Morphology Properties building block with parameters set. Parameters that need to be set are marked with a red box.

Step 3

➤ Add a **Select Population** building block, select your population of interest and method "Linear Classifier".

➤ Select the number of classes you want to identify and provide appropriate names.

➤ Start the training by adding example objects for all classes (training points).

All previously calculated object properties – including the set of STAR morphology properties – are taken into account for classification. The most appropriate properties are selected. You do not need to pay attention to the properties used.

Figure 1–77: Using Select Population, method Linear Classifier to select the microstructures with one and with multiple cells. Right panel: Select Population building block. Left panel: Some selected training points shown as an overlay on the DNA stain image. Green: Population A training points (one cell). Red: Population B training points (multiple cells).

Result

The analysis sequence should be validated with a larger set of images. It can then be used for automated analysis of any number of images (see *Figure 1–78*). The individual properties used for the classification can be viewed in the **Select Population** building block for information, documentation or to check if properties have been included which are known to cause artifacts, e.g. absolute intensity values. See section 1.15 for more details on PhenoLOGIC classification.
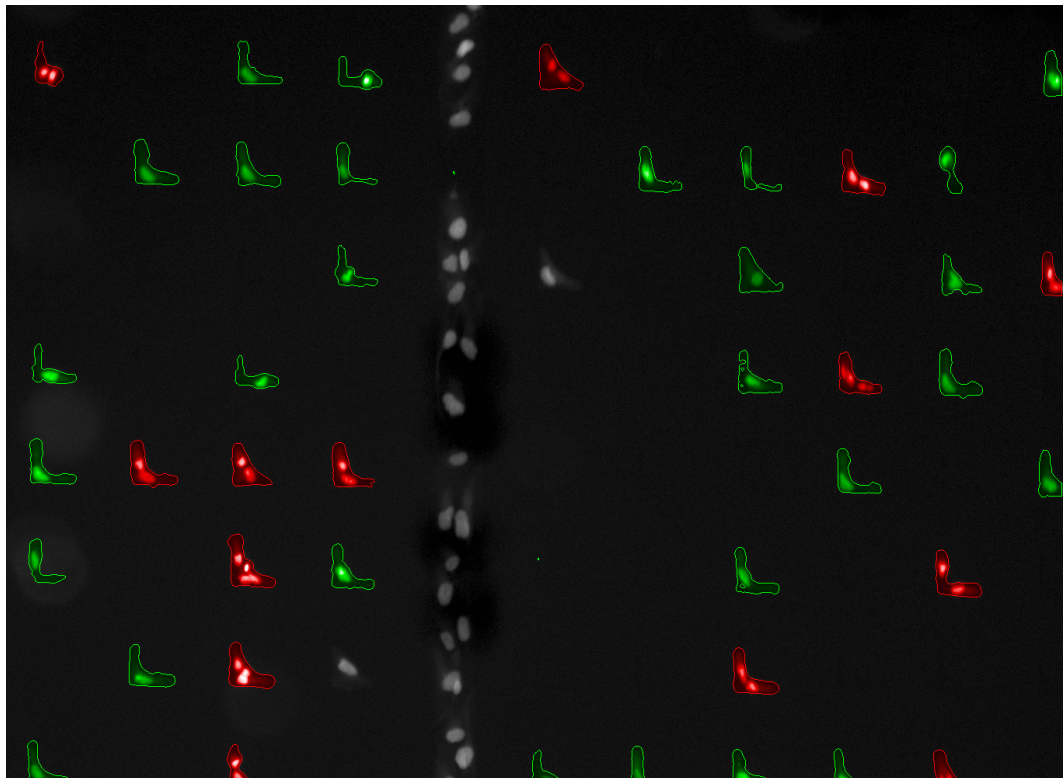
Figure 1–78: Analysis sequence from the trained **Select Population** building block applied to a larger part of the slide. Detection and classification results are shown as an overlay on the DNA stain image. Red: "multiple cells" class, green: "one cell" class.

Hints and Tips

➤ Enlarge the set of STAR morphology properties to include texture filtered and background corrected images as well, see ***Figure 1–79*** for a recommended choice. Benefit: potentially better separation of classes, works for multiple applications. Downside: longer calculation times, property tables get very crowded.
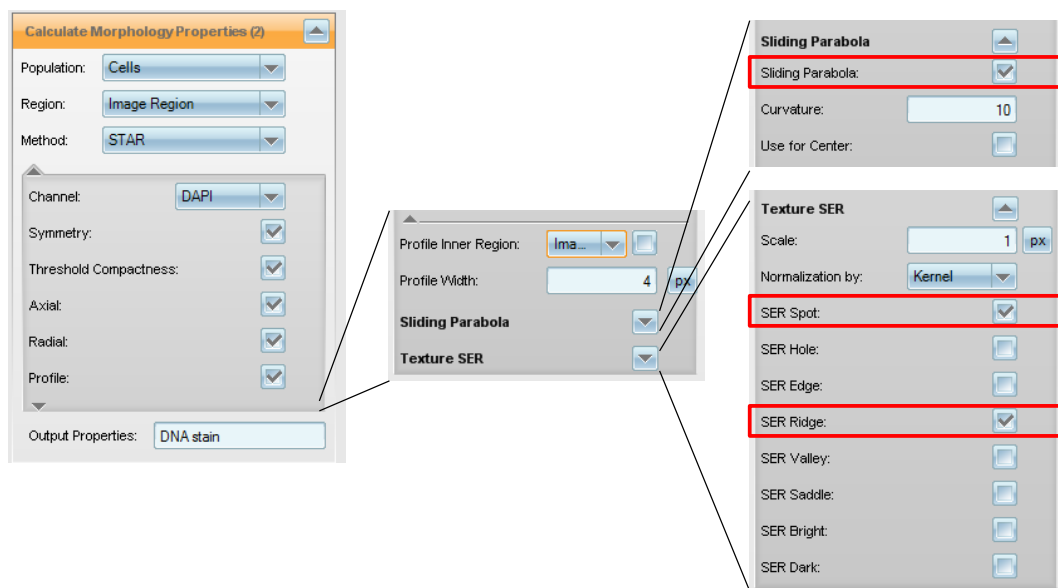
Figure 1–79: Select three checkboxes to get the larger set of STAR morphology properties as recommended. The checkboxes are accessible by opening two levels of optional parameters.

1.8.3   STAR with Z'

Step 1

Identify the objects you want to classify.

Step 2

Calculate a set of morphology properties using the STAR method with default settings. If the distribution of texture features inside the objects might be important for identifying the biological effect of interest, use the extended set including the filtered images. See *Figure 1–79*.

Step 3

Select all calculated STAR morphology features in the **Define Results** building block. Use method "List of Outputs". Choose the relevant population(s), e.g. the final list of cells that passed filtering for artifacts. Select an appropriate statistical operation, in most cases the mean value over all cells (objects).

Step 4

Check your analysis sequence with several images of positive (treated to show an effect) and negative (untreated cells) control images. Save the analysis sequence and run the analysis on a set of positive and negative control wells using the Harmony "Evaluation" screen (see *Figure 1–42*). We recommend at least 8 wells per control, more wells will give better results.

Step 5

Create a Z' plot of your control wells (see section 1.5.1). The "Z' Summary" table shows the Z' values for all readout values specified as a well result in the analysis sequence, i.e. all STAR properties. Select the property which gives the best Z' value for your assay and carefully review the results for artifacts and consistency with your expectations.

Optionally

➤   If there are several properties with similarly good Z' values, consider what you know about the biology to determine which property best matches the biological effect you are looking for (e.g. symmetry change).

➤   Validate the analysis against a dose-response curve (see section 1.5.2).

➤   Remove properties from the analysis sequence that you don't need in order to:

- Clean up and make it more readable.

- Speed up the analysis. Note that some texture properties have dependencies – it may be necessary to remove many (or all) texture properties to get a faster calculation time.

1.8.4   Some Details*

Here are some very basic facts that might be of interest as background knowledge. For more detailed information (including formulas), see Harmony Help.

The STAR properties comprise of five groups, the initials of the first four of them form the word S-T-A-R.

Symmetry – Charaterize the symmetry of the intensity distribution

A set of eight properties describe the symmetry of the intensity distribution inside the object. The property name, e.g. "Symmetry 02", includes two numbers. The first number describes the intensity decay in the radial direction (0 or 1), and the second indicates the number of repetitions in the circular direction (2 to 5). Here is an example of different symmetries that can be characterized:
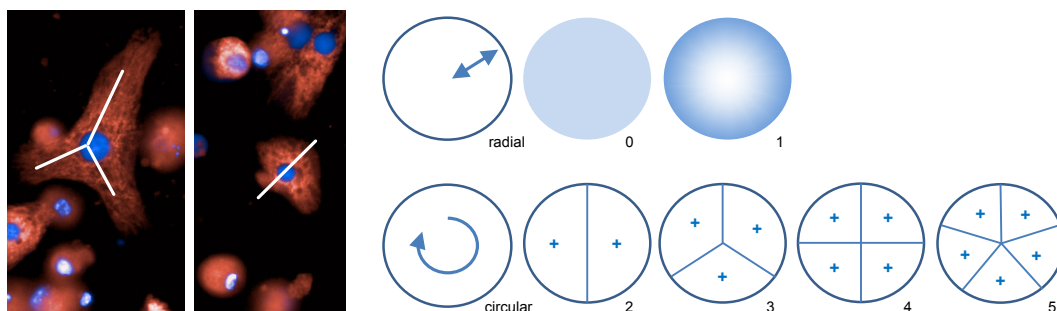
Threshold Compactness – Charaterize the compactness of bright regions

A set of four properties describe how "compact" the brightest features inside the object are. The name, e.g. "Threshold Compactness 40 %", indicates that only the regions with more than 40 % of the maximum object brightness are used. If those regions fragment into many parts with irregular borders then they have a low compactness. Here is an example of object regions with increasing compactness:
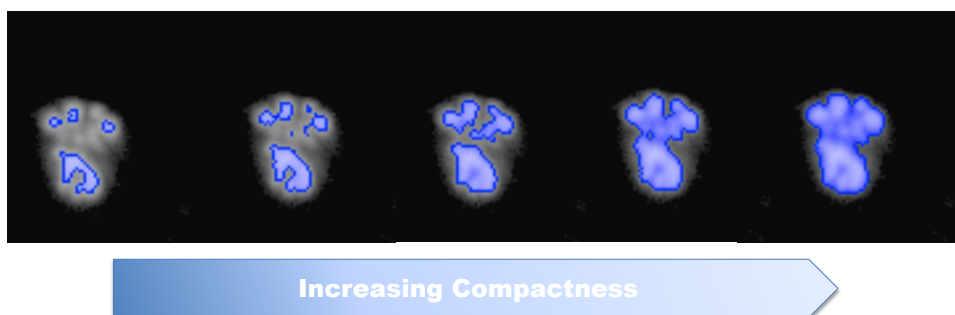
Axial – Charaterize the axis ratio

Two properties quantify the length and length ratio of the two principal axes of the object. Here is an example of principal axes with different axis length ratios:
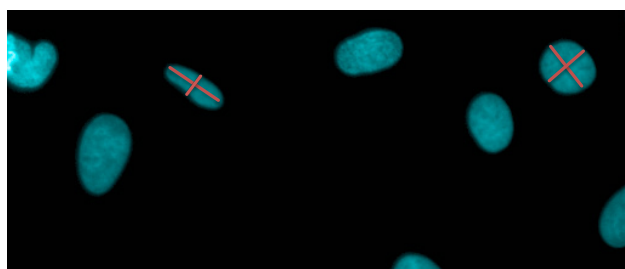
Radial – Characterize the distance of bright regions from the mass center

Three properties characterize the intensity distribution in the radial direction (yellow arrows in the sketch below). The first is a mean object radius based on the intensity values in the object weighted by mass center distance. High intensity far away from the mass center gives high values. A second value reflects the homogeneity (variation) of the intensity in radial direction.
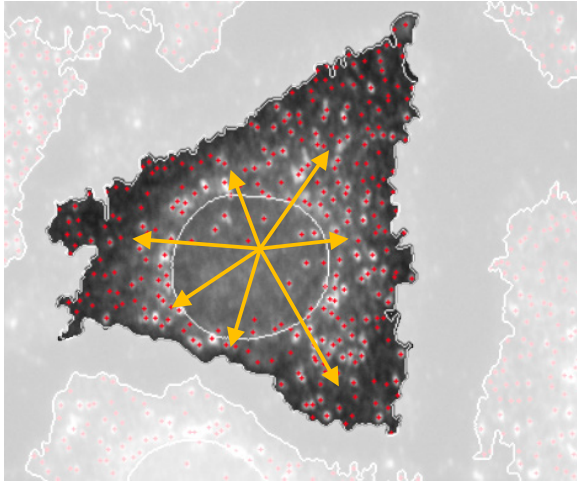
If morphology properties are also calculated on filtered images (see section "Filtered Images" on the next page), the ratio of mean radius in the filtered and original image is reported as a third property output measuring the effect of the corresponding filter (e.g. spot filter) on the radial intensity distribution.

Profile – Characterize the location of the intensity in regions

Five properties determine the location of the fluorescence intensity with respect to five different pre-defined regions in the cell (or other object). The region borders are not sharp like the regions defined by the **Select (Cell) Region** building block, but smoothly transition from a weight of 1 inside the region to 0 outside the region (see *Figure 1–80*).
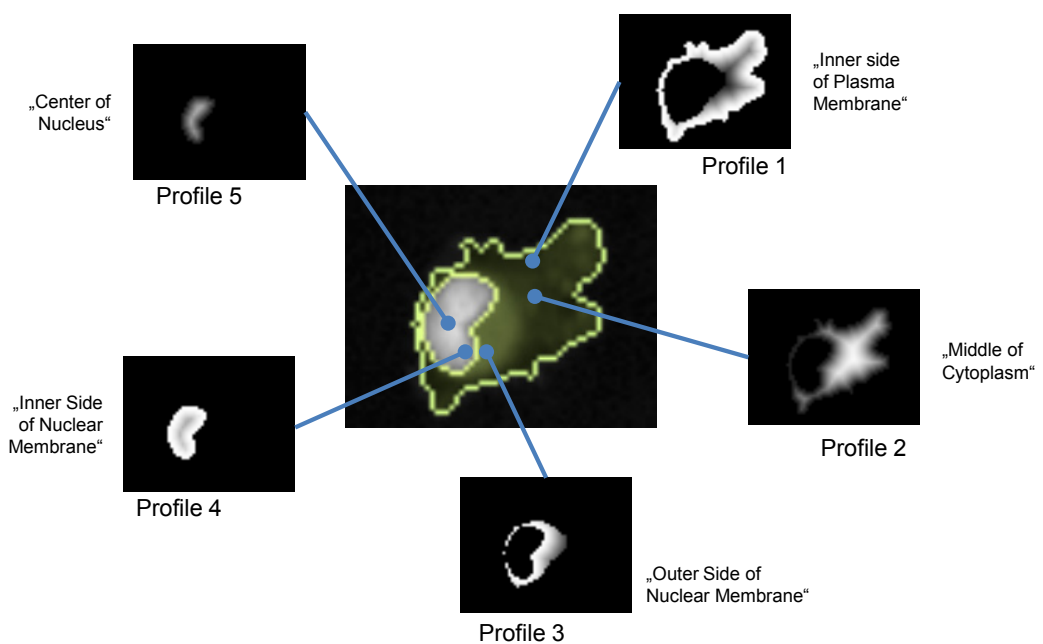
Figure 1–80: Regions defined by the STAR method's "Profile" properties. The color indicates how the intensity is weighted. White indicates a high weight of the intensity, black indicates a weight of zero.

If no "inner region" inside the object is defined, only profiles 1 and 2 are available.

The profile width can be adjusted by a tuning parameter to adapt it to the region that is known to be most significant for a specific application. See ***Figure 1–81***.

| 1px | 2px | 3px |

Figure 1–81: Adjustment of the STAR method's "Profile" regions. The illustrations show an example of the Profile 1 region for three different settings of the "Profile Width" parameter. If the effect of interest is, for example, known to be confined to a region very close to the plasma membrane, a setting of 1px could be used (left panel).

Filtered images

In addition, each of these properties can also be calculated on texture-filtered images instead of the original image, see ***Figure 1–82***. In this case the morphology properties of the structures selected by the filter (e.g. spots or ridges) are calculated.

This is very useful when a combination of texture and morphology changes best characterizes the biological effect or phenotype.
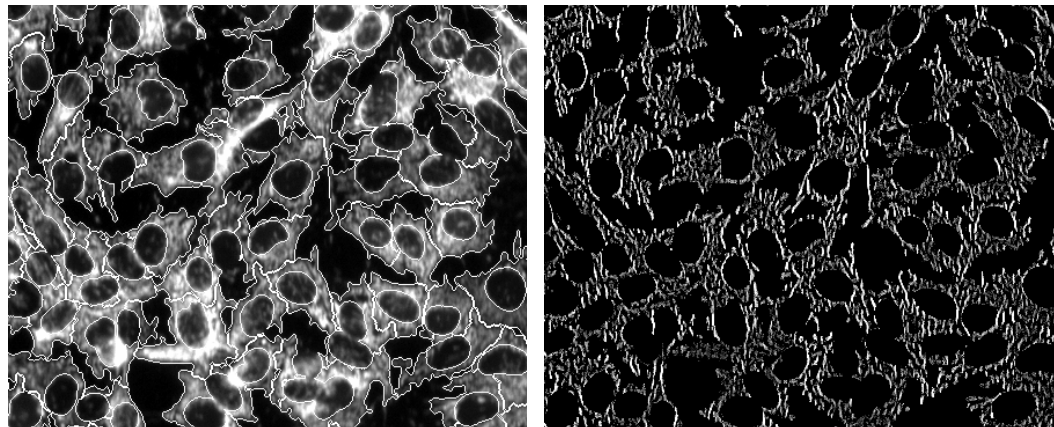
Figure 1–82: Example of a texture filtered image. Left: MitoTracker® Deep Red stained image with overlaid outlines of detected cells (white lines). Right: Same image with SER Ridge (scale 1px) filter applied. The filtered images can be inspected in the corresponding illustration panels of the **Calculate Texture Properties** building block.

To use texture-filtered images, select the desired filters in the building block options, see ***Figure 1–79***. A recommended good choice to start with is "SER Spot" and "SER Ridge".

The filter used is appended to the output property name, e.g. "Radial Mean SER Spot 1px" is the mean object radius calculated on the spot filtered image (with texture scale value set to 1 pixel).

In addition to the texture filter, a background intensity subtraction ("Sliding Parabola" algorithm) filter can also be used, see ***Figure 1–79***, top right panel.

Advanced Hints and Tips

➤ To adjust the "Scale" parameter for the texture filtered images, you can use the illustrations provided by the **Calculate Texture Properties** or **Filter Image** building blocks. The texture-filtered images used by these building blocks are identical.

➤ You can use the morphology properties to characterize the arrangement of a population of spots that has been identified inside a cell using the **Find Spots** building block. Select the cell population in the Calculate Morphology Properties building block and choose the "Spots" or "Spot Maxima" region. See *Figure 1–83* for an example.
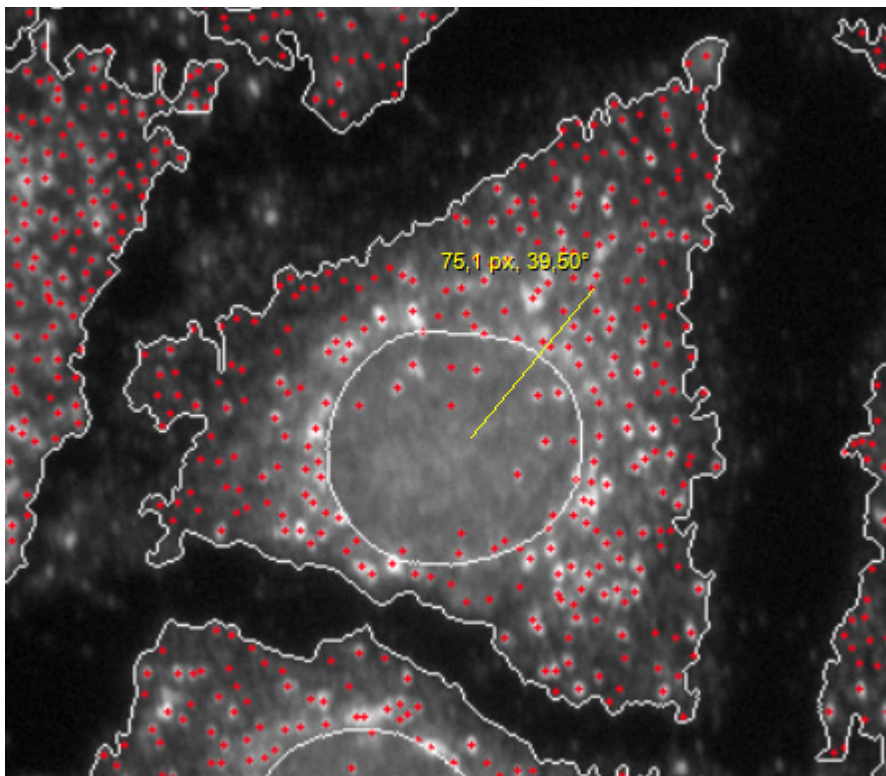
Figure 1–83: Example screenshot illustrating the analysis of the distribution of spots in a cell. The illustration shows a peroxisome stain image. The intensity maxima identified by the **Find Spots** building block are overlaid (red color). The identified "Spot Maxima" region has been resized by +1 pixel using a **Select Region** building block to make it more visible in the screenshot. The calculation of the STAR "Radial Mean" value on this region gives a value of 75 pixels. The "Measure Distance / Angle" tool in the Harmony image view has been set to display a length of approximately 75 pixels on the image (yellow overlay).

1.9   Neurite Outgrowth Analysis

The detection of neurites is an additional step in the general image analysis sequence described in section 1.3 (see *Figure 1–17*). Please refer to that section for a general description of how to set-up an image analysis sequence. The additions are described in the following section.

➤ The first step of any neurite analysis is the detection of the cell bodies (see *Figure 1–84*, middle panel).

➤ Starting from the individual cell bodies, the neurites are then detected using the **Find Neurites** building block (see *Figure 1–84*, right panel).

➤ Once the neurites have been identified, they can be further processed and quantified as needed in the subsequent steps of the analysis sequence.

➤ For an example of a ready-made analysis sequence, see the RMS "Neurite Outgrowth Analysis" in section 2.21.

➤ An example dataset containing neurite images for training and reference purposes is pre-installed in your Harmony database (see example dataset 8: "P011-N: Neurite Outgrowth", page 318).
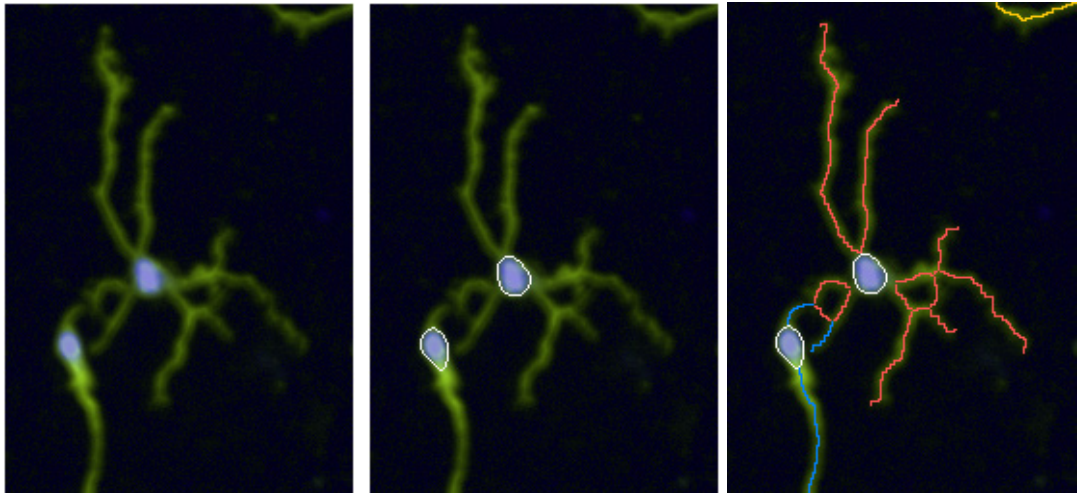
Figure 1–84: Example of neurite detection. Left: The input images are a nuclear stained image (blue) and a neurite stained image (green), shown as a color overlay. Middle: Results of the nuclei detection by the **Find Nuclei** building block (the white overlay shows the detected nuclear outlines). Right: Results of the neurite detection by the **Find Neurites** building block. The color overlay shows the two detected neurite trees (blue and red) assigned to the corresponding cell bodies. The yellow marked neurite belongs to a cell body outside the shown image area.

Find Neurites Building Block

➤ The **Find Neurites** building block is tailored to detect neurites. It provides a set of interactive illustrations and tuning dialogues to visualize the detection results and adjust the parameters if needed (see Figure 1–85).

➤ A previously detected population of cell bodies and a region (whole or part of the cell body) has to be selected first. All detected neurites are assigned to one of these objects, adding new properties and overlays to that population.

➤ The building block uses an algorithm developed by the Australian CSIRO research institute (http://www.csiro.au). The same algorithm is also available in Opera and Acapella, so analyses can be transferred between these systems.

➤ There is only one method available, "CSIRO Neurite Analysis 2". It is based on ridge tracking for neurite detection.

➤ The outdated method "CSIRO Neurite Analysis" (based on intensity thresholding of neurites) is not suported any more. It was provided for backward compatibility on 32 bit Windows systems and now has to be replaced by the new method.

➤ The neurite detection can be adapted to various assay set-ups (cell lines, staining, imaging conditions) by a number of tuning parameters. Please refer to the Harmony Help System for a detailed step-by-step description of the parameters and how to tune them.
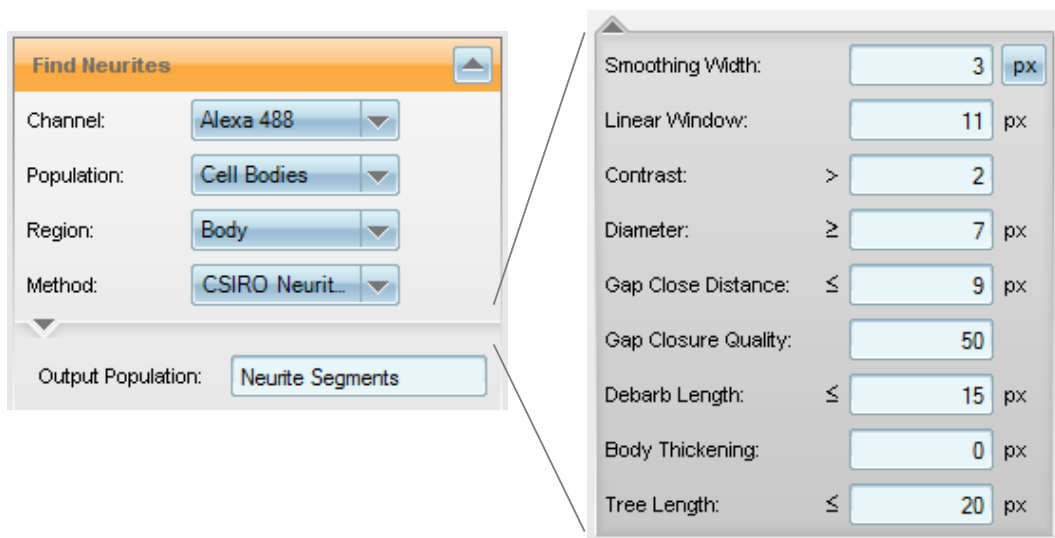
Figure 1–85: The **Find Neurites** building block. Left: Standard parameters. Right: All of the available input parameters are displayed. See the Harmony Help System for a detailed description and hints on how to adjust the parameters if needed.

Find Neurites Building Block Results

➤ **Find Neurites** adds a new region called "Neurite Tree" to each cell body in the input population (see *Figure 1–84*, right image). It can be displayed as an overlay in the image view and used as an input region for further analysis.

➤ A new population called "Neurite Segments" is created (the population name can be changed by the building block parameter "Output Population"). It contains all neurite segments found in the image (see *Figure 1–86*, left panel). Individual neurite segments can be further analyzed by successive building blocks.

➤ A set of numerical properties characterizing each cell's neurite tree is calculated and added to the cell's property table, see **Figure 1–86** and **Figure 1–87** for more details. Calculated properties are:

- Length of the longest neurite

- Total number of extremities (end points of neurites)

- Number of root points (starting points of neurites)

- Number of segments in the neurite tree

- Number of nodes type 1 (branching points)

- Number of nodes type 2 (number of segments / number of roots)

- Total length of all neurites in the tree

➤ Neurite segments which are not connected to one of the cell's neurite trees ("orphan neurites") are only listed in the "Neurite Segments" population and have their property "Parent Cell ID" set to zero. They are not taken into account in any of the default parameters.
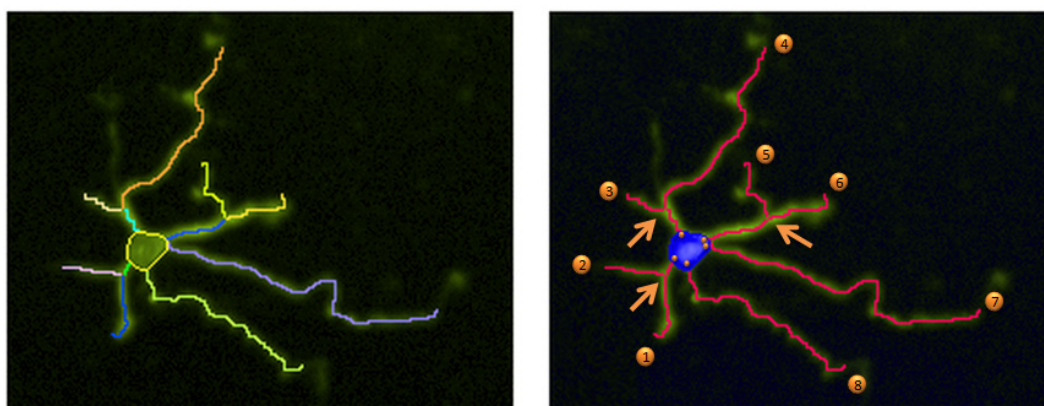
Figure 1–86: The meaning of the numerical properties that are calculated by the **Find Neurites** building block. Left: Break-down of the detected neurite tree into individual segments (marked by different colors), defining the property "Number of Segments". Right: Illustration of the following properties; "Number of Extremities" (which corresponds to the number of ends of individual neuritis), marked by numbered orange circles, "Number of Roots", marked by small orange circles inside the cell body, and "Number of Nodes type 1", marked by arrows.

Summary	Properties Cell Bodies							
Object No	Cell ID	Maximum Neurite Length	Number of Extremities	Number of Roots	Number of Segments	Number of Nodes type 1	Number of Nodes type 2	Total Neurite Length
1	1	38,7095	2	2	2	0	0	70
2	2	66,2308	3	2	4	1	1	109
3	3	27,8652	2	2	2	0	0	50
4	4	67,7942	3	2	4	1	1	114
5	5	39,8254	1	1	1	0	0	40
6	6	51,8494	2	2	2	0	0	79
7	7	102,82	1	1	1	0	0	103
8	8	21,7286	1	1	1	0	0	22

Figure 1–87: Numerical properties added to the "Properties Cell Bodies" population by the **Find Neurites** building block. The parameters can be used to characterize the topology of the neurite tree.

The Neurite Segments Population

The "Neurite Segments" population contains a list of all neurite segments that were found in the well, including orphan neurites that are not associated to any neurite tree (see **Figure 1–88**).

For each neurite a set of default properties is provided to allow filtering for specific neurite segments, e.g. only segments that are in the middle of a neurite tree.

The best place to view these properties is the "Properties Neurite Segments" table, which is shown when the "Find Neurite" building block's "Neurite Segments" illustration tab is selected (see bottom part of *Figure 1–88*).

Figure 1–88: "Neurite Segments" population with associated table of neurite segment properties.

Description of Neurite Segment Properties

Segment ID – unique identifier for the segment. Note that the Object No. changes when neurites are filtered out from the list.

Branch Level – indicates if the segment branched from another neurite. If the segment is attached directly to the cell body, the branch level is one. If it is orphan, the branch level is zero. If it branches from a neurite with level x, it is x+1.

Parent Cell ID – unique identifier of the cell in the population of cell bodies to which the segment is attached.

Parent Segment ID – the segment unique identifier from which the current segment branches. For segments that are attached to the cell body or orphan, the value will be zero.

Segment Length – in pixels. The length may be non-integer for neurites in diagonal directions.

Number of Children – number of attached neurite segments. The red segment has two children in the following example:
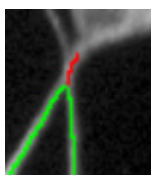
Is Extremity – has a value of one if the segment is at the outer end of a branch of the tree, otherwise it will be zero.

Length to Cells – is the distance of the shortest path from the outer end of the neurite segment to the cell body. It is equal to the segment length for segments directly attached to the cell body.

Is Inner Segment – has a value of one if the segment is neither attached to the cell body nor an "Extremity".

Neurite Tree No. – All segments that form a neurite tree have the same neurite tree number. Since not all cell bodies have neurite trees attached, this number may be different from the parent cell ID of the neurite segment. For orphan segments, the value will be zero.

> Hint: Sorting the table by **Parent Cell ID** or **Neurite Tree No.** and highlighting all segments with the same number, helps to show the association of the segments to cells or neurite trees.

Hints and Tips

➤ The "Neurite Tree" region can be used to calculate further numerical properties using the **Calculate Morphology Properties**, **Calculate Intensity Properties** or **Calculate Texture Properties** building blocks.

➤ The neurite region can be further modified, e.g. enlarged to include neighboring regions, using the **Select Region** building block. See the RMS "Neurite Outgrowth Analysis" (section 2.21) for an example.

➤ The (modified) neurite region can be used to quantify co-localized markers in other channels.

➤ The (modified) neurite region can be used in the **Find Spots** building block to find spots on neurites (e.g. synaptic vesicles) that are labeled with specific markers in a different channel.

➤ The "Neurite Segments" population can be further processed, e.g. filtered by their properties, using the **Select Population** building block. For example, orphan neurites can be found by filtering for "Parent Cell ID" that are equal to zero. Segments directly connected to the cell can be found by filtering for "Branch Level" that are equal to one.

➤ The "Neurite Segments" population is a related population of the "Cell Bodies" population. You can use the "By Related Population" method of the **Calculate Properties** building block to calculate neurite segment statistics for the cell bodies. See section 1.11.4.

➤ The **Find Neurites** building block is not limited to neurites. It can also be used to identify various dendritic structures starting from a (user-defined) selectable region.

1.10   Regions of Interest

By default, all image segmentation building blocks use the whole image for analysis. Optionally, a list of "Regions of Interest" (ROIs) in the image can be specified to restrict the analysis to specific areas. In this case:

➤   The analysis is done in each ROI separately. Results can be summarized and reported for each ROI individually.

➤   Parts of the image which are outside any ROI are ignored and have no influence on the results.

Typical applications:

➤   Restrict the analysis to a part of the image to avoid interference by unwanted regions, e.g. well borders or artifacts visible in the image. See example in section 1.10.1 below.

➤   Analyze multiple regions in the same image and report results per region, e.g. analyze individual colonies separately. See example in section 1.10.2 below.

➤   Analysis of spots in cells or other objects. See example section 1.10.3 below.

➤   Detailed analysis of sub-objects inside or attached to other objects, e.g. neurites attached to a cell body, nuclei in polynucleated cells and lipid droplets inside a cell. See section 1.11 for a detailed discussion of this type of analysis.

Regions of interest may be nested, e.g. Colony → Cell → Spot. Object relations are automatically tracked and preserved (inherited). All objects are tagged so that the software automatically "remembers" which parent object the objects belong to. When calculating the statistical properties of an object, you can refer to all of its sub-object properties and the parent object properties, e.g. to report the number of spots of a specific type in a cell.

1.10.1   Example: Excluding Well Borders

See *Figure 1–89*. The aim is to identify microtissue bodies in brightfield images using texture segmentation. In each image, a different fraction of the well border is visible which disturbs texture segmentation (well border and empty parts of the well have a very similar, smooth texture). For robust tissue detection, the texture analysis should be restricted to the well interior.
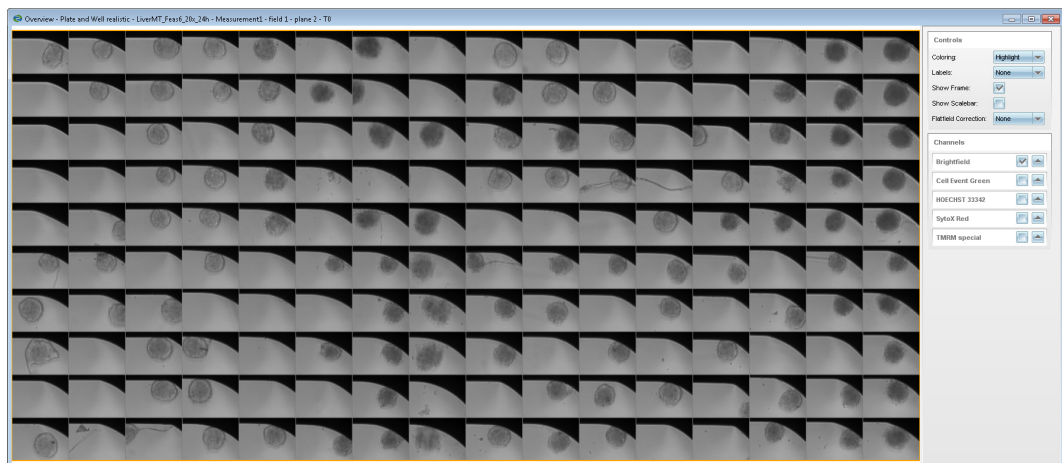
Figure 1–89: Plate overview of a 384-well plate with one image field per well. Different fractions of the well border are visible in the top and top right part of each well (dark region). The region of interest for the analyis is the interior of the well without the well border.

Step 1 – Create a Population Containing the Regions of Interest

The **Find Image Region** building block is used to identify the inside of the well by applying an intensity threshold. See *Figure 1–90*. The bright part of the image is defined as "Inside Well" region (marked by red color overlay).

Note that the building block provides some options to smooth the region (Fill holes, reject fragments smaller than an area threshold). The resulting population is renamed to "Well". It contains only a single object in this case (the well) with a single region "Inside Well".
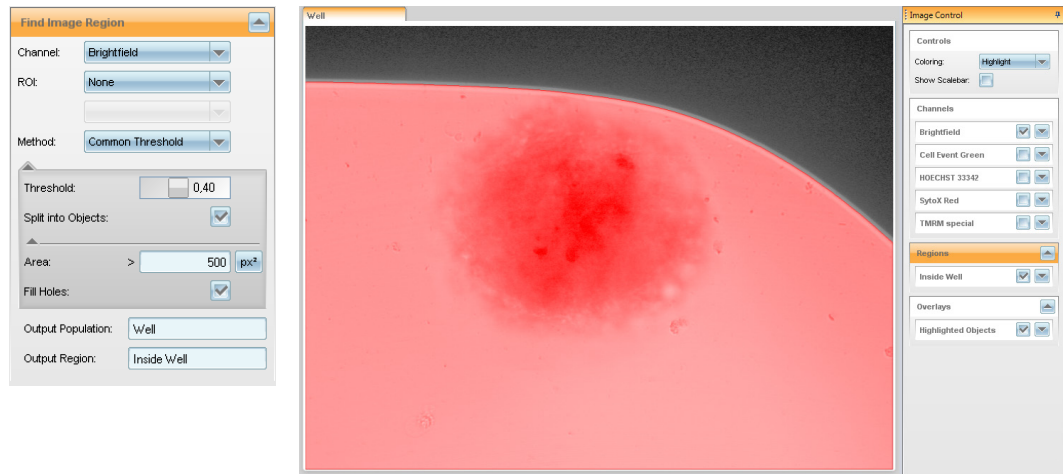
Figure 1–90: Find Image Region is used to identify the interior of a well by applying an intensity threshold. Second level options are used to smooth the region. The result is a population named "Well" containing a region "Inside Well" (marked by a red overlay on the image view).

Step 2 – Perform Analysis Restricted to the Region of Interest

In the next step, the "Well" population and the "Inside Well" region are used to define the Region of Interest (ROI) for the **Find Texture Regions** building block, see *Figure 1–91*.

Since the analysis is restricted to the inside of the well, it cannot be disturbed by the dark well border. The tissue region is robustly detected. Additional building blocks to clean up artifacts, e.g. remove small fragments and fill holes, can be added as needed.
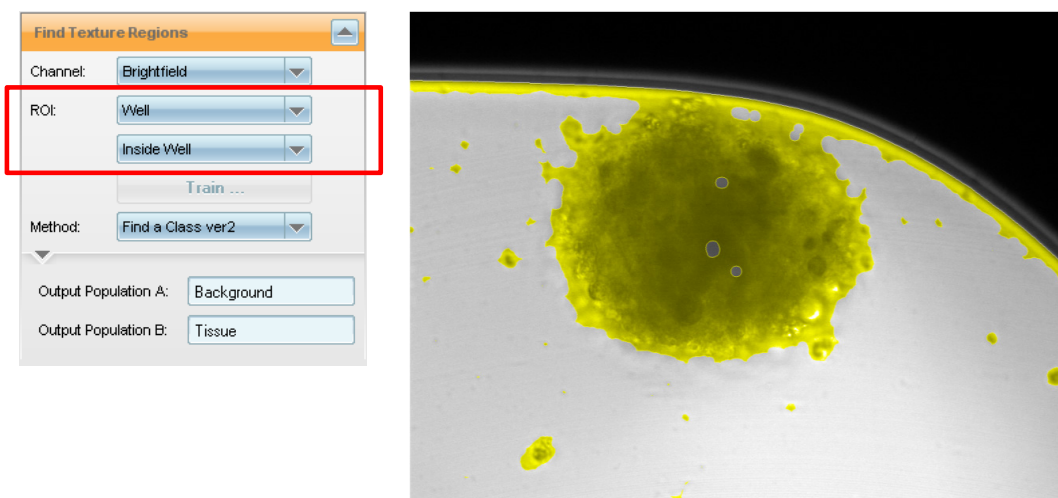
Figure 1–91: Find Texture Regions is restricted to the region of interest (ROI) created in the preceding building block. The texture segmention is restricted to the inside of the well to avoid disturbances by the well border.

1.10.2 Example: Analyze Individual Cells in a Colony

This is an example that makes use of multiple ROIs in one image. The aim is to perform detailed analysis of individual cells in multiple colonies in the image.

Step 1 – Create a Population Containing the Regions of Interest

By using a series of **Find Image Region**, **Select Region** and **Modify Population** building blocks, cell colonies are identified and a population "Colonies" with a region "Colony" is created. See *Figure 1–92*. There are multiple colonies in a single image.
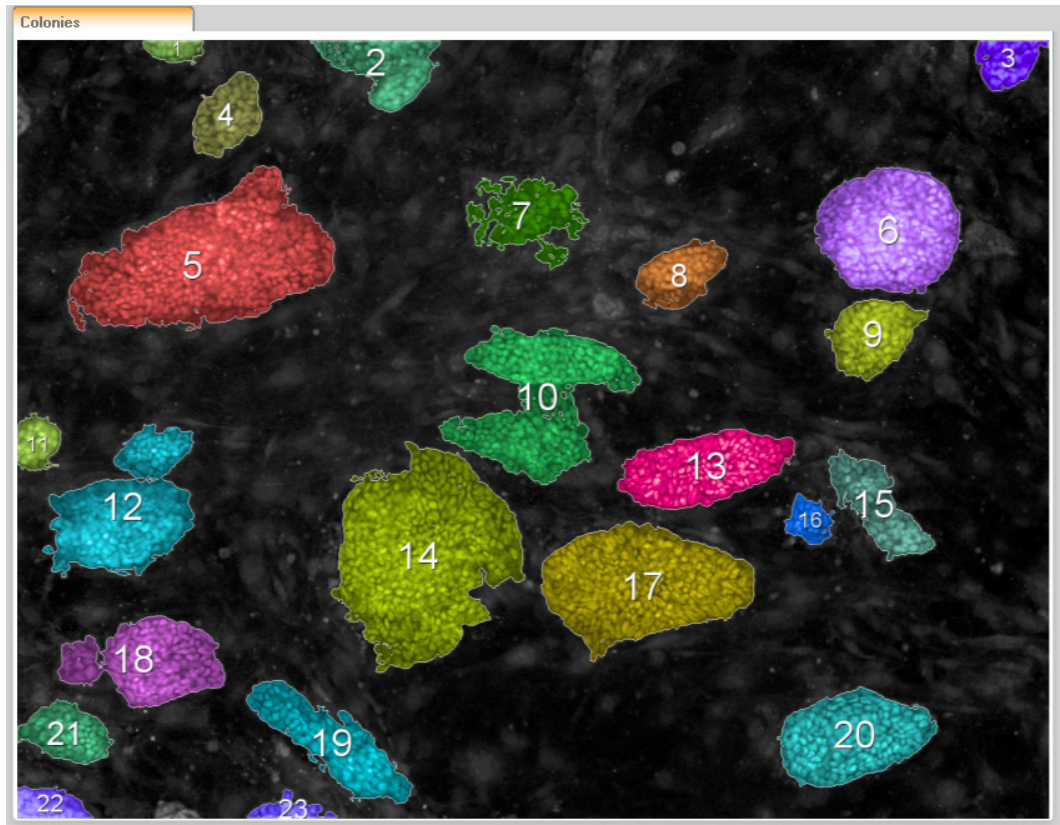
Figure 1–92: Cell colonies identified in an image. The color overlay marks the "Colony" regions; the white numbers (created by the image view overlay style "Numbers") indicate the object number of the colony in the "Colonies" population.

Step 2 – Find Nuclei in Colonies

Now the "Colonies" population with the region "Colony" is used to specify the Regions of Interest for the subsequent **Find Nuclei** building block, see *Figure 1–93*. As a result, all nuclei located in a colony are identified. Nuclei outside colonies are ignored (e.g. from the feeder layer). Each nucleus has a property "ROI No." indicating in which colony it was found.
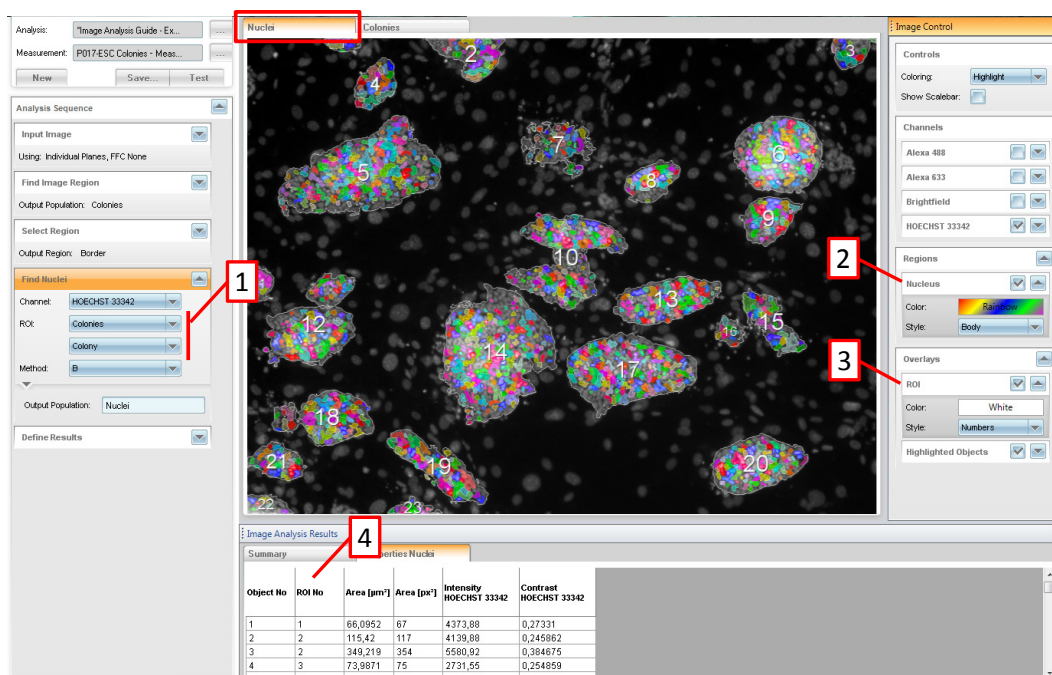
Figure 1–93: Find Nuclei applied to a set of regions of interest. 1 – The population "Colonies" is selected to define the set of regions of interest. The region "Colony" is used to define the colony outline used as ROI. 2 – As a result, a population of nuclei is created. Each nucleus object has an associated "Nucleus" region defined. The nucleus region is listed in the "Regions" box of the nuclei population. 3 – The ROI used for nuclei detection can be overlayed on the illustration for inspection. The "Numbers" style with white color is used. Note that the ROI region is listed in the "Overlays" box. It is only available as an overlay on the illustration but it is not a region of the "Nuclei" population. 4 – Each object in the nuclei population has a property "ROI No." indicating in which ROI it was found (i.e. in which colony in the "Colonies" population).

Step 3 – Further Analysis

Further analysis inside the colonies based on the "Nuclei" population is mostly identical to the analysis without a region of interest. All building blocks can be used. Each nucleus is tagged with the "ROI No." property to show which colony it belongs to. The "Nuclei" population is a "related population" of the "Colonies" population. When deriving a new population from the "Nuclei" population, e.g. by **Select Population** or **Modify Population**, the ROI information is automatically inherited to the new population.

There is one special scenario that should be noted:

When enlarging the "Nucleus" region detected inside the ROI, e.g. by finding a cytoplasm region around it or by growing the region (**Select Region** – resize method), it may spread out of the original ROI. To prevent this, **Find Cytoplasm** and **Find Surrounding Region** automatically restrict the cytoplasm (or surrounding region) to the ROI that was used to find the surrounded object, e.g. nucleus. See ***Figure 1–94***.

*Expert option: This restriction can be removed (uncheck option) or a modified ROI region can be used for restriction, e.g. an enlarged colony region could be used here to allow for some controlled spreading of objects outside the colony.

*Expert note: For **Select Region** – method "Resize Region" restrictive regions are supported as well, but not automatically inherited from the ROI regions. You may create your own restrictive regions if needed.
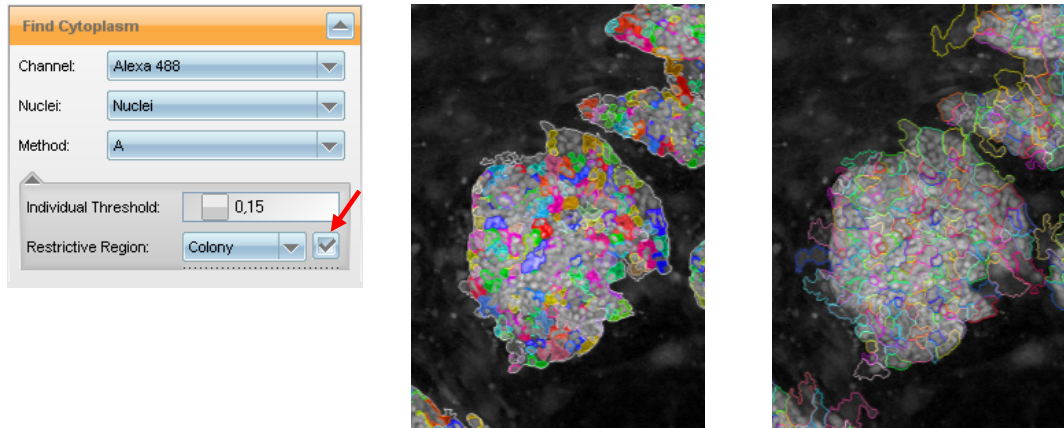
Figure 1–94: **Find Cytoplasm** around a nucleus found in a ROI region "Colony": By default the ROI region which was used to detect the nuclei is automaticall applied as a "Restrictive Region" for the cytoplasm detection as well, i.e. the detected cytoplasm cannot grow outside the ROI, see middle panel. If the restriction is not enabled (unchecked box), the cytoplasm region grows outside of the original ROI (right panel).

Step 4 – Collate Results by Colony

The result of the analysis is a population containing nuclei from all ROIs. To calculate per colony statistics, e.g. the average intensity of all cells in the colony, the **Calculate Properties** building block must be used, see *Figure 1–95*.
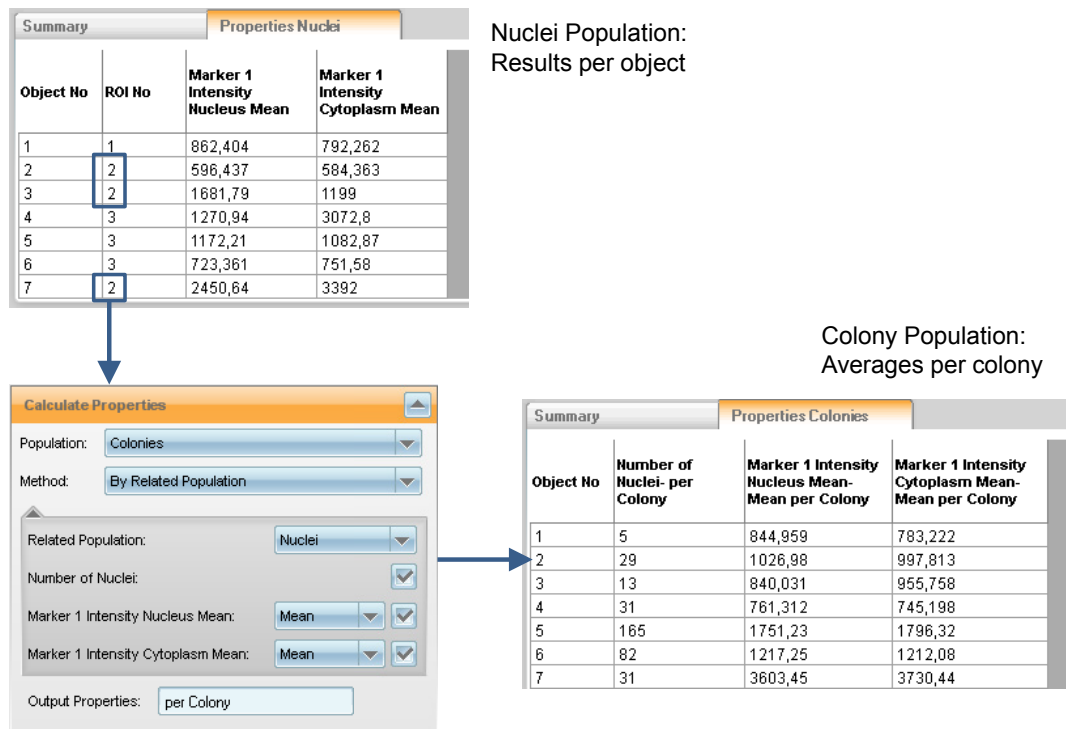
Figure 1–95: Example results for analysis of nuclei in colonies. Top panel: Results for the cells in all colonies are collected in a single population. The software indicates which ROI (colony) each cell belongs to. Cells in colony 2 are marked blue. Bottom left: **Calculate Properties** – method "By related Poplation" is used to to calculate "per Colony" averages. The colony population is selected as input population and the Nuclei population is selected as related population. A name suffix "per Colony" is specified as a suffix for the autogenerated property names. Bottom Right panel: Results generated by **Calculate Properties**. The blue arrow marks the averages for colony 2.

Step 5 – Define Results to Report

In the **Define Results** building block, it must be defined how to create "per well" statistical results from the "per cell" and "per colony" properties generated by the preceeding building blocks. For example:

➤ Use the "Nuclei" population to report the total number of nuclei in the well.

➤ Use the "Colony" population to report the minimum and maximum number of nuclei per colony in the well.

The individual "per Colony" results can be accessed by saving the "Colony" population properties table as a single cell result. Choose the "Single Cell Results" options "All" or "Selected" at the bottom of the **Define Results** building block (see section 1.5.4).

Image Analysis Guide

1.10.3 Example: Analyze Spots in Cells

The aim is to identify spots in existing regions like "Nucleus" or "Cytopasm" of a cell population and add a "Spots" region to the existing object. See e.g. the RMS "Spot Analysis" (section 2.8).

Step 1 – Segment Cells

Use, for example, the **Find Nuclei** and **Find Cytoplasm building blocks** to create a population of cells and the desired regions within these objects.

Step 2 – Find Spots

Use **Find Spots** and specify the cell population and desired spot search region as ROI. **Find Spots** returns a population of detected spots including a comprehensive set of pre-calculated commonly used spot properties (see ***Figure 1–96***).
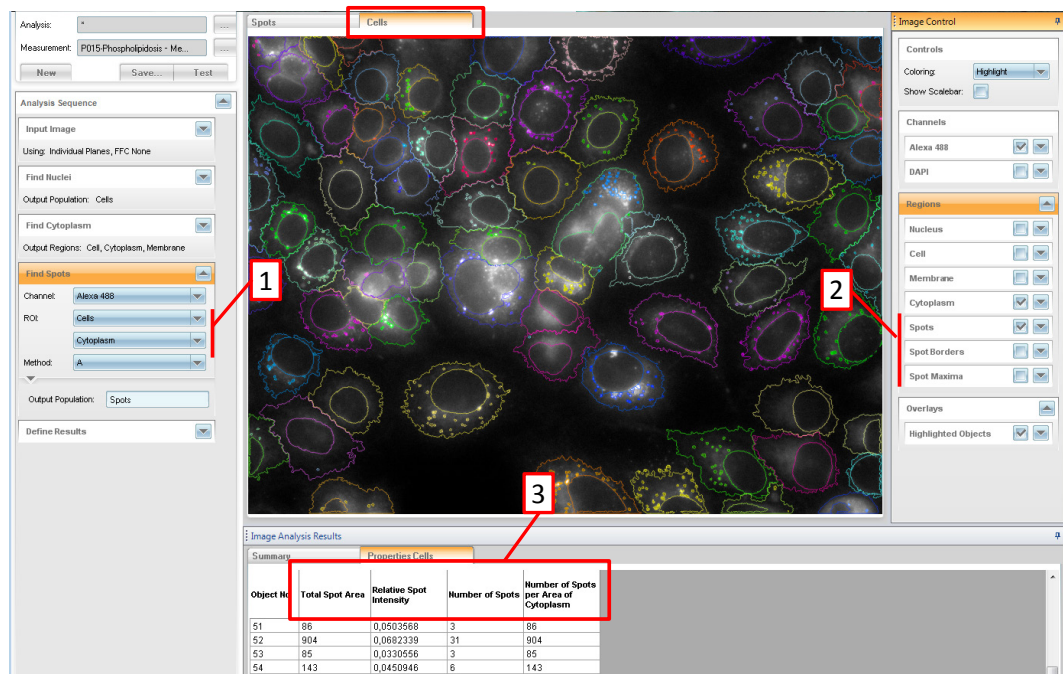
Figure 1–96: Example results of the **Find Spots** building block applied for analysis of spots in the cytoplasm region of cells. 1 – The "Cytoplasm" region of the "Cells" population is specified as a region of interest for the spot detection. 2 – Regions added to the objects in the "Cells" population. 3 – Pre-defined set of properties automatically added to the "Cells" population.

In addition, a set of pre-defined regions is added to the ROI population ("Cells"):

➤ Spots

➤ Spot Borders

➤ Spot Maxima

Furthermore, a set of pre-calculated statistical properties is automatically added to each cell in the ROI population, including

➤ Total number of spots in the object

➤ Total area of all spots in the object

➤ Relative spot intensity compared to search region intensity

➤ Number of spots per area of search region (spot density)

The "Spots" region in the cell population can be used to calculate new spot properties, e.g. measure the intensities of different marker dyes in the spot region (using **Calculate Intensity Properties**).

Note that **Find Spots** also returns a new "Spots" population containing the individual spots. This population is not needed for this type of analysis. It is sufficient to use the "Spots" region added to the "Cells" population and the pre-calcuated spot statistics. Individual spots within one cell are not distinguished in the further analysis; they are collated to one region per cell.

For advanced spot analysis including filtering or modifying individual spots inside the objects, see next section.

1.10.4 Hints and Tips

➤ You may use a kinetic population to define the ROI to get moving regions of interest in a time series. See section 1.16 for details on how to track objects and create a kinetic population.

1.11   Advanced Sub-object Analysis

It may be necessary to select and analyze individual sub-objects inside other objects, for example spots inside a cell. This section explains how this can be achieved.

The simplest way of performing sub-object analysis is for all sub-objects inside each object to be represented by one region, see *Figure 1–97*, left panel. This allows the measurement of joint properties of the sub-objects in each object and is sufficient for a wide range of applications. See example in section 2.8.

If you need more detailed information about the individual sub-objects, you can process the population of sub-objects using further building blocks, for example to calculate properties and filter or classify them. Later, when calculating statistical properties of the parent objects, you can refer to these sub-object properties, for example to report the number of spots of a specific type in each cell.

This approach was used in the cell colony analysis example in section 1.10.2. This section shows a more detailed example for spot analysis.
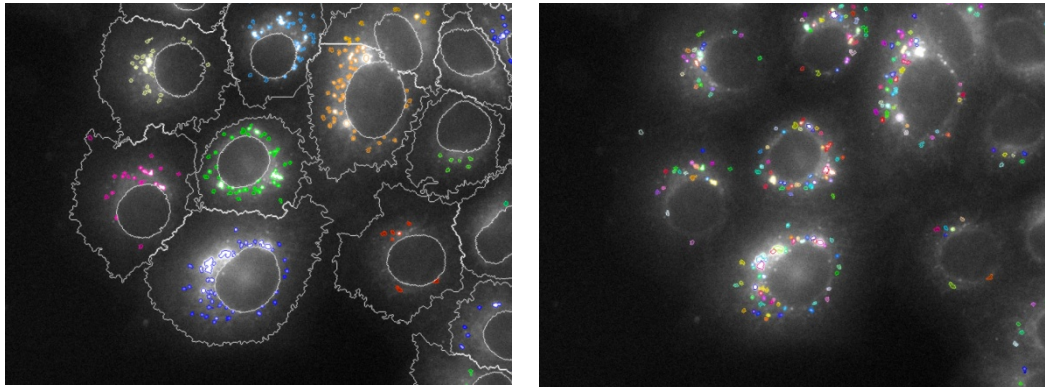
"Spots" region in the cell population: All spots in one cell are treated as one region.

"Spots" population: Each spot is treated as an individual object.

Figure 1–97: Example of detected spots in a cell. Left panel: All spots in one cell are treated as one region which covers all the spots (color coding indicates the cell that the spots belongs to). White lines show the outlines of the detected nuclei and cytoplasm borders. Right panel: The population of spots, where each spot is a separate object indicated by a separate color. Each spot can be individually addressed in further analyses, e.g. to classify the spots by their properties. Both representations of the spots are created by the **Find Spots** building block.

1.11.1   Applications

Sub-object analysis may be used for the analysis of…

➤ Multiple organelles in a cell.

➤ Individual Nuclei in polynucleated cells (see RMS "Micronucleus Analysis", section 2.27).

➤ Micronuclei in cells (see RMS "Micronucleus Analysis", section 2.27).

➤ Lipid droplets in cells (see RMS "Lipid Droplet Analysis", section 2.24).

➤ Individual cells (or objects) in clusters of cells (or objects). See example in section 1.10.2.

➤ Neurites attached to a cell body (see RMS "Neurite Outgrowth Analysis", section 2.21).

Generally, all tasks involving filtering, classifying, merging or modifying sub-objects to generate parent object results.

1.11.2   Typical Analysis Tasks

➤   Calculate the average intensity, size, texture of some selected sub-objects in a cell.

➤   Count the number of objects with a special property, e.g. bright or large sub-objects.

➤   Count the number of sub-objects which show fluorescence of a combination of multiple markers (i.e. co-localization of markers).

1.11.3   Step by Step Set-up of the Analysis

Step 1: Find Objects and Define a Region to Search for Sub-objects

For example, use the **Find Nuclei**, **Find Cytoplasm** or **Select Region** building blocks. As a result, you will get an object list of your primary objects, for example cells or colonies, and a region of interest to search for sub-objects, e.g. the cytoplasm region. See sections 1.3.1 and 1.3.2 for information on how to find objects and define regions.

Step 2: Find Sub-objects in the Regions of Interest

Use the building block **Find Spots, Find Image Region, Find Texture Regions, Find Nuclei or Find Cells** and specify your desired regions of interest. As a result, you will get a list of detected objects. Each object "remembers" a reference to its parent object which can be used later when calculating statistical properties that combine object and sub-object properties. The reference to the parent object is also visible in the column "Object No. in …" (**Find Spots**) or "ROI No." in the property table (see the highlighted column name in *Figure 1–98*).
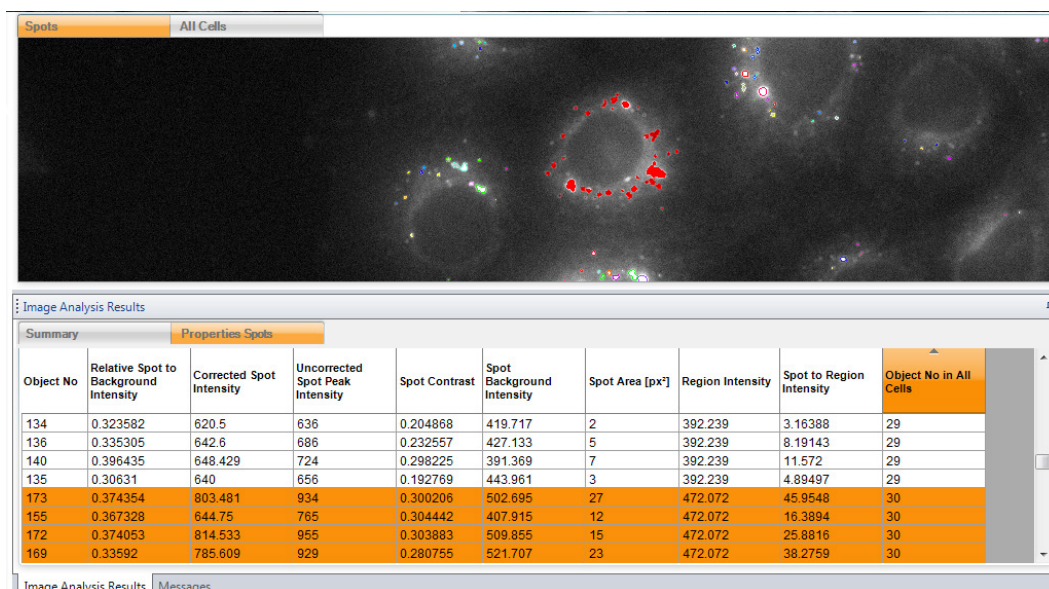
Figure 1–98: Example screenshot of a part of the Harmony GUI showing spots detected in the cytoplasm of cells (as a result of the building blocks **Find Nuclei**, **Find Cytoplasm** and **Find Spots**). The top panel shows the overlay of the detected spot borders (colored outlines) on the spot marker stain image (grey scale). All spots belonging to cell number "30" are marked red (solid fill). The bottom panel shows the spot population's property table. Method "B" was used to find the spots, which pre-calculates a set of commonly used properties for each spot (columns of the table). The last column indicates which cell (parent object) the spot belongs to. The table was sorted by the number of the parent object and all spots of parent object number "30" were selected.

Step 3: Calculate Properties of the Sub-objects

Calculate, for example, the morphology, intensity (in different channels) or texture properties. See sections 1.3.3 and 1.3.4 for all options for how to calculate object properties.

Step 4: Filter Sub-objects

Use the **Select Population** building block to select, for example, large, round or bright spots. In addition, more complex filters can be set up, for example to find spots with co-localization of different markers.

The PhenoLOGIC method "Linear Classifier" can be used here to set-up spot filtering by training using example spots. PhenoLOGIC then classifies the spots according to the given examples. See section 1.15.

A new population is created for the selected objects. The relations to the parent object are inherited to the new population, i.e. the objects in the new population "know" which parent object (e.g. cell) they belong to.
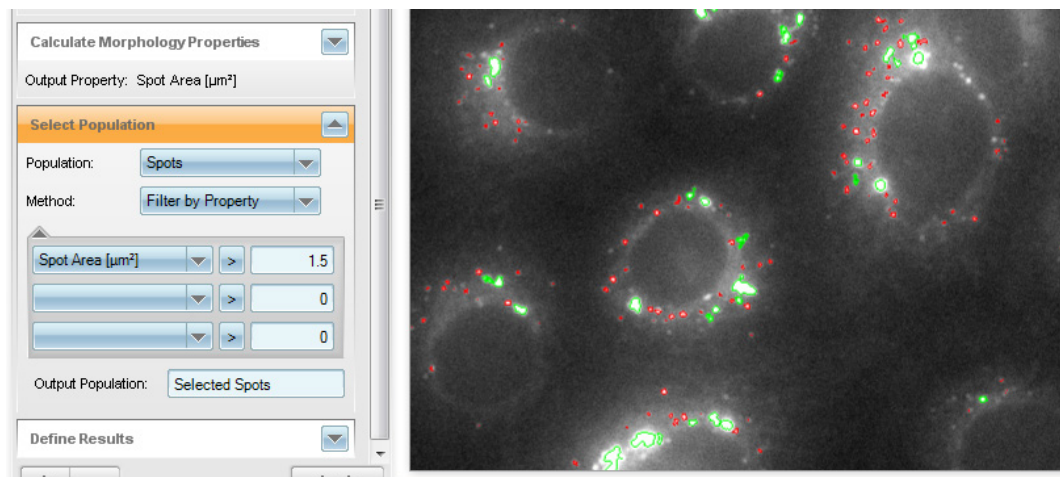
Figure 1–99: Example screenshot of the Harmony GUI showing spots selected by size (green outlines: spot area > 1.5 µm², red outlines: all other spots). A new population "Selected Spots" is created.

Step 5: Calculate Statistical Properties of the Sub-objects

Use the **Calculate Properties** building block with the method "**By Related Population**" to calculate, for example:

➤ Number of selected sub-objects per cell.

➤ Number of sub-objects of phenotype A per cell.

➤ Average size of sub-objects in a cell.

➤ Degree of co-localization of two markers in the sub-objects in each cell.
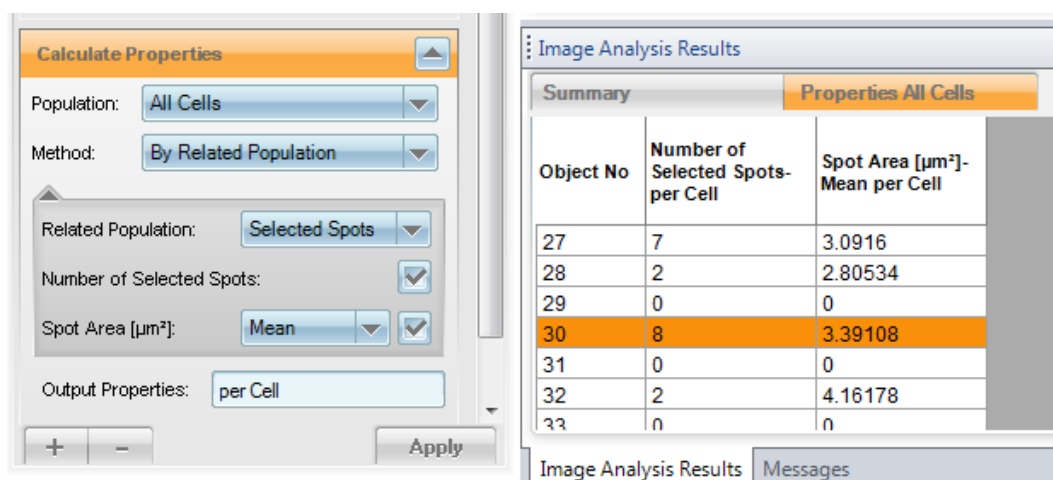
Figure 1–100: Example screenshot of part of the Harmony GUI, showing the **Calculate Properties** building block with the method "By Related Population" selected. Two properties for the population "All Cells" are calculated using the related population "Selected Spots": the number of selected spots per cell and the mean area of the selected spots in each cell. The numbers correspond to the spots displayed in green in *Figure 1–99*.

1.11.4 List of Building Blocks Generating Sub-Objects (Related Populations)

There are several building blocks which automatically generate related populations. These building blocks can also be used in steps 1 and 2 of the previous step-by-step set-up (as an alternative to the **Find Spots** building block):

➤ All "Find …" building blocks which allow specifying a region of interest. The detected objects are automatically related to the corresponding regions of interest.

➤ **Find Neurites** detects neurites attached to cell bodies. The detected neurites are sub-objects of the corresponding cell bodies.

➤ **Find Cytoplasm** – Method "E" finds polynucleated cells around previously detected nuclei. The nuclei are sub-objects of the detected cells. The method "By Related Population" in the **Calculate Properties** building block can be used to quantify the number and properties of the nuclei to the cells.

➤ **Modify Population** – the method "Cluster by Distance" creates a new population of objects from an existing one. For example, you can create a population "Agglomerates" from a population "Spots". Each agglomerate consists of a set of spots. The method "By Related Population" in the **Calculate Properties** building block can then, for example, be used to:

• Count the number of spots in the agglomerate.

• Count the number of spots of a certain phenotype in the agglomerate.

• Calculate minimum, maximum, average values, etc. of properties of the spots in the agglomerate.

1.11.5   Hints and Tips

➤ Relations work in both directions: the method "By Related Population" within the building block **Calculate Properties** can be used to access sub-object properties in the parent population (e.g. count the number of sub-objects per parent object). However, it can also be used to access the parent population's properties, for example to use the parent cell phenotype or intensity as a spot property.

1.12 Multiscale Analysis / Global Image

Multiscale analysis is a technique to analyze a set of images at two different scales:

In a first step a downscaled montage image of all image fields in a well is created ("Global Image") to identify the large scale objects of interest ("global objects"). These objects may span multiple image fields, like e.g. cell colonies, tissue slices or cell grid arrays. Note that the downscaling is needed to be able to fit the image into computer memory and analyze it.

In a second step the large scale objects are used as a region of interest for analysis of the initial set of high resolution images. See *Figure 1–101*.

Note that this is an expert feature. Even though the basic concept looks simple, the details of handling and relating results of two different resolutions often gets very challenging.

1.12.1 When to use it?

Optimally, each object of interest is imaged within a single image field at a suitable resolution to see all relevant details. If this is possible, e.g. by the choice of objective, camera binning or by using PreciScan, then it is the best solution.

If this is not possible because the objects don't fit into a single image field, the objects must be imaged with multiple fields at the highest needed resolution and analyzed using Multiscale analysis.

In addition to analysis, the global image may just be used to quickly create a well overview in **Input Image** when browsing measurements (instead of using the "Plate Overview" described in section 1.2.3).

1.12.2 How to use it?

Depending on the number of high resolution images needed to cover the objects of interest there are two different scenarios:

Analysis of the Global Image only

If only a few downscaled images per well are needed (up to four Operetta CLS or Opera Phenix images acquired with binning 2) the global image has the same resolution as the local images. It can be analyzed in exactly the same way as the original images and allows the analysis of objects crossing field borders. See step three in *Figure 1–101*. No further multiscale analysis is needed.

Full Multiscale Analysis

If the global image becomes too large to be processed by the image analysis it is automatically downscaled to the maximum possible size (currently 5 Mega pixels). The analysis then needs to be done on two different scales: objects that are too large to fit into one image field need to be identified on the global image (step three in *Figure 1–101*). Global regions of interest are transferred to the local images (step four in *Figure 1–101*). Details no longer visible on the global image need to be analyzed on the corresponding individual local images (step five in *Figure 1–101*).

1 – Imaging

25 Images per Well
Magnification 20x, Binning 2

2

Global Image
Synthetic Image, Binning 6

Field 1

Size 1080 x 1080 px², Resolution 0.6 µm

Size 1754 x 1754 px², Resolution 1.8 µm

4a

3 Find Global Objects
Select Regions
Calculate Global Properties

Field 1

4b

ROI for analysis
of high resolution
images

100µm

100µm

5 Find Nuclei
Calculate Texture Properties
Find Spots
...

- Area
- Shape
- Mean intensity
- ...

Single cell analysis results of
high resolution images in
global ROI

Image detail with analysis result

Figure 1–101: Overview of multiscale analysis using a scratch assay example: 1) 25 image fields per well with a 20x objective (binning 2) need to be acquired to resolve all single cell details and cover the whole scratch area on a 384 well plate. 2) The corresponding global image (binning 6) is created. 3) The scratch area (yellow line) and its rim region ±60µm around the border (white line) are identified. 4) The identified scratch regions are transferred to the set of the 25 local images. 5) Detailed single cell level analysis of the scratch rim on the local images is done.

1.12.3 Creating the Global Image

The global image will be automatically created when activating the "Create Global Image" checkbox in the **Input Image** building block. See *Figure 1–102*, mark 1.

For each measured channel a new synthetic global image channel is created (*Figure 1–102*, mark 3). In the subsequent analysis the global image channel can be used in the same way as the measured channels.
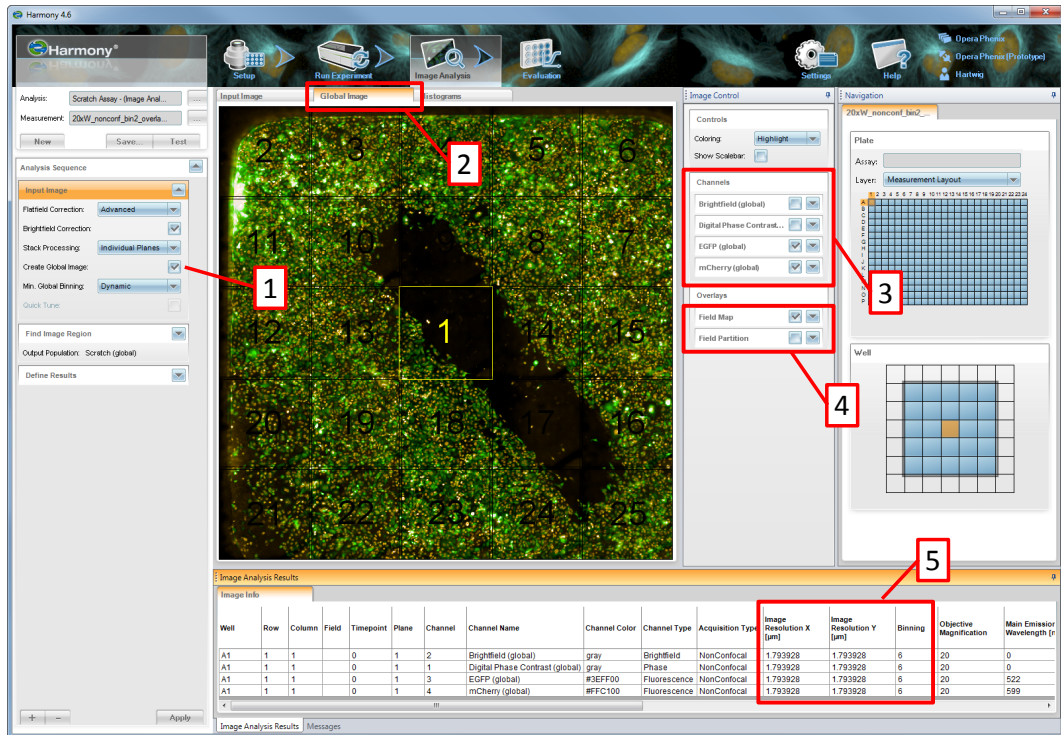
Figure 1–102: Harmony GUI with global image. 1) The "Create Global Image" check box in the **Input Image** building block activates global image creation. 2) The global image display is available on an additional tab. 3) For each measured channel a corresponding global channel is available (suffix "(global)"). 4) Two extra overlays are provided for global channels: i) the field locations and currently selected field ("Field Map") and ii) a unique tiling of the global image ("Field Partition"). 4) If the Global Image tab is selected, the "Image Info" table shows the metainformation of the global image instead of the local images. E.g. resolution and binning information can be found here.

Downscaling is done by binning the image, i.e. by replacing square blocks of pixels by their average intensity value for each individual channel. For example a binning factor of 3 replaces 3×3 pixel squares by a single pixel with the average intensity value. This reduces the image width and height by a factor of 3, but also reduces the image resolution by a factor of 3. The total number of pixels, and therefore memory consumption, is reduced by a factor of 9.

Note that the camera binning during imaging is a sum of the intensity of the pixels, resulting in 4x higher intensity or 4x lower exposure times to get the same intensity. Binning during downscaling is a mean of the intensity. See Application Guide section 2.2.1 "Influence of measurement options on experiments".

Background – How is the Global Image Created?

➤ Flatfield correction is applied as specified in Input Image.

➤ Brightfield correction is applied if specified and applicable, and brightfield intensity levels are made uniform across fields.

➤ PentaPattern based skewcrop correction is applied, correcting for deviation of the actual magnification from nominal magnification, geometric image distortions and camera rotation with respect to mechanical table movement axes. Potential inaccuracies would result in imprecise matching of adjacent image fields.

➤ Local images are binned. The binning factor is automatically determined so that the resulting global image does not significantly exceed the size of an unbinned Opera Phenix image (about 5 Mega pixels). Analysis of larger images may become very inefficient due to computer memory limits.

➤ Image fields are cross-faded in overlapping parts of fields to avoid intensity jumps and to smooth remaining inaccuracies.

➤ Image fields are automatically re-arranged on the global image to avoid large empty regions if the image size can be significantly reduced ("Squeezing", "Packing"). See below.

Creating the global image may be very expensive in terms of processing time, network traffic and memory usage. All individual local images need to be transferred into the computer memory and compressed into the global image. For example, a four channel measurement with 100 fields needs to transfer and process 400 images per well.

Note that the images usually become slightly smaller due to the skewcrop corrections. To ensure a continuous global image, contiguous fields should be imaged with at least 2% overlap. Better results would be obtained with a 5% overlap (see Application Guide).

Please note that for Operetta measurements no PentaPattern based skewcrop information is available, resulting in inaccurate image matching at field borders. The global image needs to be checked to see if the matching is good enough for the application of interest. However, determining a ROI for the analysis of local images should usually not be affected by small inaccuracies.

Global Image Compression

If the field layout of a well contains large gaps between the image fields, the resulting global image will be mostly empty and the image fields are unnecessarily excessively binned. On the other hand, removing all empty regions in the global image would result in minimal image size but completely obscure the original topology of the layout making visual interpretation difficult. To get the best compromise the following compression modes are automatically selected:

➤ "Realistic": no compression, the original placement of fields is used. See Figure 1–102 for an example.

➤ "Squeezing": all full horizontal and vertical empty ribbons are reduced to half an image field width or height. This very efficiently collapses grid structures while preserving rows and columns of fields. See **Figure 1–103**, right side for an example.

➤ "Packing": best arrangement of fields in terms of resulting image size. Blocks of touching fields are preserved, including diagonally touching fields. This ensures that objects extending over multiple fields are not separated in the montage image. See **Figure 1–103** left side for an example. Individual blocks are separated by a gap of at least half an image field width or height to ensure that expanded segmentation regions do not spread into an incorrect neighboring image field during analysis.

Squeezing is used in instances where a 10% reduction in image size is achieved compared to the realistic image. Packing is used in instances where a 10% reduction in image size is achieved compared to both of the other methods.
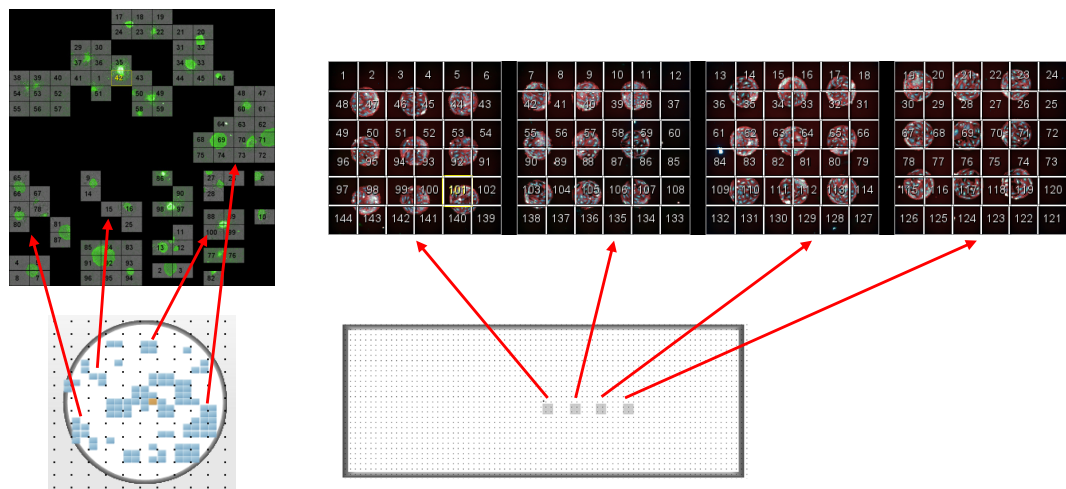
Figure 1–103: Illustration of the global image compression modes. Left: "Packing" example of cell colonies in a 6 well plate. The global image size is reduced by 32% compared to realistic mode. Note that a single very large block of fields (diagonally connected) covers the top half of the montage preventing further compression. Right: "Squeezing" of a gridded cell spot sample (Microstem). Empty regions between the blocks of fields are reduced to half a field width.

Differences to Local Images – Potential Pitfalls

Global images may have arbitrary dimensions (width/height).

Global images may have undefined image regions where no intensity information is available as it has not been measured. These areas are displayed with intensity zero (black coloured pixels) in the image view (see *Figure 1–103* for examples). Note that this might be misleading as the intensity in these areas is unknown (not measured). Assuming zero intensity will be incorrect in most cases. To ensure correct analysis results, these areas must be excluded from the analysis by using a proper ROI. For this purpose the region "Imaged Area (global)" is automatically created for each global image. See section 1.12.4 (page 113).

If used in combination with PreciScan, the global image may have a very different shape and binning for each individual well, see *Figure 1–104* for an example. To avoid biasing the results by a variation in binning, the input "Minimal Global Binning" in Input Image and the Define Results output "Global Image Binning" is provided. See section 1.12.5.

A single global image pixel may correspond to multiple image fields in instances where fields are overlapping. To enable a single analysis of an individual global image pixel a "Field Partition" region is automatically provided for all global images. See section 1.12.6.

C4 **C7** **C11** **C13**
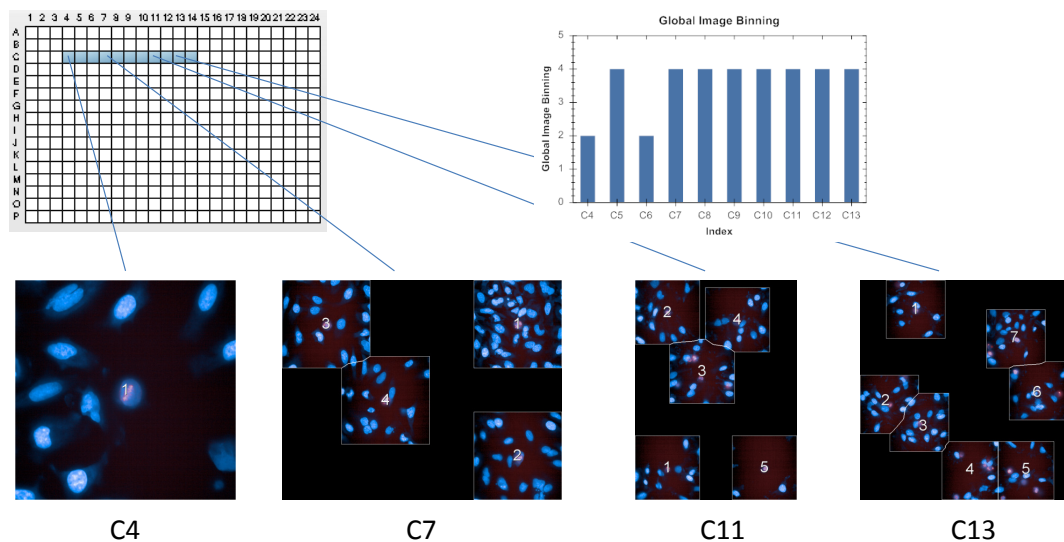
Figure 1–104: Example of different global images from a PreciScan rescan. Depending on the content of the rescan the global images may have very different sizes and binnings for different wells. (Global images are shown correctly scaled to compare their sizes.) The bar graph (created on the Harmony Evaluation Screen) shows the "Global Image Binning" values for all wells. Wells C4 and C6 have a different binning than the other wells.

1.12.4 Analysis of Global Images

Global images can be chosen as an input channel in virtually all existing building blocks, e.g. to find objects on the global image or to filter the global image.

Generally, the analysis is identical to the analysis of "local" images. However, there are some subtle differences and limitations that might make the analysis hard to understand, especially when mixing different scales. These differences are explained in the next sections.

Note that when operating on a global image or population, all results (new images and populations) will be "global" as well.

The following table summarizes the key differences between global and local scale to keep in mind when setting up the analysis:

	Local Scale	Global Scale
Image Scope	A single image field	All image fields in a well / slide
Image Resolution	Resolution used for image acquisition	Additional binning to fit the image to 5 MPixels
Image Size	Fixed size, images are square for Phenix and CLS and rectangular for Operetta	Variable, may include undefined regions
Populations	Object regions are restricted to a single image field	Object regions may extend across field borders

Table 1-8: Overview of local and global image and population key properties.

Mixing of Local and Global Scale in one Building Block

Local and global image and population analysis cannot be mixed in the same building block. Once a global image is chosen as input, all other inputs of the building block (additional channels or populations) can only be "global" as well. In other words, a single building block can only work on a single scale – either the full resolution local images or the downscaled global images. All generated results – populations and images – will also be at that scale. See *Figure 1–105* for an example.
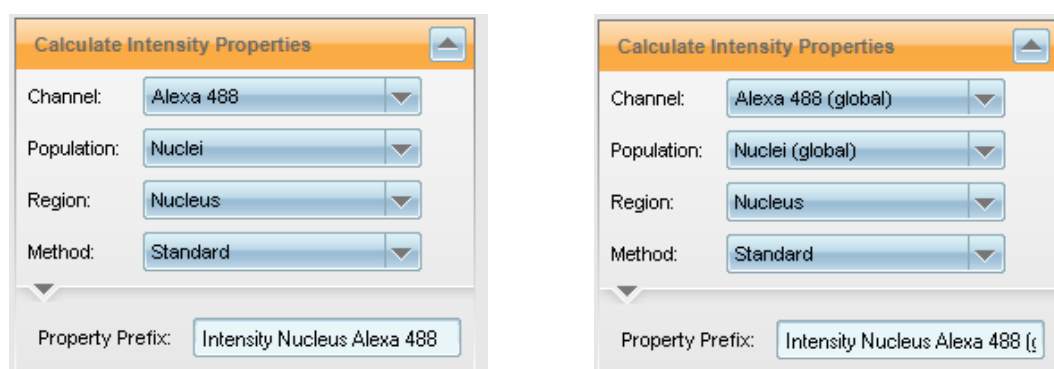
Figure 1–105: The analysis in one building block may be either local (left screenshot) or global (right screenshot). Mixing both scales is not possible. That means e.g. the intensity in the "Nuclei (global)" objects cannot be measured on the "Alexa 488" local image.

The only possible way of using global information in a local analysis is to use it as a ROI to restrict the local analysis to a sub-region of the local image. Note that the results are strictly local and no information is transferred back to the global population. This also includes relationships: while the local nuclei are related to the corresponding global population, there is no relationship from the global population to the local. See *Figure 1–106* for an example.
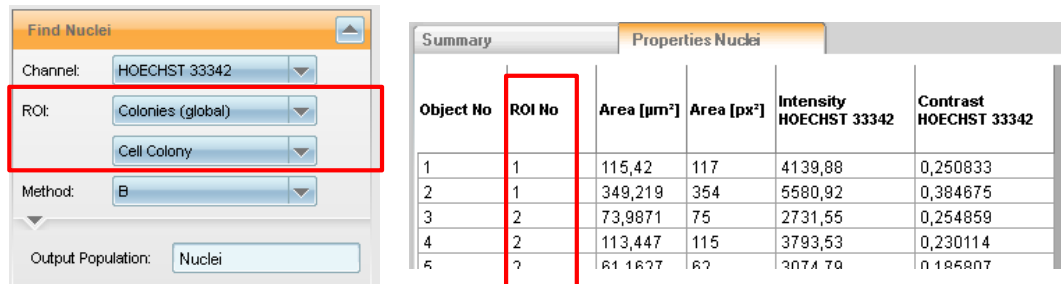
Figure 1–106: Example of finding objects (nuclei) on local images using a global ROI (colonies). The output population "Nuclei" is "local": detected nuclei regions cannot cross field borders. For each nucleus the ROI number (colony) in which it was found is reported for followup processing.

Mandatory use of a ROI or Restrictive Region

If a global image is chosen as input for a building block an explicit ROI must be used, i.e. the ROI "None" cannot be chosen. The default ROI is "Imaged Area" to exclude regions of the global image which do not correspond to any measured image field and therefore have unknown intensities (displayed as zero intensity in the image view). See *Figure 1–107* for an example. Instead of the default, any other global region created by the preceeding building blocks can be used, e.g. to restrict the analysis to the center part of a colony.
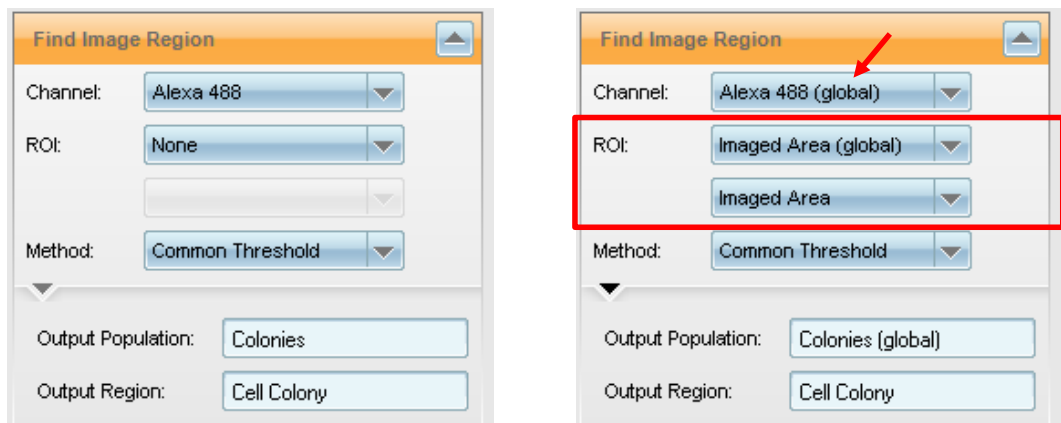
Figure 1–107: Example of the **Find Image Region** building block operating on a local image (left panel) and on a global image (right panel). In instances where a global channel is selected (red arrow) a ROI must be specified (red box). The default ROI is the whole imaged area of the global image. Note that the imaged area usually does not include the whole global image, see e.g. *Figure 1–103* or *Figure 1–104*.

For the same reason a "Restrictive Region" must be used in building blocks that modify existing regions. These regions must be prevented from spreading outside the valid image region, e.g. in **Select Region** method "Resize [µm]" or in **Find Cytoplasm**. The default restrictive region is "Imaged Area (global)".

PhenoLOGIC – Building Block Find Texture Regions

The training can be done either on local images or global images. When switching from a local to a global image, the training must be reset, i.e. training points set on the local image cannot be transferred to global images and vice versa. The user must take care that the training is done on appropriately binned images. E.g. if the training is done on global images with binning 4, the resulting classifier is optimized for this binning value and might not work as expected for other binnings.

Find Micronuclei

The building block **Find Micronuclei** does not support global images. This should not be a limitation because micronuclei are small objects that need to be identified on high resolution images anyway.

"Maximum Projection" Stack Processing Mode in Input Image

The maximum projection works on global images as well, but does not provide a separate XYZ-view for the full global image. The XYZ-view only shows the currently selected image field. No "Plane Map" channels are provided for global images.

Object Tracking

Track Objects does not support object tracking on global images, i.e. global populations cannot be selected as an input population. Note that tracking local objects that were found in a global ROI is possible, e.g. cells in a colony. There is no way to track objects across local image field borders.

1.12.5 Minimal Global Binning Adjustment – PreciScan

The behavior of the automatic binning of the global image can be controlled by the "Min. Global Binning" value in the **Input Image** building block.

The default value ("Dynamic") always uses the optimal value for the current well. In instances where all wells are imaged with the identical field layout, this is also the optimal value for the whole plate.

In instances where PreciScan is used this may result in different binning values in different wells, which would strongly bias e.g. texture analysis results. See e.g. *Figure 1–104*.

In this case an explicit "Min. Global Binning" value can be manually specified forcing all global images to be consistently binned by at least this value, even if they happen to contain very few fields. Only in cases where the global image would exceed the memory size limit, a higher binning value is used to allow processing of the image.

The actual global bining value used is reported in the well results. Wells with higher binning than the specified "Min. Global Binning" value can be easily identified. In any secondary analysis outside of Harmony these datapoints can be handled as needed for the specific application: discarded, specially annotated (flagged) or used as valid datapoints after validating that the binning does not influence the analysis results.

To identify the most suitable value for a specific application you might:

➤ Identify a "worst case well" with the highest expected number of fields in your application. Dynamic global image binning will provide an optimal binning value.

➤ Set up the image analysis and check to ensure it is working sufficiently.

➤ Fix the "Min. Global Binning" to this value.

Note that the available "Min. Global Binning" values in the drop down menu depend on the binning of the original images and follow a logarithmic scale (binning 2 or 3 makes a significant difference, while binning 53 or 54 makes virtually no difference).

1.12.6   Field Partition – Avoiding Double Counting of Objects*

If image fields have overlapping regions, the same object may be visible in two image fields, see e.g. the nuclei marked by red arrows in the middle panel of *Figure 1–108*. When analyzing the global image, no special attention is needed. Each nucleus appears only once in the global image (Figure 1–108, right panel) .

When analyzing the corresponding local images each field is analyzed individually and all results are collected in the resulting local population. Therefore objects visible in two image fields will occur twice in the local population. These objects are double counted and their properties will get double weight in statistical operations (e.g. when calculating well averages). Depending on the design of the specific experiment this might significantly bias results and needs to be corrected.
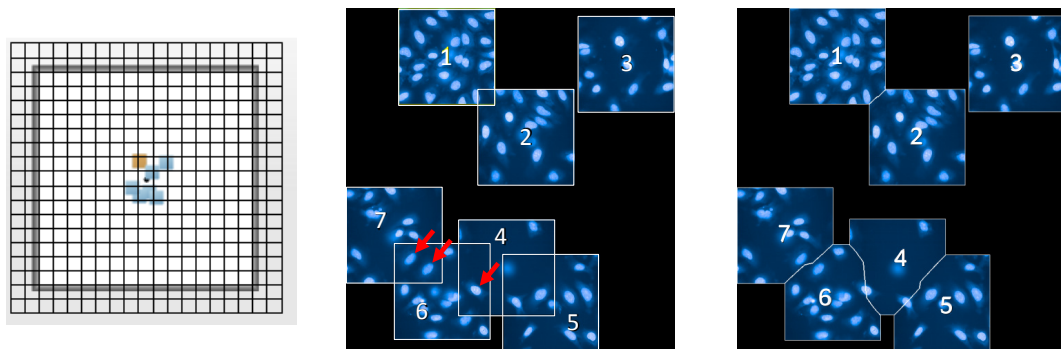
Figure 1–108: Example of a PreciScan measurement with some strongly overlapping fields. Left panel: well layout. Middle panel: resulting global image with "Field Map" overlay, Right panel: global image with "Field Partition" overlay. The red arrows mark examples of nuclei appearing in two image fields.

In order to remove double objects from a local population the "Field Partition" overlay of the global image and the **Select Population** building block's method "Select by Mask" can be used, as shown in the example in *Figure 1–109*:

The "Field Partition" overlay (as shown in the right panel of *Figure 1–108*) maps each pixel of the global image to a single local image field. Overlapping field regions are equally split between the fields.

The "Select by Mask" method allows the selection of objects that are inside (or outside, by selecting the "Use Inverted Mask" checkbox) a specified region. The criterion to define "inside" can be adjusted in the advanced options section of the building block ("Select by"). By default, objects are considered to be inside a region when the geometrical center of the object is inside the region.

By using the resulting "Output Population" from the Select Population building block as an input population in subsequent building blocks, each object will only be analyzed once, even though it is possible for an object to appear in multiple overlapping fields.
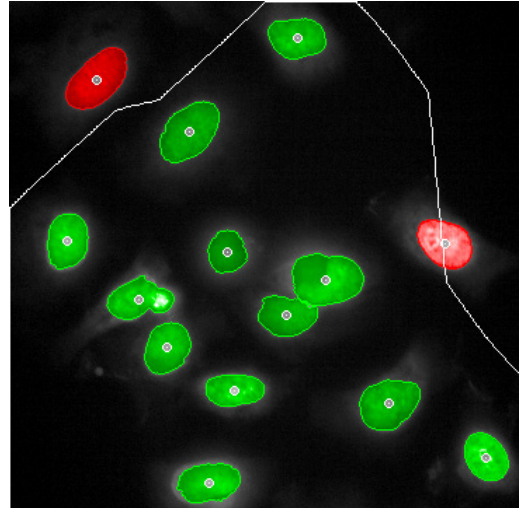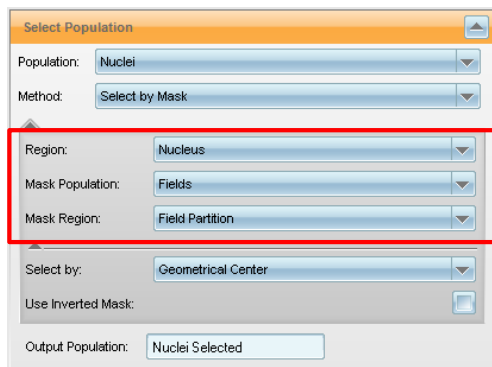
Figure 1–109: Example of using the "Field Partition" overlay of the global image to remove double counted objects from a local population. Left panel: Building block with a local nuclei population selected. Filtering based on the "Nucleus" region of the objects and the "Field Partition" region of the image fields is selected. Note that "Fields" automatically becomes available as a "Mask Population" when global images are available. Right panel: Resulting illustration of field number 6 of the example shown in **Figure 1–108**. The geometrical center of the objects is marked by a circle. The red colored objects are removed from the population because the geometrical center of their nuclei region is located outside the Field Partition region of the current field (indicated by the white line).

1.12.7　Hints and Tips

➤　For considerations regarding the impact of image binning on the resolution of image details, see sections 1.4 "Objectives" and 2.2 "Experimental Setup – Optimization and Finalizing Steps" in the Application Guide.

1.13   PhenoLOGIC™ Machine Learning – Concept

➤ A new class of image analysis methods based on machine learning.

➤ Principle: Identify examples of objects or regions by selecting them with the mouse in a "training" phase.

➤ Once the training is complete, the computer identifies all similar objects or regions in the "image analysis" phase.

Figure 1–110 shows an example which illustrates how machine learning works. The aim of the analysis is to measure the area covered by HT1080 cells in all wells of a micro plate, using brightfield images (label free). To set-up the analysis:

➤ Select an image.

➤ Decide how many classes you want to detect, here we have chosen 2 (cell layer and background).

➤ Select training points for each class by clicking into the image. The selected training regions are marked by red and green circles in the image.

➤ The computer segments the rest of the image according to your training samples (red and green colored regions in the image).

➤ You can adjust training points and parameters until you are satisfied with the result.

➤ The trained building block can be used to analyze all other images of the experiment.

The training can be refined at any time by using more training points, more training images or adjusting tuning parameters of the algorithm (e.g. typical size of the regions of interest).
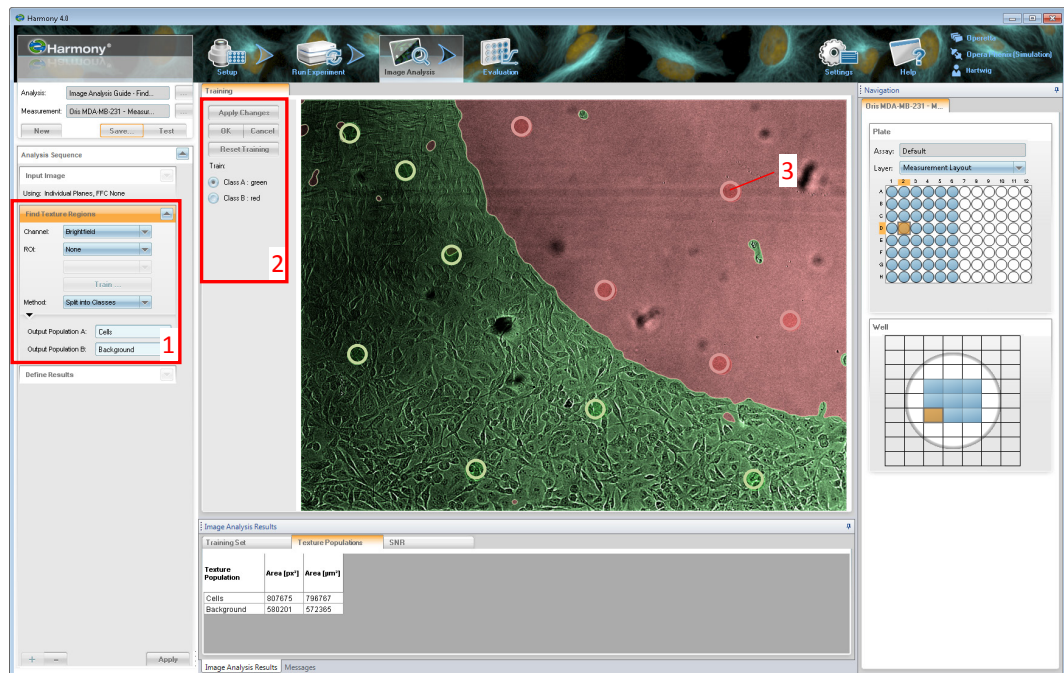
Figure 1–110: Example screenshot of the Harmony GUI showing a machine learning method. A brightfield image of a sample of migrating HT1080 cells is selected. Cells are migrating from a closed cell layer in the bottom left area of the image to an empty region in the top left. The color overlay shows the segmentation results (cell covered region – green, cell free region – red). The red and green circles indicate the training samples used to train the **Find Texture Region** building block. 1 – Building block using PhenoLOGIC machine learning. 2 – Training control elements. 3 – Training sample.

1.13.1   Overview of Algorithms

Two completely different categories of analyses are currently supported by PhenoLOGIC methods:

Image Segmentation

Finding regions or objects of interest in an image, similar to the example shown above. See *Figure 1–112* (*C.elegans*) and *Figure 1–114* (stem cell colonies) for more examples. This is done using the **Find Texture Regions** building block, described in section 1.14.

Object Classification

Classifying existing objects according to their previously determined properties, such as size, intensity, texture or morphology. See *Figure 1–118* or the RMS "Phenotype Classification" (section 2.23) for examples. This is done using the "Linear Classifier" method in the **Select Population** building block, described in section 1.15.

Please note that the PhenoLOGIC methods require an additional software license. If this license is available on your system, this is indicated by the PhenoLOGIC logo in the Harmony window headline, as shown in *Figure 1–111*.

Please contact your PerkinElmer customer support for information on how to obtain a license.
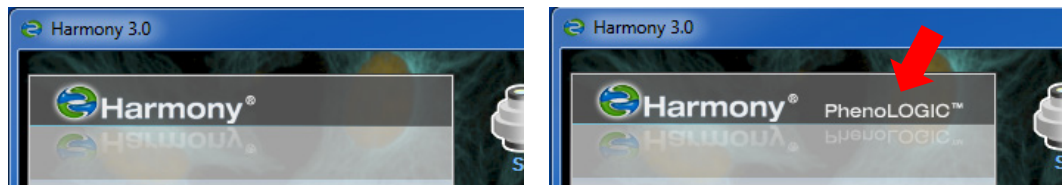
Figure 1–111: If the PhenoLOGIC license is available, this is indicated by the PhenoLOGIC logo at the top left hand side of the Harmony window. Left panel: PhenoLOGIC license is not available. Right panel: PhenoLOGIC license is available.

1.14 PhenoLOGIC™ – Texture-based Segmentation

Texture-based segmentation is a method used to find objects and regions in images by their different textures. See *Figure 1–60* for an example of different textures.

- ➤ The Segmentation is independent of the image intensity.
- ➤ The different textures are trained by machine learning.
- ➤ Texture segmentation is well suited for brightfield images (label free).

Requirements

- ➤ The objects or regions must be large enough to be identified by their characteristic texture (= intensity pattern). As a rule of thumb, the object or region size should be at least 20 x 20 pixels.
- ➤ The object classes must have different textures and not just different intensities. To find regions by intensity, use the building block **Find Image Region** instead.
- ➤ Be aware that texture segmentation gives less accurate detection of object and region borders than a specific, high contrast fluorescent staining of the regions of interest used with a specialized building block such as **Find Nuclei**.

When to use it?

- ➤ When fluorescent staining is not wanted, or is not possible.
- ➤ When regions of interest are not clearly distinguishable by their intensity, i.e. there is no match between high intensity = wanted object, low intensity = background.
- ➤ To quickly set up an image segmentation by selecting some training points.

1.14.1   The Find Texture Regions Building Block

Texture-based segmentation is done using the building block **Find Texture Regions**. There are two different methods available.
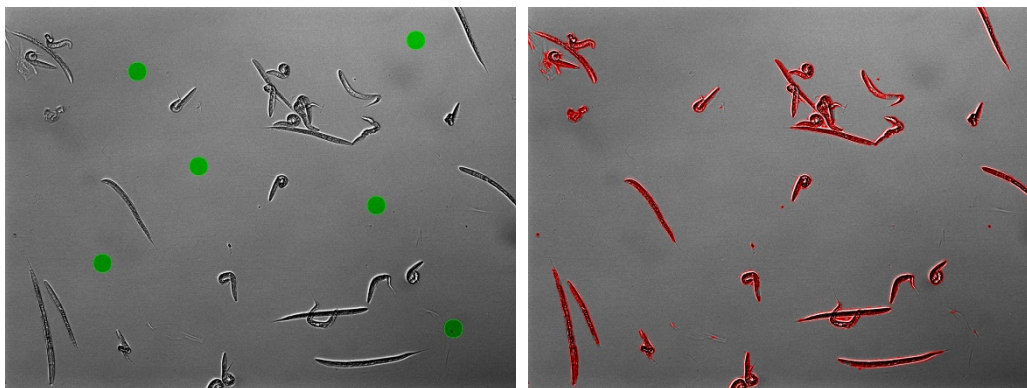
Figure 1–112: Example of the detection of *C.elegans* on 10x high NA brightfield images. Left panel: Original image with training points. Right panel: Detection result of the trained **Find Texture Region** building block.

Method "Find a Class"

This method is built to find one homogeneous texture class, e.g. a cell-free region in the image. The rest of the image is assigned to a second class, independent of its texture.

Note that "Find a Class" is an improved replacement for "Find a Class (legacy)". The new method is up to 10x faster, especially for larger Region Scale values. The old method "Find a Class (legacy)" is still supported in saved old analysis sequences, but cannot be added to new sequences any more.

Figure 1–112 shows an example where the aim is to detect *C.elegans* worms on brightfield images. There are two different classes to detect: "Background" and "Worms". Since the background has a far more homogeneous texture than the worms, it is selected as the texture class to be detected.

> Keep in mind: Sometimes, as in this example, it is easier to detect the background instead of the objects of interest.

By clicking on the image, the training points are set (marked by green circles in the left panel of *Figure 1–112*). By applying the training set (using the "Apply Changes" button in the training controls, see *Figure 1–110*, 2), a classifier is calculated and used to segment the whole image. All regions with a texture similar to the training sample are assigned to the background class (green) and the rest of the image is assigned to the "Worms" class (red, shown in the right panel *Figure 1–112*).

The segmentation characteristics can be influenced by some tuning parameters in order to adapt it to different application needs. See *Figure 1–113* for a screenshot of the GUI of the building block.

➤ The "Tolerance" can be increased to assign more of the image to the detected class. Use this value if you have the impression that the selection is too "picky".

➤ The "Texture Scale" specifies the characteristic scale of the texture features that are used for classification. A low value (~0…3 px) is appropriate for fine patterns, higher values (~3…20 px) for coarser patterns. If the segmentation

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does not meet your needs, increase and decrease the scale in 0.5 px or 1 px steps and choose the value which gives the best results.

If you have trouble selecting a suitable value, try using the "Split into Classes" method, which offers a special tuning aid for the texture scale (see below).

Expert hint: The scale parameter is identical to the one used in the building blocks **Calculate Texture Properties** (method "SER Features") and **Filter Image** (method "Texutre SER"). You can use these building block's illustrations as an additional aid to adjust the scale value. See section 1.7.

➤ "Region Scale" determines how big the smallest features are that you are looking for. Higher values create smoother border lines and average out noise and small details. Lower values show more details but also more noise and artifacts. See *Figure 1–117* for an example.

➤ The "Training Region Radius" can be used to adapt the diameter of the training region created by one mouse click on the image (green circles in *Figure 1–112*). It can be made smaller to fit into smaller image features, but be aware of the principal texture segmentation resolution limits for small objects. Use it with extreme care!

➤ The "Split into Objects" checkbox can be used to split the detected texture regions into discrete objects. It only determines how the result is reported, the segmentation is not influenced. See *Figure 1–115*.

➤ The "Include Intensity Information" checkbox allows using intensity information in addition to texture information for classification. Use with extreme care, see hints and tips section 1.14.2.
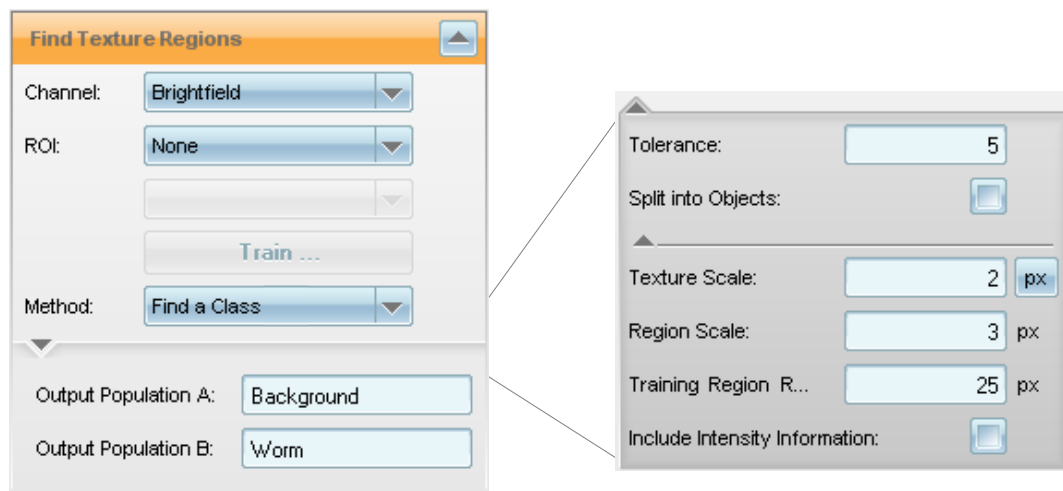
Figure 1–113: The **Find Texture Regions** building block with method "Find a Class" selected. Left: Standard parameters. Right: All of the available input parameters are displayed. The parameter settings used in the *C.elegans* detection example in *Figure 1–112* are shown.

Method "Split into Classes"

This method can split the images into two or more classes. Use it:

➤ If more than two classes are needed.

➤ If there are two classes and "Find a Class" does not give sufficient results.

See ***Figure 1–114*** for an example. The task is to find mouse embryonic stem cell colonies growing on a feeder cell layer. Two classes have to be detected "Colonies" and "Background" (feeder layer) so training points for samples of both classes have to be selected (red and green circles).

The tunable parameters are the same as for "Find a Class" except that the "Tolerance" parameter is missing. Instead, the number of classes can be selected (up to six).

Please note that this method has an extra tuning aid for the adjustment of the "Texture Scale" parameter, see ***Figure 1–116*** and hints in the next section.
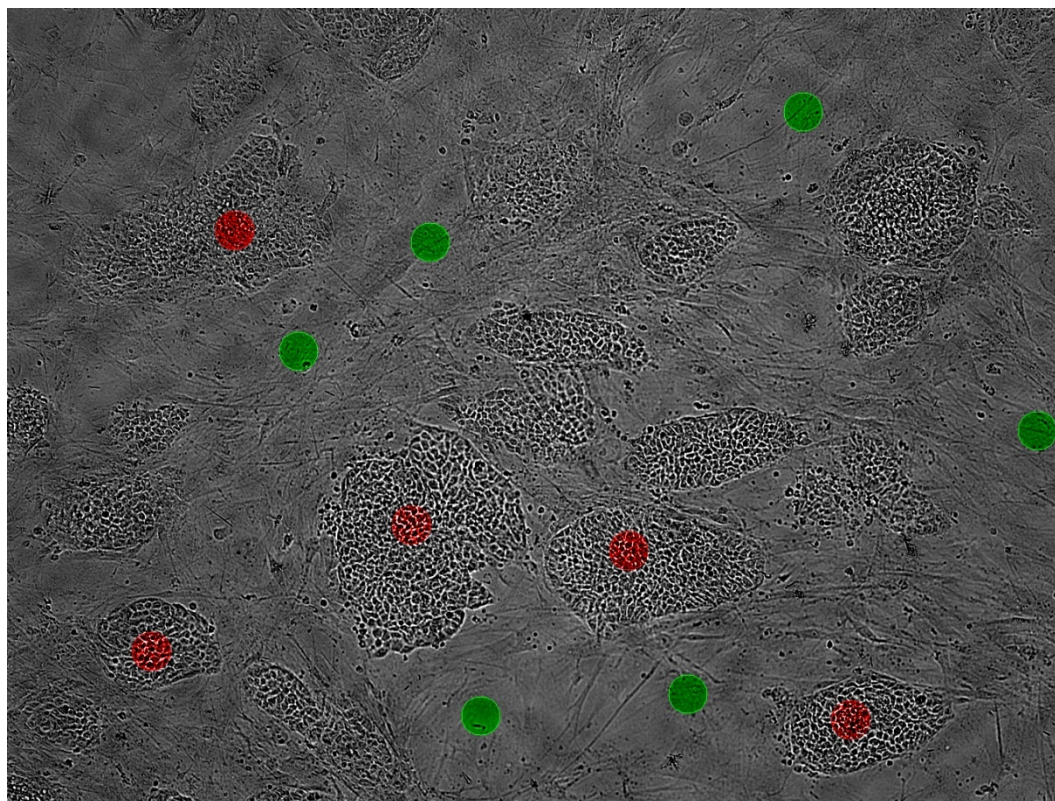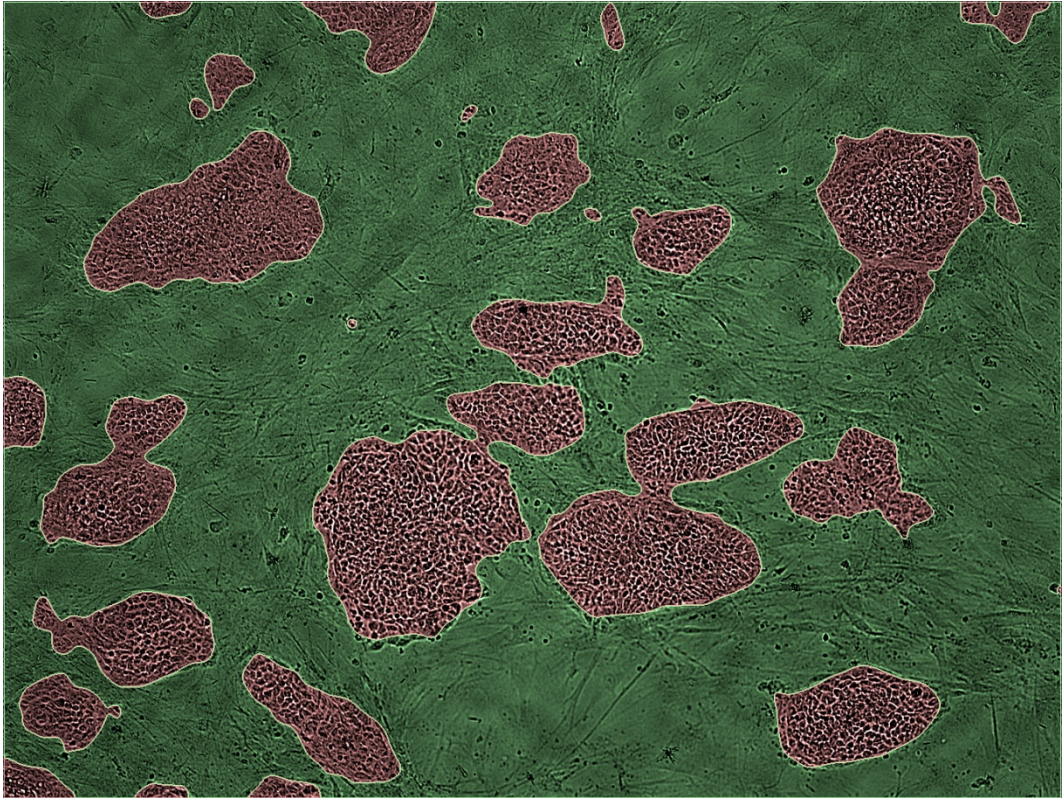
Figure 1–114: Example of a detection of mouse embryonic stem cell colonies on brightfield images. Top panel: Original image showing the training points for two texture classes, "Background" (green) and "Colony" (red). Bottom panel: Detection result of the trained **Find Texture Region** building block. The image is taken from the example dataset "P017-ESC Colonies"; see the Appendix, page 320, for details.
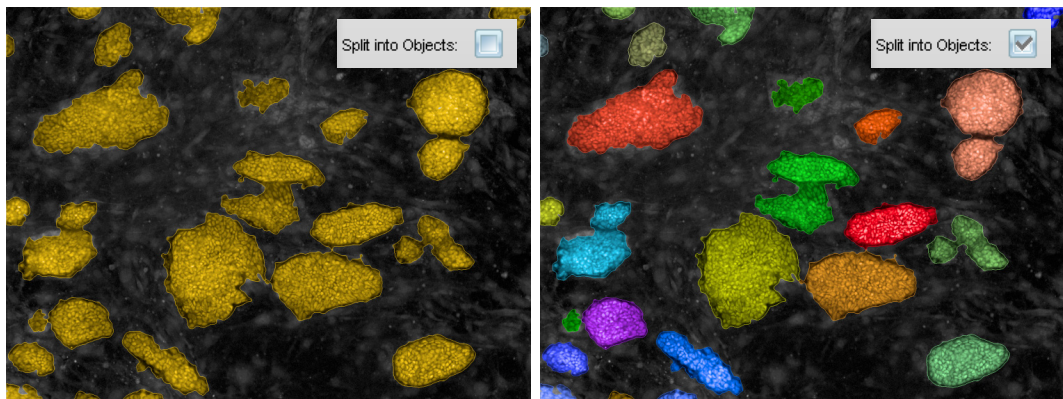
Figure 1–115: The "Split into Objects" option splits the detected texture region (left panel, amber color overlay) into separate objects (right panel, each color indicates an individual object). Regions which are not connected are split. The default splitting can be refined using the **Modify Population** building block if a customized analysis is needed, e.g. to separate touching objects.

1.14.2   Hints and Tips for Selecting Training Points

Where should I put the training points?

➤ Each texture class varies slightly in different parts of the image. The recommended strategy is to capture various samples for each class. Include a bit of every slightly different variant within the class.

How many training points are needed?

➤ Start with some representative samples for each class (e.g. 2-5 samples).

➤ More samples do not necessarily mean better results.

➤ The calculation time does not depend significantly upon the number of selected samples, so do not try to use fewer samples to speed up the analysis.

How can I improve the results if I am not satisfied with them?

➤ If the problem is only in certain parts of the image then try to resolve it by adding training points within that particular area.

➤ If the region tends to be generally too narrow then increase the "Tolerance" parameter. Otherwise, decrease it (available in the "Find a Class" method only).

➤ If the border line between the classes is too "noisy" or too "straight" then tune the scale parameters. Typically, the "Texture Scale" has a real optimum while the best value of the "Region Scale" is a compromise between better-by-noise-but-worse-by-details and worse-by-noise-but-better-by-details.

➤ For tuning the "Texture Scale" parameters, the method "Split into Classes" provides extra information in the results tab to find the optimum value. See *Figure 1–116* for information on how to use it.

➤ If you detect lots of small patches of a given class all over the image, which are smaller than the features you are looking for, increase the "Region Scale" to get rid of the artifacts. See *Figure 1–117* for an example.

➤ If you only have two classes: Test the other method ("Find a Class" or "Split into Classes").

Why does Find Texture Regions not find the regions I want to detect?

Only the texture of the region is used for classification, NOT the shape, NOT the intensity (depending on "Include Intensity Information" option) and NOT the location (inside something, next to something). If you wonder why an object or region is classified in an unexpected way, ask yourself: how do I know what class it is? Is it only the texture or is it additional information as well (shape, location, pre-existing knowledge, other marker dyes) that is unknown to the **Find Texture Regions** building block? Try to use this information for detection, e.g. by intensity.

When should I use the "Include Intensity Information" option?

Generally, intensity varies from sample to sample, field to field and object to object a lot. Therefore, texture features which are normalized by intensity are more reliable for segmentation than the original intensity. In most cases, the switch "Include Intensity

Information" should be kept off. However, intensity information is valuable in cases when:

➤ The textures of the different classes are similar or vary a lot within a class, AND

➤ Intensities between the classes are significally different.

It is worth trying out both options and inspecting the results visually, but you should definitely use many images.

Please be aware that this option makes the analysis results dependent on absolute image intensities, which may vary e.g. due to sample preparation. Test to make sure that the analysis works for all possible intensities and that the intensity difference between classes is much bigger than the intensity variations between batches, plates and wells.

Image Analysis Results		
Training Set	**SNR**	**Texture Populations**

Texture Scale	SNR Classes A-B
0	5.59521
0.5	5.34927
1	4.87754
1.5	4.34402
2	4.02008
3	3.7862

Figure 1–116: Tuning aid for the selection of the best "Texture Scale" parameter of the **Find Texture Regions** building block. The signal to noise ratio (SNR) measures how well the selected training points for classes A (e.g. "Background") and B (e.g. "Colony") are separated for different texture scale settings. For optimization, try different settings (the SNR values are recorded in the table) and choose the best one. In most cases there is a clear optimum; in this specific case the finest possible texture scale "0" is the best choice.
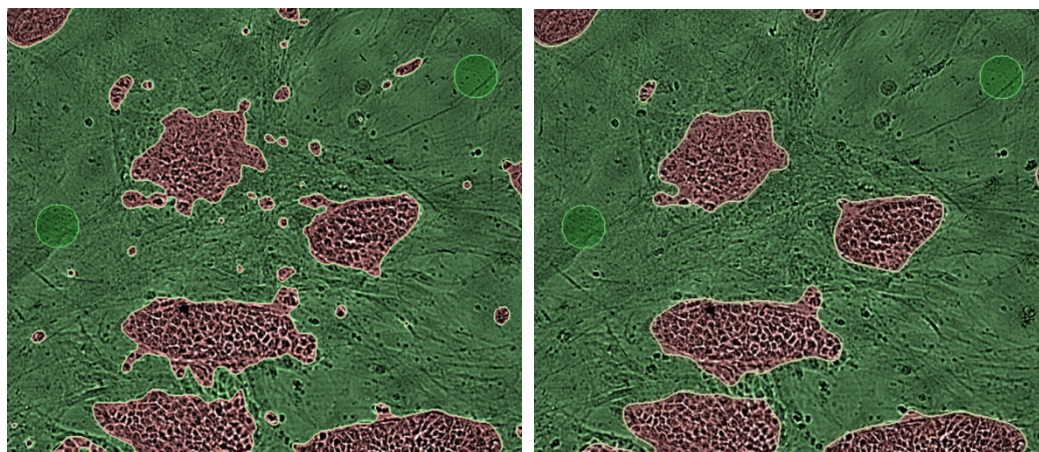
Figure 1–117: Example of the influence of the "Region Scale" parameter. Left panel: Region scale set to 6 px (default value). Small patches of the red class are detected in the green class region, which is considered as an artifact. The colonies of interest are much larger. Right panel: Region scale set to 10 px. Only larger structures are detected. In addition, border lines are straighter which averages out smaller features.

1.15   PhenoLOGIC™ – Classification

➤ A new method to interactively train a building block to classify objects such as cells, colonies or spots.

➤ Built into the **Select Population** building block (method "Linear Classifier").

➤ Replaces the manual set-up of conditions like "mean pHH3 marker intensity inside the cytoplasm region > 2300 counts" AND "cell area < 200 µm^2".

➤ Ideally suited to make best use of STAR morphology or texture properties.

Requirements

➤ The objects must be identified prior to classification.

➤ A set of properties (e.g. marker intensities, morphology, texture) must be calculated, which will be used to classify the objects.

When to use it?

➤ When objects need to be selected in the **Select Population** building block.

➤ When there is no obvious property available that clearly indicates the class of the object (like a highly specific fluorescent marker).

➤ To quickly set-up object filters with a few mouse clicks.

➤ To determine multiple classes (up to 6) at a time.
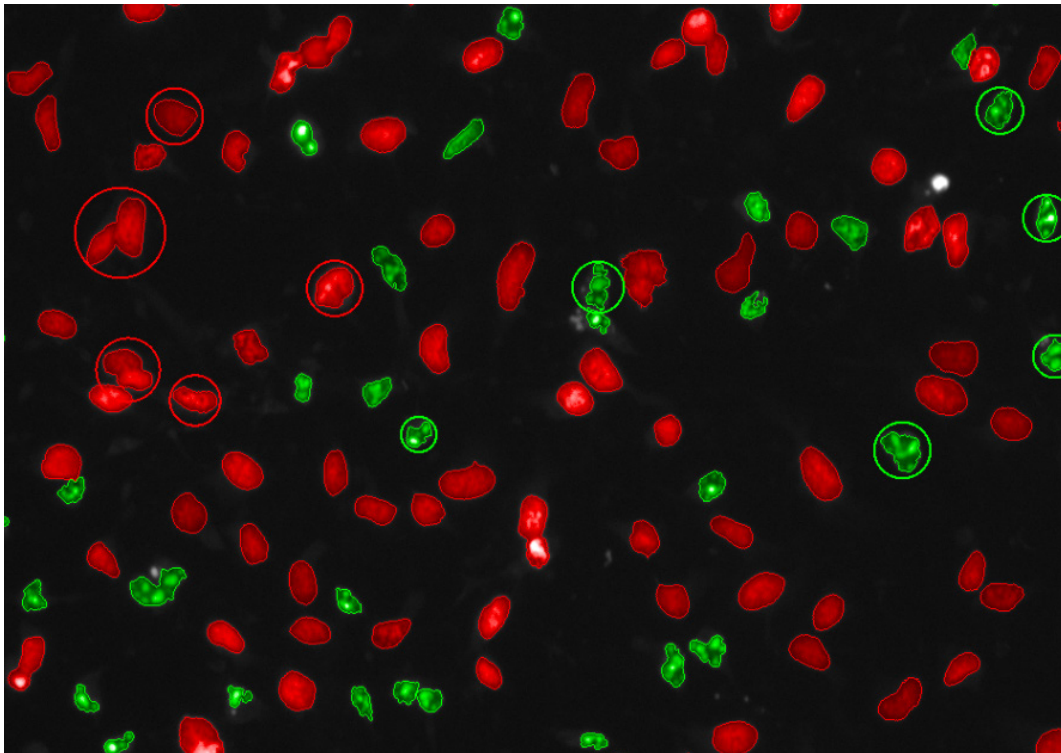
Figure 1–118: Detection of fragmented nuclei by a trained linear classifier (**Select Population** building block, method "Linear Classifier"). The DRAQ5™ stain channel is shown with an overlay indicating the detected nuclei. Red circles: selected nuclei for training of the "normal cells" class. Green circles: selected nuclei for training of the "apoptotic cells" class. Red and green color overlay: classification results from the trained classifier.

1.15.1 Step-by-step Set up of the Analysis

For general instructions on how to set up an analysis sequence, please see section 1.3. The following description focuses on details that are specific for object classification by the PhenoLOGIC "Linear Classifier" method.

Step 1 – Identify and segment the objects you want to classify

For example: nuclei, cells, worms, colonies, spots or micronuclei. See section 1.3.1.

Step 2 – Calculate a set of properties that characterizes the objects

For example: fluorescence intensity of different markers, area, morphology, texture or properties of sub-objects like spots. See section 1.3.3.

PhenoLOGIC is especially useful in combination with texture or STAR morphology properties. Use the **Calculate Texture Properties** building block if there is (or if there might be) a difference in the texture between the classes. See section 1.7. Use the **Calculate Morphology Properties** – Method STAR building block, if the morphology changes. See section 1.8.

Step 3 – Classify the objects

Use the **Select Population** building block method "Linear Classifier", see *Figure 1–119*.

➤ Select the number of classes you want to discriminate.

➤ Select at least 80 example objects for each class, see *Figure 1–118*.

➤ It is recommended to "blindly" select good representative cells, not looking at previous classification results. Take examples from multiple image fields and wells.

➤ Use "Apply Changes" to create the classifier based on the selected training examples and to apply it to all cells in the image.

➤ Check if you are satisfied with the classification results. To see the classification results switch on the "Last Classification" overlay.

➤ If you are not satisfied, select more training examples and apply the changes again. For unbiased training, you might want to switch off the "Last Classification" overlay while adding more points. If this doesn't help, start over with "Reset Training".

➤ If you are still not satisfied with the results, have a look at the "Hints and Tips" section 1.15.3.

➤ Exit the training by pressing "OK".

Step 4 – Process and report the results

The **Select Population** building block returns one population per class which contains all objects belonging to that class. You can add more building blocks to:

➤ Calculate additional properties of the objects.

➤ Filter out objects (e.g. to remove artifacts).

➤ Calculate statistical information (e.g. percentage of cells in "Class A").

➤ Report on the final readout values in the building block **Define Results** (see section 1.3.6).
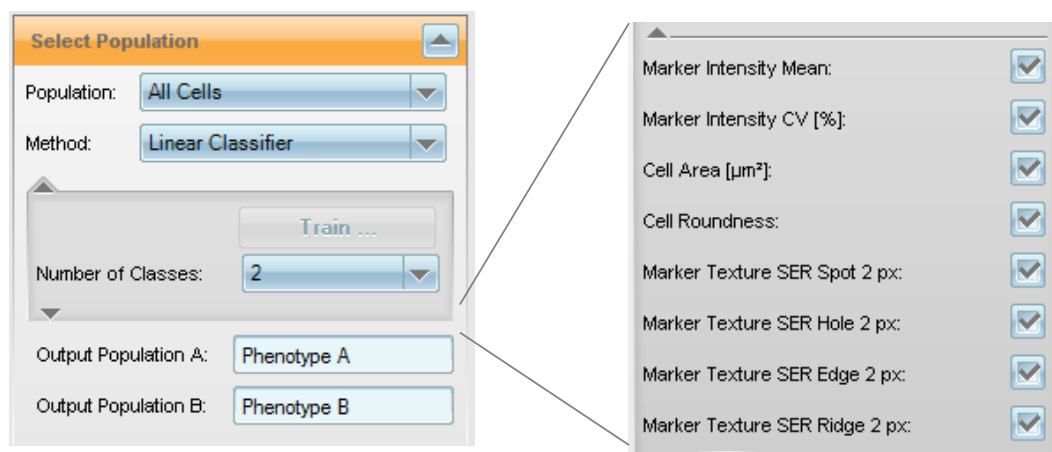
Figure 1–119: The **Select Population** building block with the PhenoLOGIC method "Linear Classifier" selected. Left: Standard parameters. Right: All of the available input parameters are displayed. The list shows all properties calculated by the preceding building blocks. All of these will be taken into account to select the best ones for classification. By de-selecting the checkboxes some properties can be disabled, e.g. if they are known to cause artifacts.

1.15.2   The Linear Classifier Method – Details

PhenoLOGIC is designed so that users do not need to worry about how it works. Sometimes, however, it is useful to know the principles behind it, for example to follow up artifacts, to extract more information than the final result, or to tailor it to very specific situations.

How does it work?

Based on the selected training objects, a linear combination of properties is identified which best separates the training samples.

In *Figure 1–120*, each training sample is represented by a solid dot; the color indicates the training class. The value of the classifier's linear combination is shown on the x-axis, a value greater than zero indicates class B (red), less than zero class A (green). Other objects in the training images are represented by a small circle and the color of the circle indicates the classified class for that object. The vertical red line divides the two classes.

Selected linear combination, goodness 3.65
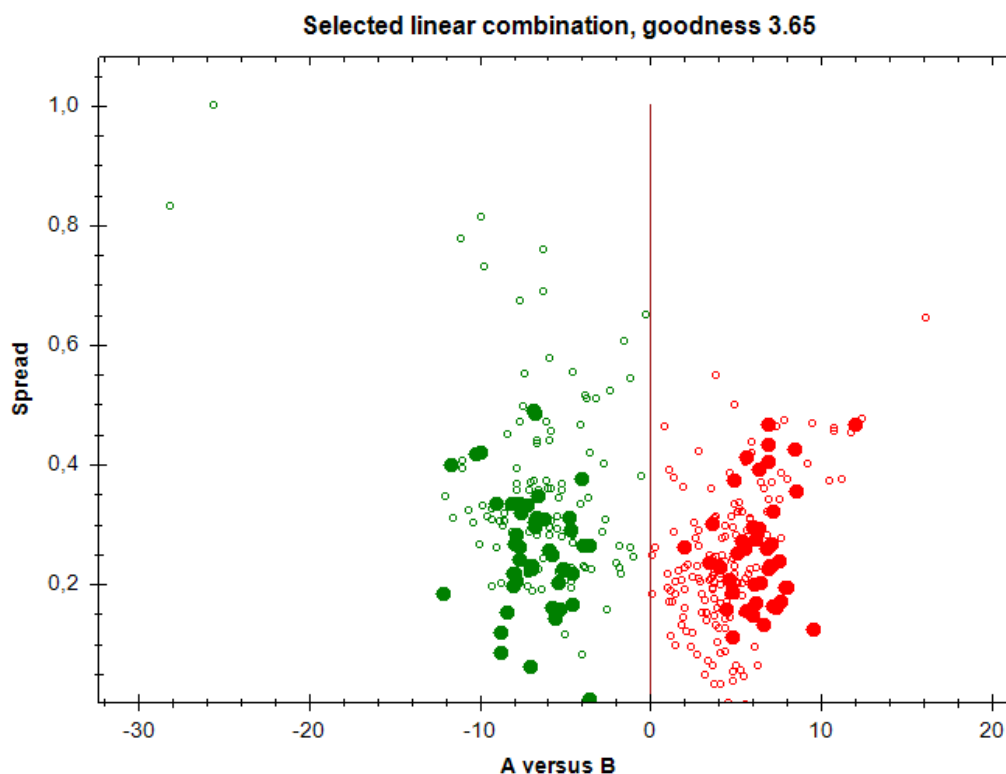
Figure 1–120: Scatter plot illustration created by the "Linear Classifier" method of the Select Population building block.

On the y-axis ("Spread") a different linear combination is shown which gives the maximum spread of the data points in y-direction for best visualization. The absolute numbers on the y-axis have no specific meaning.

In the example, we can see that the training points are very well separated by the classifier. The other data points (small open circles) form two groups that are also well separated by the classifier. A few data points are very close to the classifier line, these are either transient objects changing their state or artifacts of the classification.

This illustration is provided on the "A versus B" tab during the training. It is a good tool to judge:

➤ If there are clearly separated populations or if there is a continuous group of points.

➤ How many "outliers" there are.

➤ How well the training points represent the whole population.

If there are more than two classes, one tab is shown for each pair of classes so that the mutual separation of classes can be evaluated.

During the training phase, you can modify the selection of training points, re-calculate the classifier and examine changes on the scatter plot until you are satisfied with the separation of the classes. After finishing the training (OK or Cancel), only the final results are shown. You can re-enter the training at any time using the "Train…" button.

What do the numbers mean?

The details of the selected linear combination can be found in the corresponding table "A versus B" in the image analysis results window, see *Figure 1–121*.

Training Set	A versus B	
Goodness	**Offset**	
3,65	-1,81584	
Properties (ordered by relevance)	**Linear Coefficient**	
Marker Intensity Mean	-0,00961324	
Marker Texture SER Spot 2 px	1473,79	
Marker Texture SER Edge 2 px	97,5387	
Marker Intensity CV [%]	-0,129957	
Cell Area [µm²]	0,00146558	

Figure 1–121: "A versus B" table with linear classifier details corresponding to the scatter plot illustration *Figure 1–120*.

This information allows us to:

➤ See which object properties are used for classification and how relevant they are.

➤ Document and use the linear classifier formula in Microsoft® Excel® or other software.

In this example, five properties are relevant for the classifier. The two most important ones are the change of a mean marker intensity and the change of small spot texture property. In addition, a second texture property, an intensity variation and the cell area are used. This information allows us to identify which object properties best describe the biological effect (in this case, marker intensity change and spot texture change). They provide insight into what is happening in the object and allow plausibility checks to avoid artifacts (in this case, it is plausible that the mean marker intensity matters most).

The Classifier Formula

The exact formula for the classifier is:

$X = -Offset + Linear\ Coefficient\ 1 * Property\ 1 + Linear\ Coefficient\ 2 * Property\ 2 ....$

The linear coefficients and the offset value are listed in the table. Please note: The magnitude of the coefficients does not correspond to the absolute contribution of the properties to the classification because the properties are not normalized. The "relevance" for classification is reflected by the order of the properties in the table (most relevant ones first).

You can use the formula to document how the objects were classified. This is a feature that is unique to PhenoLOGIC™ and is not possible with other "black box" algorithms. You can also use the formula to process exported single cell data with third party software.

How many properties are used?

The best number of properties and the influence of each property on the classification result are automatically determined by the PhenoLOGIC™ algorithm. The number of properties is statistically balanced against the robustness of the classification: More properties always give better training results, but also increase the danger of adapting too tightly to small effects only seen in the training set ("overfitting"). This can give worse results for other data sets, though the training results look better.

How "good" is the classification?

The "goodness" of the separation is reported in the table and the title of the scatter plot. This is the signal to noise ratio based on the distance of the training points from the classifier line.

This value expresses, to some extent, the quality of the separation. It does not, however, provide information about the distribution of classification results – outliers, separation and shape of the populations (focused, splattered, multiple focus regions). The scatter plot is essential to fully understand the quality of the classification.

Optional Parameters

The optional parameters of the "Linear Classifier" method include a full list of all properties which are taken into account for building the classifier (see *Figure 1–119*, right panel). If one property is known to cause artifacts, or you do not want it to be used for classification, it can be excluded by de-selecting the checkbox. As an example, intensity properties may be problematic, see the "Hints and Tips" in section 1.14.3 for more information.

1.15.3 Hints and Tips

➤ In the illustrations during training, you can adjust the overlay styles used for the trained objects and classification results. Select the most appropriate overlay styles for your images. Good options are, e.g.:

- For small objects or small numbers of objects: For the "Trained Objects" use "Circle" style and for the "Last Classification" result use "Body" style, see *Figure 1–118*.

- For larger objects or large numbers of objects: For the "Trained Objects" use "Body" style and for the "Last Classification" results use "Border" style, see *Figure 2–48*.

➤ Select only training points that clearly belong to one of the classes and avoid "borderline" cases. Leave it to the algorithm to judge these cases and check that it matches your expectations.

➤ Examine the scatter plots to judge whether you have clearly separated populations or a continuous transition between the classes. Clearly separated populations are shown by two clouds of points separated by a gap. With a continuous transition, the clouds are connected without a gap. Both cases will occur depending on your assay set-up. If you expect clearly separated classes but this is not reflected in the scatter plot, then:

- Modify the training.

- Calculate more properties of the objects in the preceding building blocks.

➤ For an example RMS, see "Phenotype Classification – Cytoskeleton" section 2.23.

➤ Once the training is complete, you may remove the calculation of properties that are not used by the classifier to speed up the analysis.

➤ Linear Regression: If there is a continuous transition between two classes, you may manually enter the linear classifier formula into a **Calculate Properties** building block to determine the detailed transition state of each object (x-position in the scatter plot shown in *Figure 1–120*). See *Figure 1–122* for an example.

The linear regression value for the first two classes (A and B) is automatically calculated and added as a property "Regression A-B" to the input population.

PhenoLOGIC classification can be used for kinetic populations, i.e. tracked moving objects (see section 1.16). In this case please make sure that you first select a proper time window and then select all training points using this time window. Don't change the time window during the training. In order to re-enter the training, select exactly the same time window you used for the initial training.

Figure 1–122: Example of a linear regression: the linear classifier formula values shown in the property table in *Figure 1–121* have been manually entered in a **Calculate Properties** building block, method "By Formula" (1), to add the linear classifier value as a property to each classified object (2). The object property table was sorted by the classifier value (by clicking on the column title) and an object with a classifier value near zero has been highlighted (orange circle) by clicking on the corresponding line in the table. This object is very close to the transition line between class A and B.

Why not calculate all possible object properties and then use the linear classifier to select the best?

In principle this works and you can do this in Harmony, but there are two potential drawbacks:

➤ You might miss the chance to include your pre-existing knowledge into the analysis. This makes it less robust and prone to artifacts. For example, you know from looking at the images and your assay biology that the nuclear stain intensity of the phenotypes will vary, but that this is not relevant for the classification in your assay. Using it in the "black box" analysis might make use of this property, even though you already know that it is an artifact.

➤ It can significantly increase calculation times. This is particularly true of the texture and STAR morphology parameters.

Why is a linear classifier used?

It is very robust, widely applicable and has very good performance for all assay types presented here. It is relatively easy to understand how the results are created (no "black box"). In our research, the performance gained by more sophisticated algorithms for specific situations did not justify the trade off in robustness.

How many training points do I need?

There is no simple general truth for this. Having more than 100 training points per class should work well for most applications.

Here is an example to highlight how the number of training points influences the classification quality:
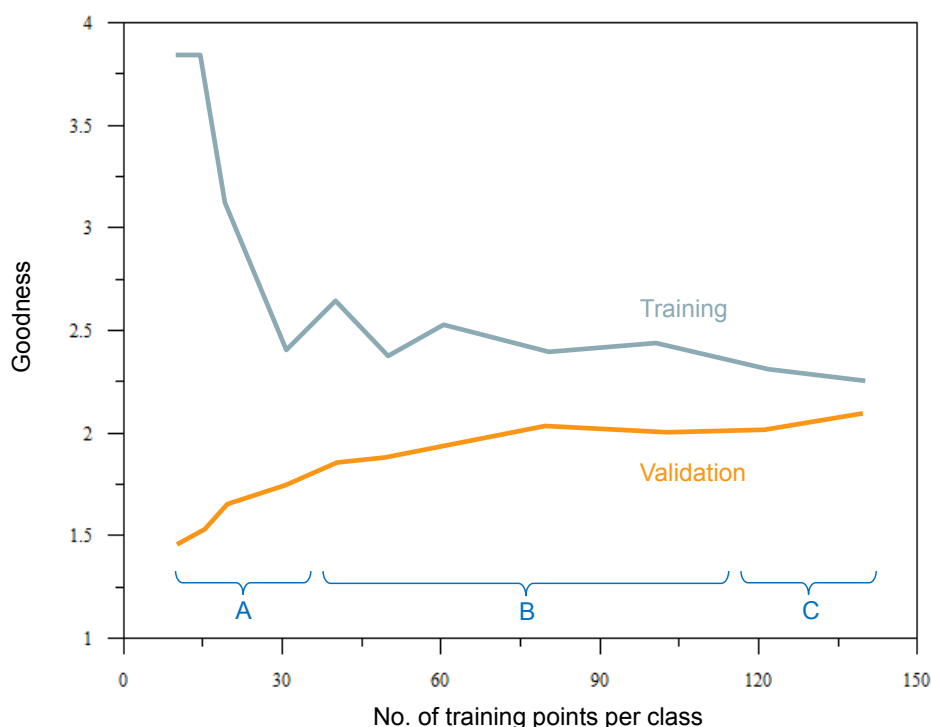
Figure 1–123: Example illustrating the dependency of the classification quality on the number of training points.

STAR morphology properties for a set of about 2000 cells of two different cell types were calculated. The software was trained using the linear classifier method with various numbers of randomly chosen training points from both classes (training set). The goodness values for the training set are shown in the blue curve in ***Figure 1–123***.

The classifiers were then applied to the rest of the cells that were not used in the training (validation set) to identify the cell type of each cell. The goodness values are shown in the orange curve in ***Figure 1–123***.

For a low number of training points (region "A"), the training set can be very well described by the classifier (high goodness value in the training), but applying this classifier to the full dataset gives low goodness values (validation). The training points do not reflect the full dataset well enough, the classifier has adapted to features only seen in the training set, but not the full dataset.

In region "B" the training set better reflects the full sample. The training goodness value is lower, but the validation shows that the classification of the full dataset is better.

In region "C" the training goodness converges smoothly towards the validation goodness. Adding more training points only very slightly improves the classification results.

➤ The number of training points should be in the "higher B" or the "C" region.

➤ Don't be confused if the goodness value decreases when adding more training points. The classification results will improve.

➤ The training behavior might look very different for different datasets. It depends on many factors such as:

- Variations in the dataset.

- Systematic artifacts in the dataset.

- How well – or if at all – the classes can be discriminated by the specified set of features.

- How diverse the classes are.

- If there are more classes visible in the dataset than specified in the training (e.g. mitotic cells).

➤ If you see a qualitatively different behavior in your training:

- Make sure that the set of training points does not contain points where you are not 100% sure that they belong to the specified class.

- Try to extend the set of calculated properties. Maybe the properties do not provide sufficient information for the classification. How do you visually distinguish the classes? Is there anything you see in the image that is not reflected by the calculated set of properties, e.g. morphology or texture features?

- Try to reduce the set of calculated properties. Are there any properties that are definitely not relevant for the judgment of the class? Use this knowledge and remove these properties.

1.16　Object Tracking

Object tracking is indispensable for studying the kinetics of biological processes on a per object basis. Object tracking generates a connection ("track") for a particular object over a number of time points. Object tracking can, for instance, be used to:

➤ Follow biological processes over time on a single cell basis (e.g. translocation of transcription factors or cell division).

➤ Analyze the migration of cells (e.g. Chemotaxis or Chemokinesis assays).

For an example of a ready-made analysis sequence using object tracking, see the RMS "Cell Tracking – Migration Analysis" (chapter 2.29) and RMS "Cell Tracking – Cell Division" (chapter 2.30).

Requirements

➤ A population of objects must have been identified, e.g. by Find Nuclei.

The measurement must consist of at least 2 time points with an appropriate imaging interval that results in a sufficient overlap of the same object over 2 consecutive time points (see *Figure 1–124*). Please refer to the Application Guide, chapter 2.4.6 "Measurements for Object Tracking" for recommendations on measurements for object tracking experiments.
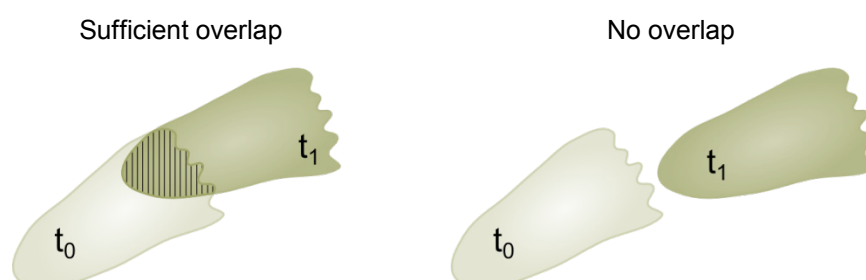
Figure 1–124: Object Tracking in the Harmony Software is based on the overlap of the same selected region of an object between two adjacent time points. The hatched area indicates the object overlap in the left example.

1.16.1　Step by Step Set-up of the Analysis

Step 1: Finding Objects

Before tracking objects, the image has to be segmented using building blocks such as **Find Nuclei** or **Find Cells**, which create an object list. The quality of the initial segmentation is crucial for successful object tracking as segmentation errors can result in lost object tracks later on. Potential reasons for segmentation errors that occur during time series measurements are as follows:

➤ Increasing cell density due to cell proliferation

➤ Bleaching of fluorescent dyes that are used for segmentation

Therefore, it is highly recommended to check the image segmentation parameters on early and late time points in the time series. If the images contain artifacts that interfere with object tracking, it can be beneficial to remove these artifacts from the object list using the building block **Select Population**. For more information on image segmentation and object list manipulation see section 1.3.

Most common nuclear dyes exhibit comparatively low photo bleaching and are excellently suited for image segmentation due to their well-defined staining pattern. However, nuclear dyes are often toxic and therefore not well suited for longer live cell measurements. An alternative is digital phase imaging, which does not result in any intensity loss due to photo bleaching and minimizes phototoxicity.

Step 2: Tracking Objects

Next, add the **Track Objects** building block, define an input population and region, and select an appropriate time window. The selected region will be used to measure object overlap between adjacent time points of the time window. After the calculations for object tracking have finished, the determined object tracks will be shown in the image display area.

Defining a very large time window will result in longer calculation times. It is recommended to use up to 10 time points to optimize tracking in the image analysis process.
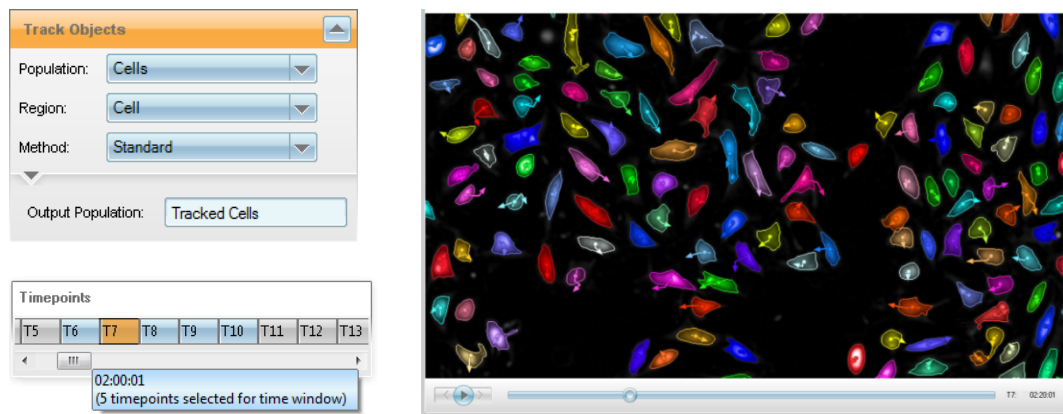
Figure 1–125: Example screenshots of the Harmony software user interface showing the **Track Objects** building block (upper left), the selected time window (lower left) and the image display area with the tracks overlaid on the cell masks (right).

If the quality of the cell tracking is insufficient consider the following steps:

➤ Improve image segmentation by adjusting parameters, e.g. in the **Find Nuclei** building block. Focus rather on clear object separation than on perfect detection of object outlines.

➤ Reduce the number of seeded cells to reduce the number of segmentation errors.

➤ Shorten the imaging interval to reduce the number of tracks lost.

Step 3: Calculate Properties of the Tracked Population

Once object tracking is properly set up, the numerical properties of the tracked objects can be calculated. This includes standard properties like object morphology, intensity or texture, and time-dependent properties like current speed. The latter are calculated by the **Calculate Kinetic Properties** building block. *Figure 1–126* illustrates how the properties are calculated by this building block.

➤ **Current Step Size** = $(s_{-1} + s_{+1})/2$

➤ **Current Speed** = $(s_{-1} + s_{+1})/(t_{+1} - t_{-1})$

➤ **Current Turning Angle** = α

➤ The coordinate system underlying the **Current Direction** is depicted in the right schema in *Figure 1–126*.
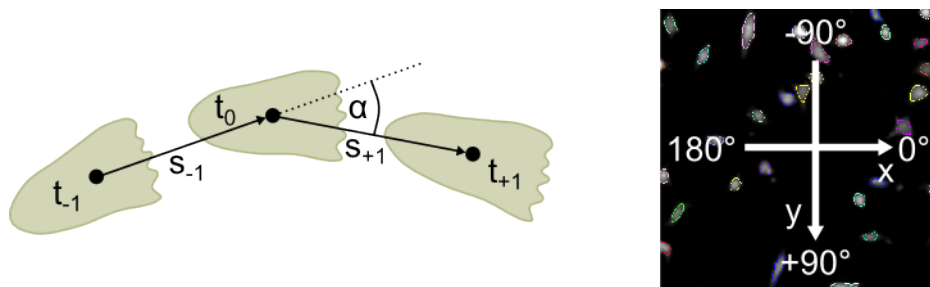
Figure 1–126: Left: The schema depicts three time points of a cell trajectory. Time points -1 and +1 are needed to calculate kinetic properties for the time point 0. Right: Orientation of the image axis. The origin of the image coordinate system is in the upper left image corner.

There are three time-dependent object properties that are already calculated by default by the **Track Objects** building block:

➤ **Age [s]** is the time that has passed since the object was first observed.

➤ **Current Displacement X** and **Current Displacement Y** are the X and Y components of a vector from the first observation to the object position at the present time point.

Step 4: Calculate Properties of the Entire Track

In addition to object properties that are calculated per object and time point, properties for the entire object track can also be calculated, e.g. maximum object intensity over time or accumulated distance. These properties are stored in a separate results table. To calculate such properties, add the **Calculate Track Properties** building block. This building block has two available methods:

➤ **Standard** method can calculate different properties reflecting the object movement over the entire observation time.

➤ **Time Aggregation** method calculates a statistical property for the entire track derived from object properties that have been calculated at each time point, e.g. the maximum object intensity over time or the mean object roundness over time.

Figure 1–127 illustrates how the properties are calculated by the **Standard** method:

➤ **Displacement X** and **Displacement Y** are the X and Y components of a vector from first observation to the last observation.

➤ **Displacement** is the length of a vector from the first observation to the last observation.

$$Displacement = \sqrt{DisplacementX^2 + DisplacementY^2}$$

➤ **Accumulated Distance** is the length of the object track.

$$Accumulated\ Distance = \sum length\ track\ segments$$

➤ **Straightness** $= Displacement/Accumulated\ Distance$

➤ **Speed** $= Accumulated\ Distance/Observation\ Time$
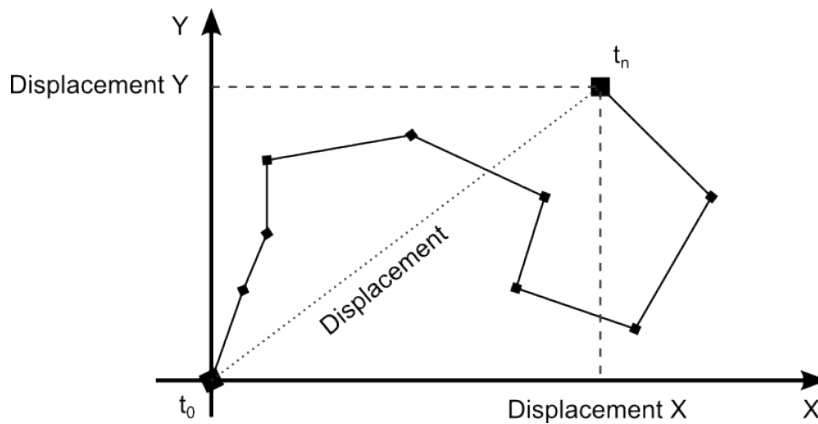
Figure 1–127: Schema of an object track. The black line indicates the object movement and the observation time points are indicated by diamonds. The calculation of the standard track properties is always based on the movement relative to the first time point where the object was detected (t_0).

There are several track properties that are calculated by default by the **Track Objects** building block:

➤ First and last time point of object observation

➤ Start and end time of object observation

➤ Number of time points at which the object was observed

➤ Duration [s] that the object was observed

➤ Start type (see *Figure 1–128*) and End type (see *Figure 1–129*)

➤ Generation, Parent ID and Root ID provide genealogical information for each object (see section 1.16.2 for further information)

Start		Start from the beginning
Border		Enter at the image border
Cosmos		Appear out of nothing
Split		From split (e.g. mitosis)
Merge		From merge (e.g. cluster formation)
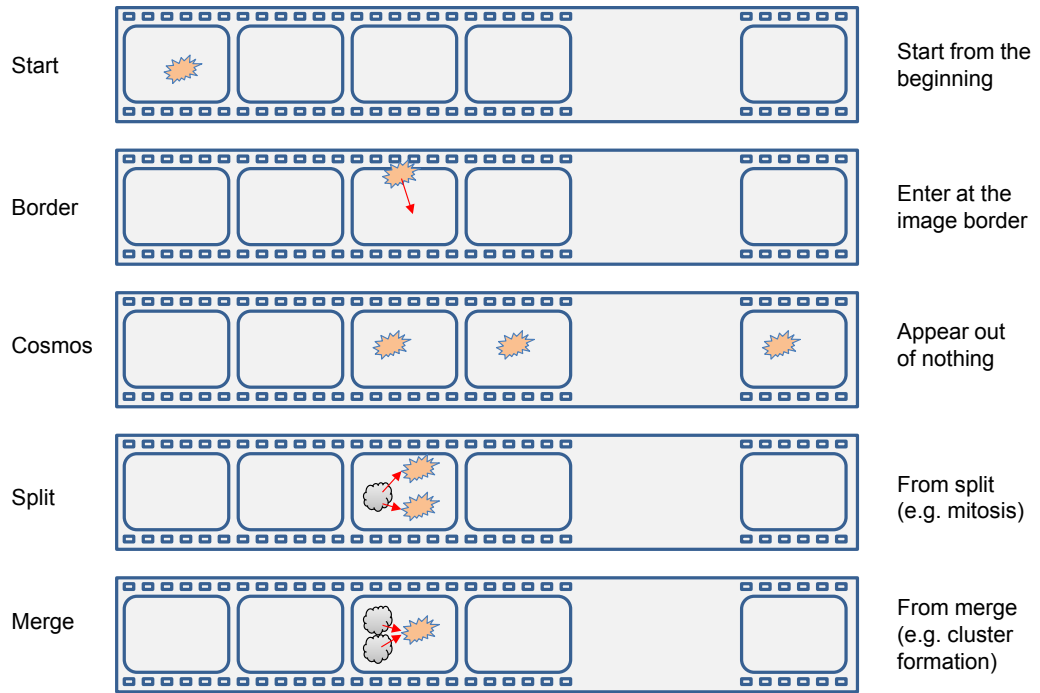
Figure 1–128: Scheme of the different start types of an object track. The depicted start type on the left always refers to the start type of the red objects.

End		End on the last frame
Border		Leave at the image border
Cosmos		Disappear (e.g. apoptosis)
Split		Splitted (e.g. mitosis)
Merge		Merged (e.g. cluster formation)
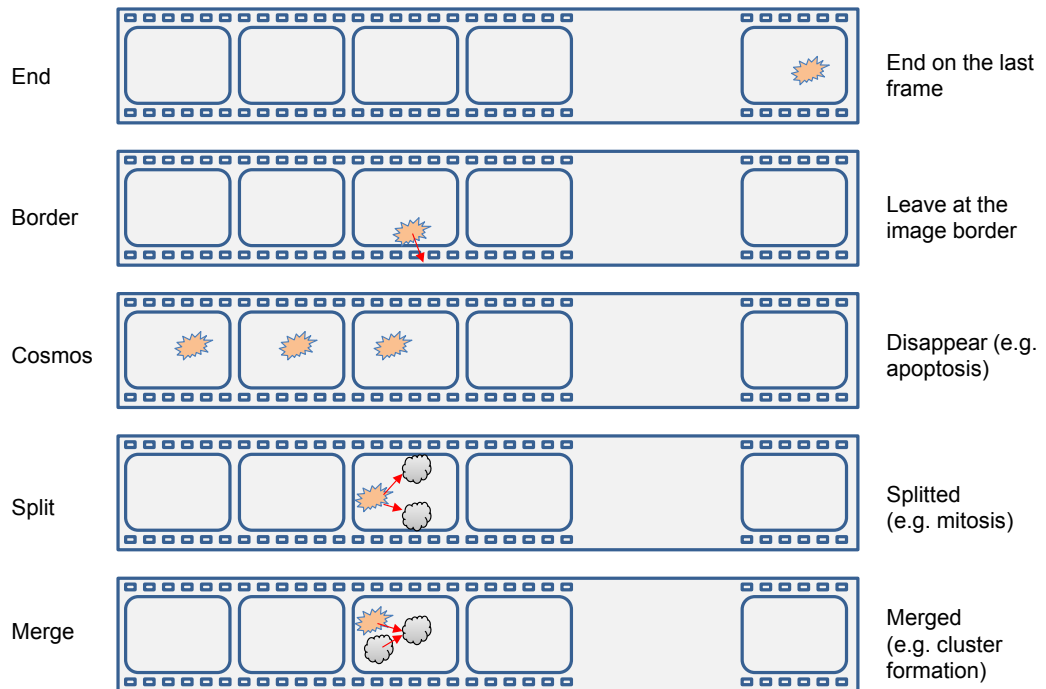
Figure 1–129: Schema of the different end types of an object track. The depicted end type on the left always refers to the end type of the red objects.

Step 5: Define Results

The **Define Results** building block is described in detail in section 1.3.6. Having populations of tracked objects will result in small extensions of this building block:

➤ The List of Outputs method will provide two blocks of properties for the tracked population. One block contains properties calculated for each time point (object results) and the other block contains properties calculated for the entire object track (track results).

➤ The Formula Output requires you to select the class of results you want to use to calculate a derived output. You can select either track results or object results.

➤ The image analysis results per object and per track can be accessed via tabs in the image analysis results area.

Figure 1–130 shows an overview on the **Define Results** building block and the result tables.

Figure 1–130: Screenshots from the Harmony Software showing the **Define Results** building block (left) and the obtained results tables (right). The blocks *Population: Tracked Cells* and *Tracks: Tracked Cells* contain the 2 classes of properties that were calculated on one tracked population (1). The drop down list in the Formula Output allows you to select which class of properties should be used for calculation (2). All calculated track properties and properties per object are stored in separate data tables (3).

1.16.2 Object Tracking – Details

Basic Principle

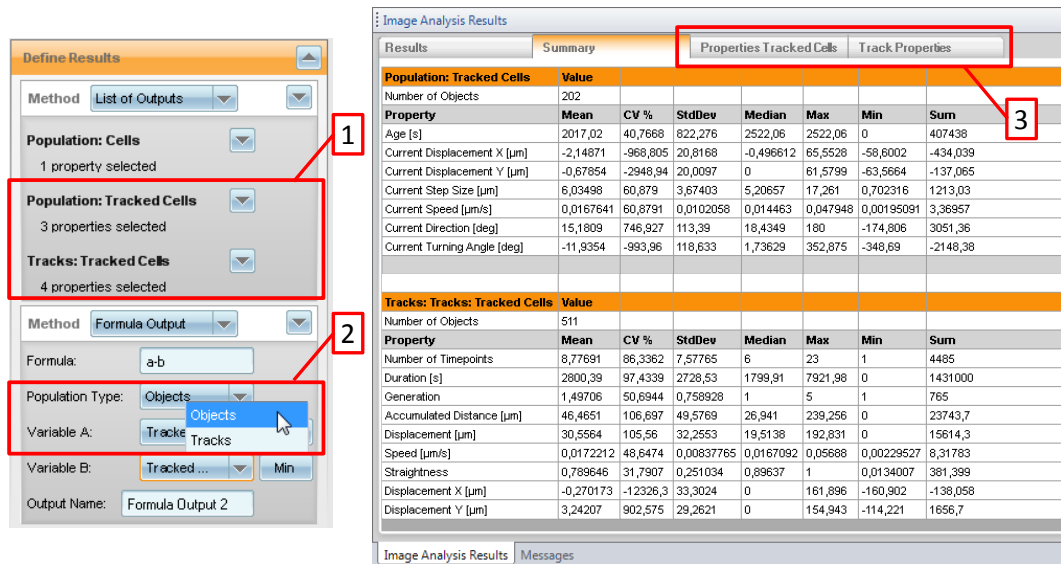During image segmentation an **Object Number** is assigned to each object based on its position in the field of view. Due to object movement, the assigned ID can change over time. The **Track Objects** building block correlates the objects through all time points and assigns a new object number that is constant over all time points to each object (see *Figure 1–131*). Consequently, the track that is associated to each object will have the same **Track ID**.

The output population generated by the **Track Objects** building block is a **Kinetic Population**. In the Harmony software kinetic populations have some special properties:

➤ The **Select Population** building block does not create sub-populations of kinetic populations. Instead, using this building block it is possible to create an object flag, which indicates whether the object meets a user-defined criterion or not.

➤ Kinetic populations have two associated result tables. One table contains the results for an entire object track, the other contains results per object and time point.

➤ Upon generation, kinetic populations lose all the associated object regions apart from the one that was used for object tracking.

➤ Kinetic populations do not inherit any properties from their parent population.

➤ Kinetic populations cannot be related to their parent populations. Consequently, it is not possible to transfer properties from the parent population via the **Calculate Properties** building block using the method *By Related Population*.
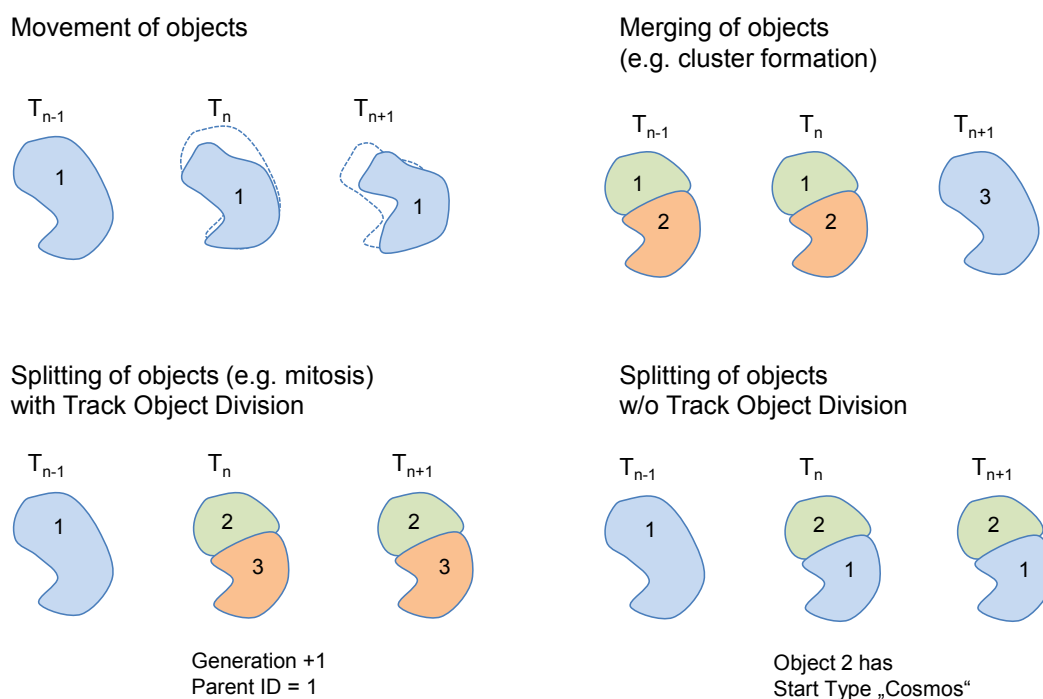
Movement of objects

Merging of objects
(e.g. cluster formation)

Splitting of objects (e.g. mitosis)
with Track Object Division

Splitting of objects
w/o Track Object Division

Generation +1
Parent ID = 1

Object 2 has
Start Type „Cosmos"

Figure 1–131: Assignment of object numbers for different scenarios.

Parameters of the Building Block Track Objects

By default, the following three parameters are activated in the building block to allow genealogical analysis, to improve tracking quality and to remove noise from the results table:

➤ **Track Object Division**: If activated, each daughter object receives a new object number and the object number of the parent object is saved as a property of the daughter object in the track properties table (see *Figure 1–131*). This information can be used to perform a genealogical analysis (see *Figure 1–131*). Assignment of the parent object number will only work if an objects splits into two daughter objects. Splitting into more than two objects will not be considered as a regular object division and so daughter objects will be classified as starting from *cosmos*. If Track Object Division is deactivated, the smaller daughter objects will receive a new object number and start from "cosmos". No genealogical information will be stored (see *Figure 1–131*).

➤ **Correct Detection Errors**: If activated, the Harmony software will correct segmentation errors by comparing the segmentation of an object at a given time point with the object segmentation on the two adjacent time points (see *Figure 1–132*). Error correction is based on the assumption that splits or merges of objects that last for one time point only are always errors.

➤ **Discard Single Timepoint Tracks**: If activated, the Harmony software will remove tracks from the results tables that have a duration of one time point. Such short tracks are usually created as a result of segmentation errors or artifacts in the image (e.g. cellular debris, serum ingredients). However, if a short track is defined as a parent object, it will not be removed from the result tables.
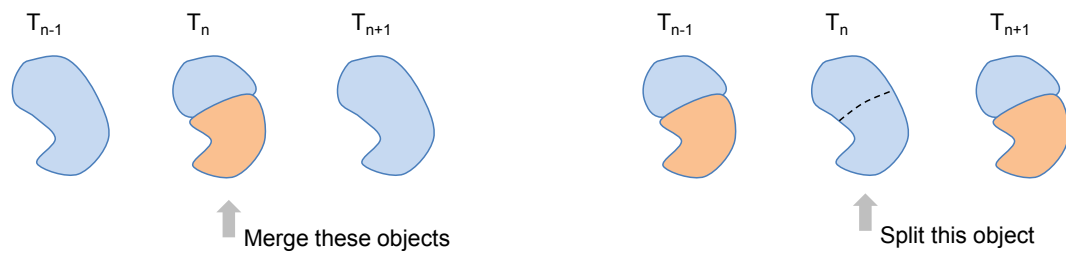
Image Analysis Guide

Figure 1–132: Correction of segmentation errors during object tracking.

1.16.3 Object Tracking – Advanced Hints and Tips

Working with Flags and Sub-populations

The **Select Population** building block does not create a true sub-population of a kinetic population. Instead, it will flag each object at each time point to indicate if the criteria of the **Select Population** building block have been met. For instance, all cells that respond to compound treatment by an increase in a fluorescence signal could be flagged. The flag will only be set to 1 for the time points where the signal exceeds the threshold defined in the **Select Population** building block. Use the **Calculate Track Properties** building block to mark the entire track of cells that have responded with a fluorescence increase:

➤ Select method *Time Aggregation.*

➤ Set the aggregation type of the flag property to "Max".

➤ The entire object track will be flagged as a responder if the threshold of the **Select Population** building block was exceeded at at least one time point.

Alternative Approaches to Image Segmentation

In a standard assay, the first step is usually to segment an image, e.g. by a combination of the building blocks **Find Nuclei**, **Find Cytoplasm** and **Find Spots**. If the **Track Objects** building block is used after the image segmentation is complete, the tracked population obtained will only contain the region that was used to measure the overlap between adjacent time points. All other regions are lost because objects might be splitted and merged by tracking error correction.

As a consequence, this approach is no longer possible to calculate ratios of properties between different cellular regions, e.g. the ratio of nuclear and cytoplasmic transcription factor NF-κB as in *Figure 1–133*. To avoid these limitations, you may:

➤ Add your first image segmentation building block (frequently **Find Nuclei**).

➤ Add the **Track Objects** building block, thereby generating a tracked population.

➤ Proceed with segmentation (e.g. **Find Cytoplasm**) using the tracked population.

The drawback is that the smaller *Nuclei* region may perform worse in the overlap-based tracking and a higher sampling rate might be necessary.

Alternatively, you can:

➤ Segment the region best suited for tracking, e.g. the cell region.

➤ **Track Objects** using that region. This will create a kinetic population only containing this region, e.g. the cell region.

➤ Use the kinetic population as Region of Interest (ROI, see section 1.10) for further segmentation of the tracked objects, e.g. use **Find Nuclei** or **Find Spots** in the cell region.

The drawback of this strategy is that some segmentation steps may need to be done twice – before tracking and after tracking – resulting in slower analysis.

As a third option, you may create new regions in the tracked objects by using regions of the original population as a mask within the kinetic population's tracking region, for example:

➤ Use **Find Nuclei** and **Find Cytoplasm** for segmentation.

➤ Use Track Objects to create the kinetic population, e.g. using the Cell region as tracking region.

➤ Use the **Select Region** method Restrict by Mask to "cut" the original nucleus region (before tracking) out of the cell region of the tracked cells. As a result, this region is added as a new region to the tracked cells population.

This method is very fast. The drawback is that regions in objects which have been corrected by the error correction (splitted or merged) may be inaccurate. They are not re-segmented in the splitted or combined object but are a combination of the segmentation in the original objects.
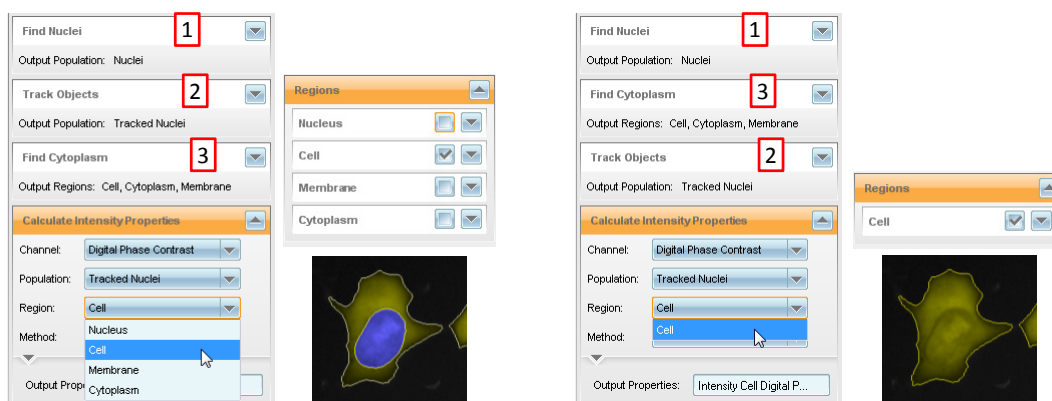

Figure 1–133: Comparison of two analysis sequences based on identical building blocks but in a different order. While there are 4 available regions in the tracked population, if the **Find Cytoplasm** building block is used after tracking (*left*), there is only one region available, if the very same building block was used before object tracking (*right*). In this example, the regions *Nucleus* and *Cell* were used for object tracking in the left and right example, respectively. In the left example, the ratio between nuclear and cytoplasmic fluorescence can be calculated, whereas this is not possible in the right analysis sequence.

Interconnecting Track Results and Single Object Results

Object tracking generates two classes of numerical results:

➤ Single object results that are calculated per object per time point.

➤ Track results that are calculated once per object for the entire time series.

Both classes are stored in different data tables that are interconnected by the Track Number and Object Number properties, respectively. On the Harmony software's Evaluation Screen both result tables are accessible. Highlighting a track result in one table will automatically highlight all corresponding lines in the single object results table (see section 1.5.5). Likewise, a combination of row, column, plane, field and object number can be used to interrelate both exported data tables in any external secondary analysis software (e.g. TIBCO Spotfire®).
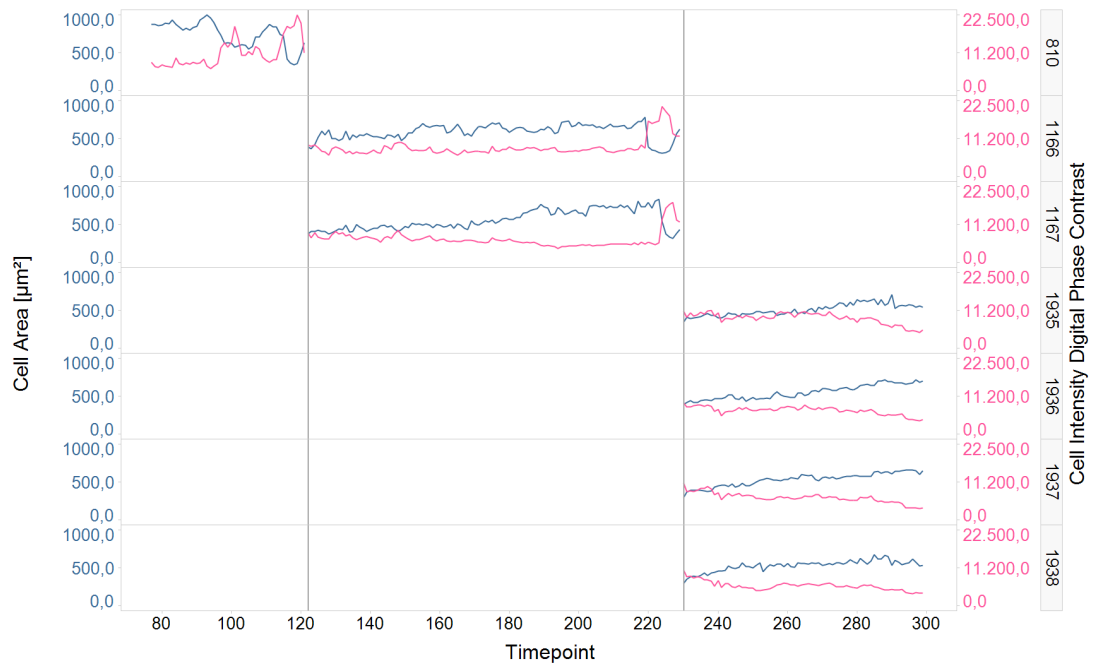
Figure 1–134: Analysis of three cell generations using TIBCO Spotfire® software. The vertical grey lines indicate cell division. Plotted are the cell area (blue) and the cell intensity in a digital phase contrast image (red). Note how the cell area decreases and the phase contrast signal increases prior to mitosis. The two daughter cells of the 2nd generation divide synchronously. Object numbers are depicted in the grey rectangles to the right. The plot combines information from the single object results table (area and intensity versus time) with information from the track results table (object number, generation, root ID).

1.17   3D Analysis

The ability to acquire multiple images in the same image field but at different z-heights (image stack) allows the capturing of 3D information. This section gives advice on how to analyze these stacks and how to adjust the acquisition parameters for best performance.
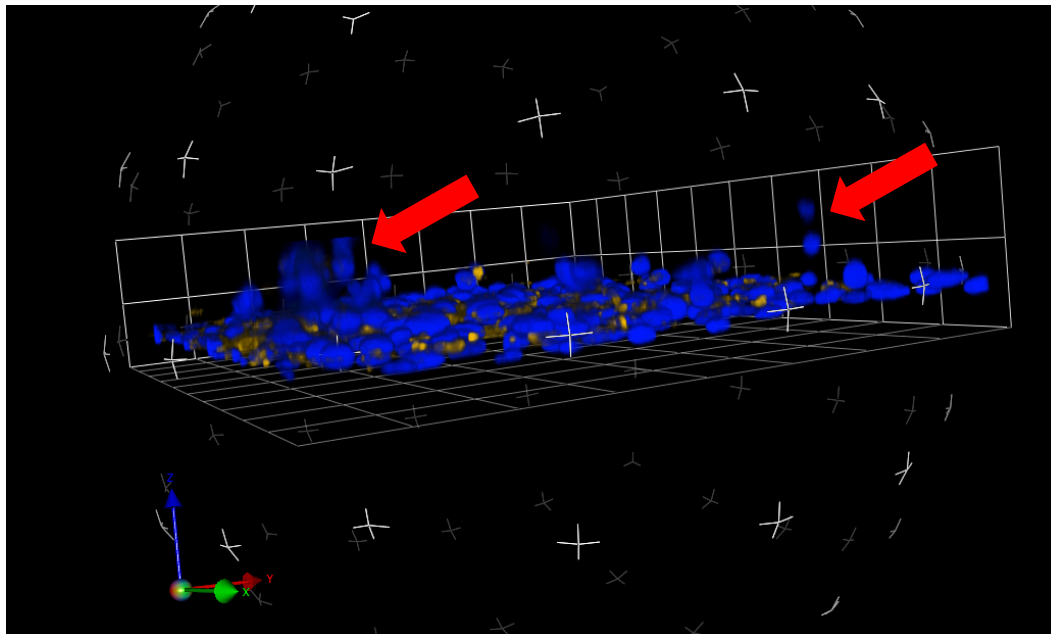

Figure 1–135: Volocity software 3D view, showing cells invading into a gel. The plate bottom is located in the x-y-plane indicated by the green and red arrows of the orientation indicator in the bottom left of the image. Cells growing adherently are topped with a gel matrix into which some cells are migrating (in the z-direction). At the locations marked by red arrows, groups of cells move into the gel. Hoechst 33342 stained HT1080 cells were imaged with a 40x High NA objective in confocal mode using the Operetta system with 50 z-planes with 1 µm spacing, and exported into Volocity software for 3D visualization.

3D Data Visualization

Before analyzing the 3D data, it is very important that you study the images carefully and make sure that the expected effects are clearly visible in the images. Be aware that the resolution in the z-direction is far more limited than in the x-y direction, especially for non-confocal images and low NA objectives (see ***Figure 1–136*** for an example).

Here are the options to explore your images:

Using Harmony:

➤ Plane-by-plane using the stack navigation control in any of the screens. See, for example, ***Figure 1–138 –*** (2).

➤ Stack montage overview (similar to the well montage in ***Figure 1–12*** but with stack planes).

➤ XYZ View: When "Stack Processing" in the **Input Image** building block is set to "Maximum Projection" a new "XYZ View" tab is added to the image view. It shows an xy-, xz- and yz-section through the image stack. Section planes can be interactively moved by mouse clicks.

In-depth interactive 3D visualization and analysis using the Volocity® 3D Image Analysis Software:

➤ Drag and drop a selection (wells, fields, stack planes, time points, etc) of your measurement from the Harmony navigate control into Volocity software. You could, for example, grab the well in *Figure 1–138* – (1) and drag it into Volocity to transfer the selected fields and stack planes.

➤ Export a measurement and import it into Volocity using the "File>Import" menu in Volocity. See section 1.5.7 on how to export images.

A useful tool for assessing what is visible in the images are the xz and yz-sections through the image which are created by Volocity, see *Figure 1–136*. Use these illustrations to find out the following:

➤ What features are visible in the images.

➤ How many planes you need to capture all information without redundancy.

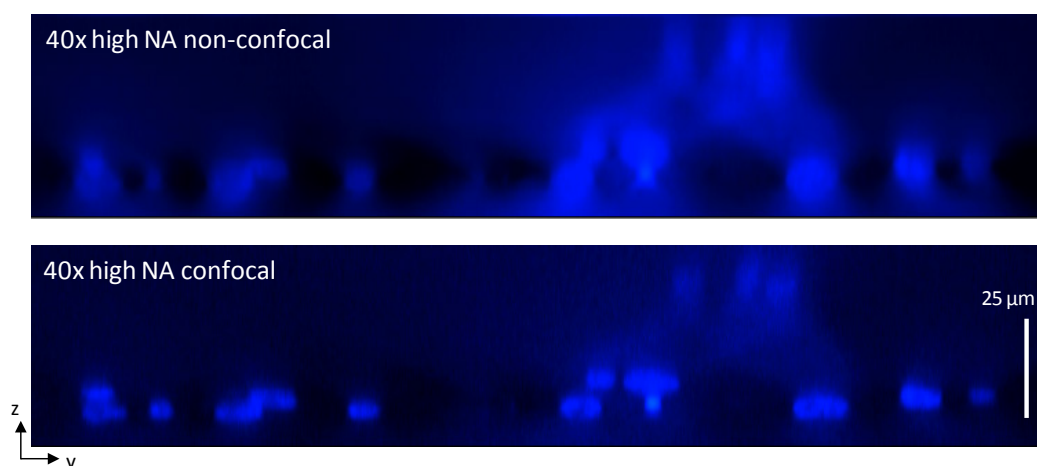➤ What is a suitable z-range to use.

Figure 1–136: Example of the z-resolution of confocal versus non-confocal imaging. The image section is shown in the y-z direction (z=up, y=right) through the 3D image shown in *Figure 1–135*. The sections can be interactively created by Volocity.

Considerations to Optimize Image Quality for Analysis

➤ For the theoretical z-resolution of the different objectives, see "Objectives" table in the appendix of the Application Guide. In practice, you will usually find more blurring in the z-direction due to the use of air objectives and limitations of the plate bottom flatness of microplates. Use an image stack, and Volocity, to see how your particular set-up looks.

➤ High NA objectives give better z-resolution.

➤ Confocal images have much better z-resolution.

➤ The deeper you focus into the well, the worse the z-resolution gets due to the principle air objective limitations, see *Figure 1–137*.

➤ Consider the plate bottom flatness of the microplates. For example, for a 10x objective the Operetta field of view is 1.351 mm wide. Within that distance, the plate bottom of some plates, and so the absolute height of your stack planes, could vary by up to 10 µm in the worse case scenario. See section "Plate Planariy" in your instrument's Application Guide for information on plate planarity.
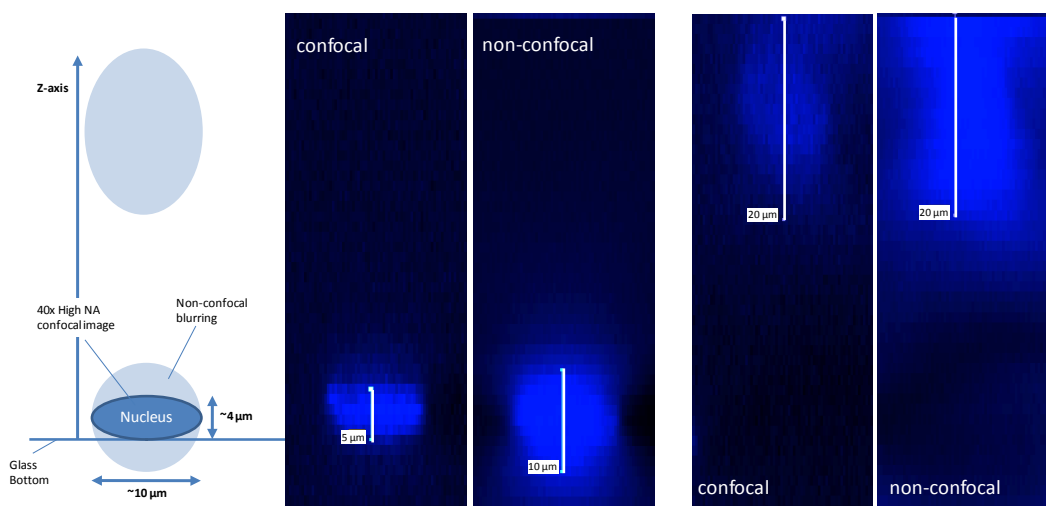
Figure 1–137: Limitation of the z-resolution at higher planes. The image shows detail of two Hoechst 33342 stained nuclei at different heights over the plate bottom, imaged with a 40x high NA objective on the Operetta system. The sections were created using Harmony stacks of 50 planes with 1 µm spacing, and were exported to Volocity software.

3D Data Analysis Options and Strategy

There are two different ways of analyzing 3D data with Harmony. Both are based on reducing the data to two dimensions and then running the 2D analysis:

➤ Plane-by-plane analysis to quantify image changes as a function of z-height, for example to quantify cell invasion into a gel. See *Figure 1–138* for an example.

➤ Projection of the z-stack to one plane. This is used to increase the depth of focus by projecting objects (e.g. nuclei) in different z-heights back to a single image plane before analysis. See *Figure 1–140*. Object z-position information can be obtained by identifying on which plane the highest intensity pixel was found.

Note: Full 3D segmentation of the image stack into 3D objects is not provided by the Harmony building blocks. Please use Volocity for this task.

The full 3D segmentation is usually only needed to study certain samples in detail during assay development and set-up, target identification or follow-up studies on HTS hits.

For a statistical compound screening approach which aims at the analysis of 200…2000 cells per compound and dose, and usage of batches of 96 or 384 well plates, a simplified assay set-up is more suitable.

Analysis of Representative Individual Planes

See *Figure 1–138* for an example of a cell migration analysis based on the acquisition and analysis of a few representative z-planes. The migration of cells from the plate bottom into a gel is characterized by counting the nuclei in 3 different z-heights above the plate bottom. *Figure 1–136* shows that confocal imaging is essential for this approach and gives information about which height the planes are placed best.

For a screening application, make sure the effects of interest are measured using as few image planes as possible to achieve short measurement times. For example, when using 50 image planes instead of one, the measurement time will increase by a factor of 50.

We recommend starting with a high number of planes (e.g. 30...50) and using Volocity to get a detailed overview of the cells and which features can be optically resolved, especially in the z-direction. Next, select a set of representative planes that are sufficient to quantify the effect of interest. For example, for counting the cells in different heights using 3...5 planes at a distance equivalent to two times the z-resolution of the chosen objective should be sufficient.
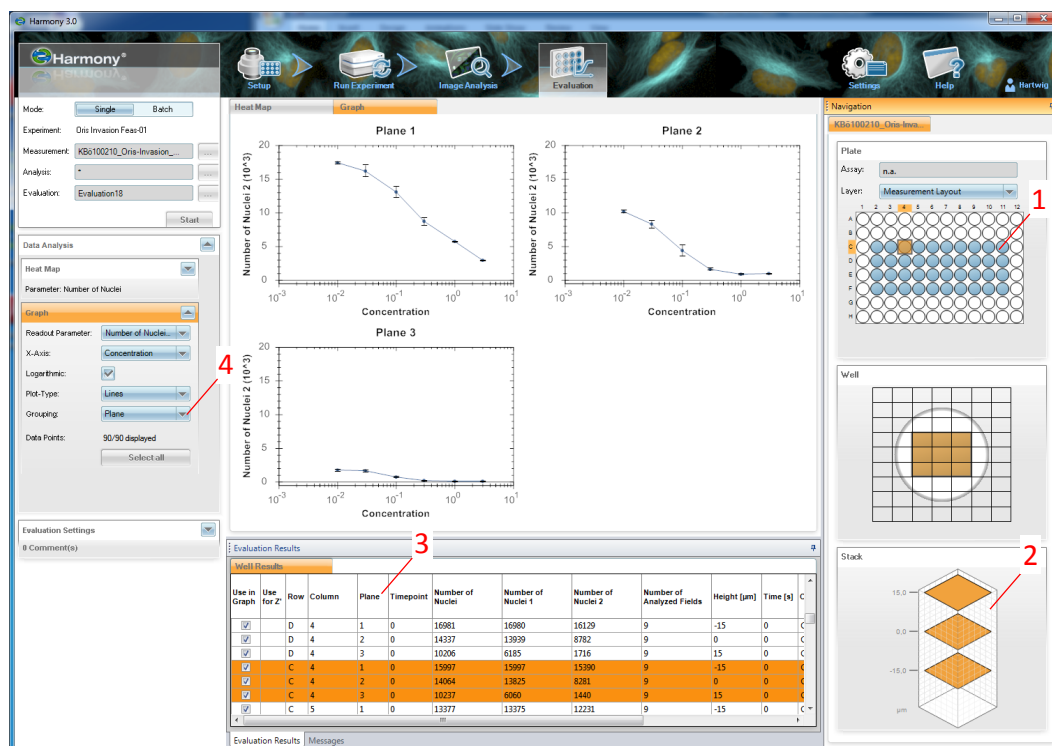

Figure 1–138: Example Harmony GUI screenshot of a 3D analysis of cell invasion into a gel. Images are acquired in 3 different heights over the plate bottom level and nuclei are counted in each plane. The plate contains four replicate dose response curves of compound treatment, with an increasing concentration from left to right (1). The "Navigation" control allows the selection of the image planes (2). The well result table lists the individual plane results as separate lines (3). The graph view is set-up in this example to show the dose response curve for the number of cells for each of the planes in a separate graph, the "Grouping" parameter is set to "Plane" (4). Two effects are visible here, the cytotoxic effect of the compound (decrease in cell number with increasing concentrations) and the decrease of cell migration into higher planes, which starts to decrease even at lower concentrations (number of cells in the top plane "Plane 3" drops faster than the cell number in the bottom plane "Plane 1").

Single Cell Results

Single cell results are also available for z-stacks. See *Figure 1–139* for an example. Single cell results were recorded by the analysis sequence during evaluation (expand section "Object Results" in the Define Results building block and select "All" or "Use Selected Well Results" for one or multiple populations, see section 1.5.4) and exported (see section 1.5.7). Microsoft® Excel® was used to create the illustration of cell invasion in one image field (675 x 509 µm): each cell is represented by a data point and the height of the cell is color coded. Yellow indicates cells which are adhered to the plate bottom and red indicates cells which have migrated to the top plane, 26 µm above the glass bottom. It can be seen that cells migrate in groups at various locations within the image field.

Further statistical analysis can be done to characterize the cell migration and to study the influence of many compounds on the migration in a high throughput screening (HTS) assay.
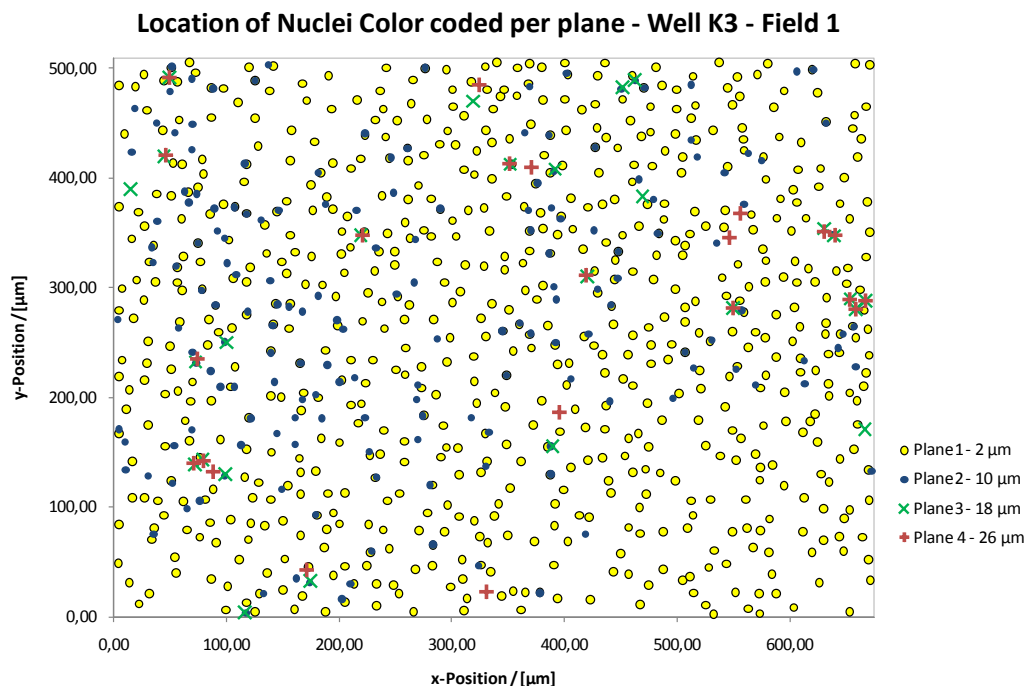
Location of Nuclei Color coded per plane - Well K3 - Field 1

Figure 1–139: Example of cell invasion analysis on a single cell basis. One image field is shown with each dot representing one cell. The color encodes the plane in which the cell was detected. Images were acquired with a 20x high NA objective on the Operetta system.

Analysis of a Maximum Projection of the Stack

➤ The maximum intensity projection creates two new images from a stack of images, see *Figure 1–140*:

- Projection Intensity: Each pixel of the intensity image is set to the maximum intensity found in the corresponding pixels in the image stacks.

- Plane Map: Each pixel of the plane map image is set to the number of the plane in which the maximum intensity was found.

➤ Maximum intensity and plane map are available for further analysis as input channels in the following building blocks.

Typical Workflow:

➤ Select stack processing option "Maximum Projection" in the **Input Image** building block to create a maximum intensity projection of the selected stack planes.

➤ Select stack planes to take into account for the projection in the navigate control, see *Figure 1–138*, number (2).

➤ Add object segmentation building blocks to perform object segmentation on the maximum intensity image.

➤ Use a **Calculate Intensity Properties** building block to calculate the average plane map value as a property for each object. This corresponds to the z-position of the object. In the example shown in *Figure 1–140*, this would, for example, result in about plane 12 (yellow-orange) for the mitotic cell and about plane 7 (orange-green) for the other cells.

➤ The z-position property can be directly used as a read out, e.g. to quantify the average degree of invasion in z-direction, or for further calcuations, as a

criterion for filtering cells using **Select Population**, or creation of the population of invading cells.

➤ If exported as a single cell result, the z-position property can be used for further visualization and analysis of the single cell results in TIBCO Spotfire®, see *Figure 1–141* for an example.

Hints and Tips

➤ If objects are stacked on top of each other, they will partly or fully overlap in the projection image. In this case object segmentation will miss or merge these objects. If this artifact is not tolerable for your application, this strategy cannot be applied. Use single plane analysis instead.

➤ Since the maximum projection approach only needs to do the segmenation on one image, it is much faster than analyzing individual planes.
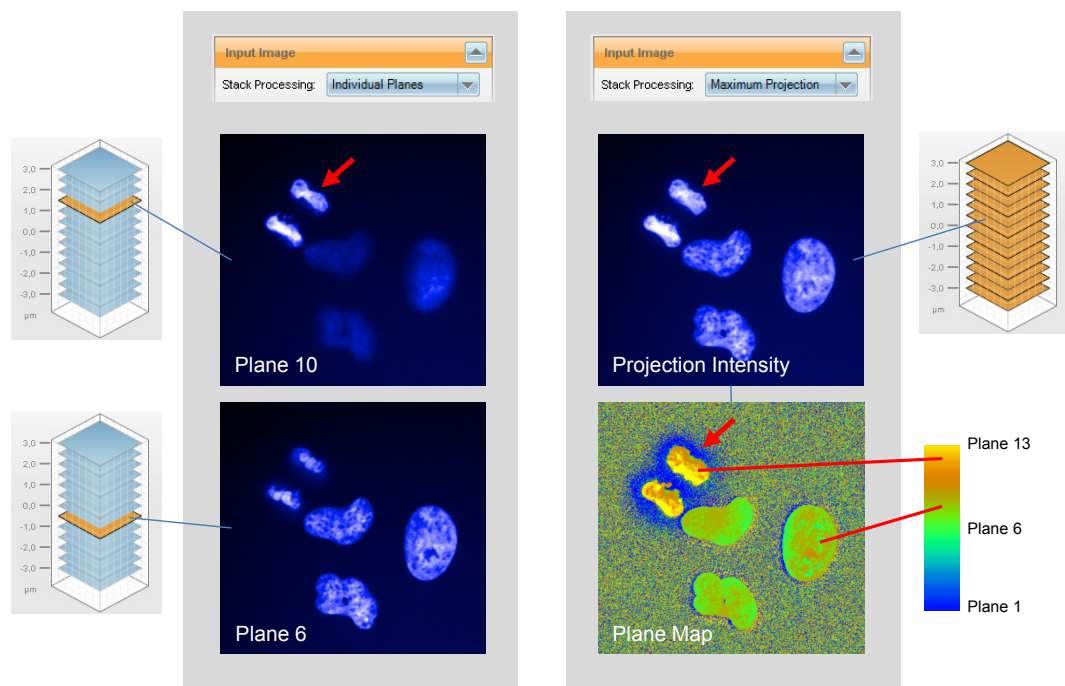

Figure 1–140: Example of using the "Maximum Projection" option in the **Input Image** building block. The left panel shows a detail of images acquired at two different stack planes (60x high NA, confocal mode, 13 planes with 0.5 µm spacing, HeLa cells, Hoechst 33342 stain). The z-height distance between the two images shown is 2 µm. The right panel shows the results of a maximum projection of the full stack. The top image shows the intensity channel, the bottom image shows the plane map channel indicating in which plane the brightest intensity was found. The plane map is shown as a pseudocolor image using the "Cold-Hot" palette (see section 1.2.2). Note that it is clearly visible that the mitotic cell marked by a red arrow is located in the top planes while the other cells are located in the middle planes. Also note that the areas with very low fluorescence intensity (background) show a noise pattern in the plane map. The plane number with the highest intensity is random if there is no significant intensity signal.
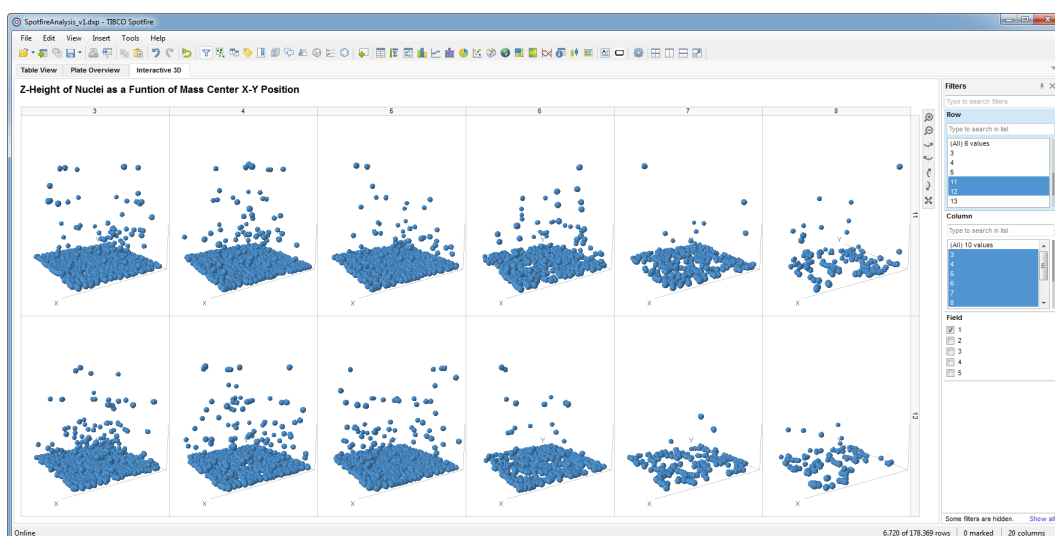
Figure 1–141: Example of using TIBCO Spotfire to visualize the cell invasion data shown in ***Figure 1–139***. Each dot represents a single cell detected in the maximum intensity projection image. The z-axis shows the average z-plane position of the cell determined from the maximum projection's plane map channel. The filter panel on the right was used to select 12 wells containing a dose response titration of a toxic compound (increasing concentration from left to right). The top and bottom rows of scatterplots are replicates of the same titration. Rotation of the 3D scatterplots and selection of cells can be interactively controlled using the mouse. Marked cells can be used in further user defined calculations and visualisations.

1.18 Various sized Spots – Spot Co-localization

The detection of spots of homogeneous size, as well as various sized spots and patches is used for many different analysis tasks in Harmony:

- ➤ Detection of cell organelles.
- ➤ Detection of lipid droplets.
- ➤ Detection of bacteria.
- ➤ Detection of micronuclei.
- ➤ Detection of protein agglomerates.
- ➤ Detection of defined bright objects inside darker regions.
- ➤ Detection of defined dark object inside brighter regions.

Spot detection is performed by the **Find Spots** building block. In this section, you will find some useful hints in addition to the information available in the Harmony Help system, accessible at any time by hitting the F1 key while the **Find Spots** building block is selected.
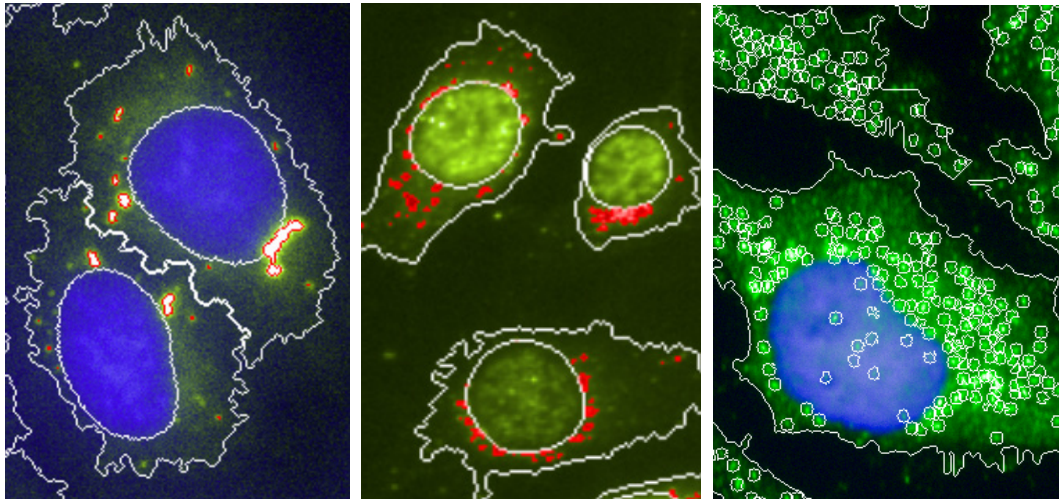

Figure 1–142: Three examples for the use of the Find Spots building block. Left panel: Detection of lipid droplets. Middle panel: Detection of Huntington protein aggregates around the nucleus. Right panel: Detection of peroxisomes.

Methods

There are four different methods available for spot detection which are named "A" to "D". Each method follows a different, multi step, strategy to reliably find the spots even when imaging conditions vary due to cell-to-cell, day-to-day variability, etc. during screening.

The fastest and most reliable way of selecting the most appropriate method is by trial and error on one example image: use the choice that works best in the first trial.

There is one guideline for selection:

Methods "A", "B" and "D" detect spots and bright patches of any shape (see *Figure 1–142*, left and middle panels)

Method "C" focuses on round spots with the radius specified by its input parameter "Radius" (see *Figure 1–142*, right panel).

Pre-calculated Spot Properties

All methods calculate a set of spot properties that characterize each spot. In addition, they add different properties to the objects (cells in most cases) in which the spots were detected, e.g. the number of spots and their average intensity.

Many of the pre-calculated spot properties are normalized or relative values, which makes them independent of background intensity fluctuations. See *Figure 1–143* for a graphical illustration of the properties. Which property is best suited, depends on the specific application. Use the property which gives the best Z' value, see section 1.5.1.
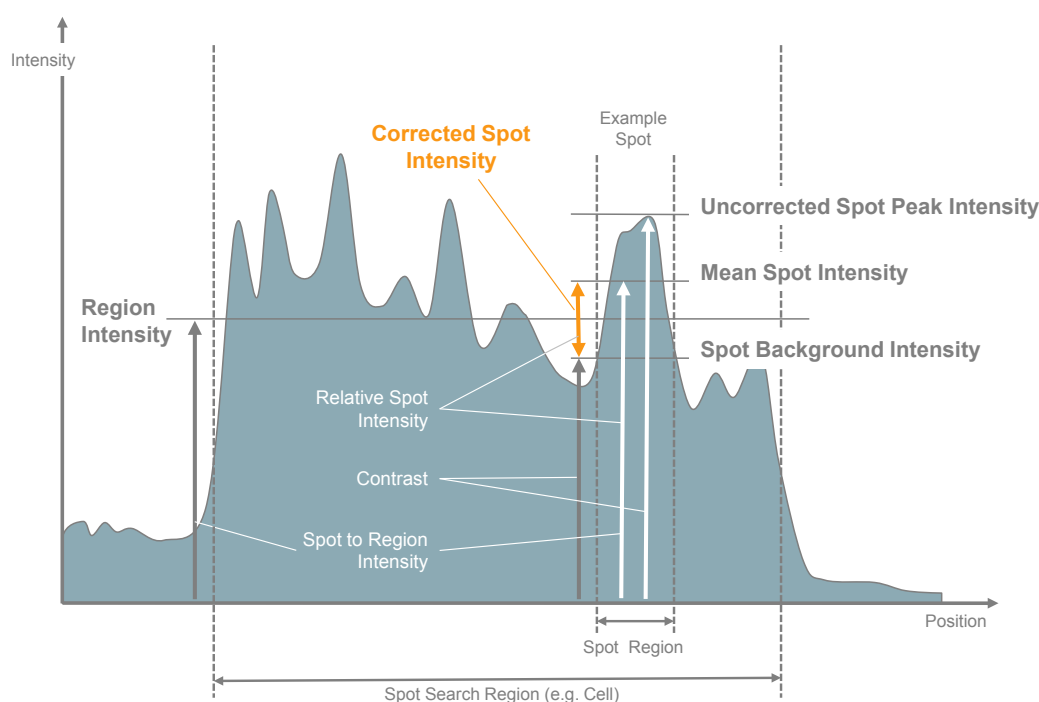

Figure 1–143: Illustration of the Find Spots pre-calculated spot properties. Four basic intensities are calculated - two of them characterizing the spot intensity, one characterizing the local spot background and one characterizing the spot search region - denoted by white and grey vertical arrows. The "Corrected Spot Intensity" (orange font) is most relevant for many applications. In addition, three intensity relations are calculated, which are independent of the absolute intensity of the image (small white font). See the Harmony Help for additional information

➤ Additional arbitrary spot properties can be calculated using additional building blocks.

➤ The spot population can be filtered using the **Select Population** building block to select spots with special properties, or remove artifacts.

➤ To determine cell properties based on the spots located inside the cell, use the Calculate Properties building block method "By Related Population", see section 1.10.

➤ PhenoLOGIC classification can also be used for spot classification or classification of cells based on the properties of spots inside them, see section 1.15.

➤ To allow secondary analyses at the single spot level, all or selected spot properties can be included in output of the analysis sequence (to be configured in the **Define Results** – "Object Results" section).

➤ To find micronuclei, a special variant of the **Find Spots** building block is available: **Find Micronuclei**. This building block takes special care to detect micronuclei which have been erroneously detected as part of the nucleus by the preceding nuclei detection. It also calculates a special set of properties

tailored to micronuclei selection by common criteria, such as their distance to the nucleus and fractional intensity.

➤ To detect dark spots in bright regions, use the **Filter Image** building block's method "Invert Image" to create an inverted image, then use **Find Spots** on the inverted channel.

Spot Co-localization

To determine the co-localization of different markers in spots, first find the spots in one of the channels and then use the **Calculate Intensity Properties** building block to determine the marker intensity in the other channel at the spot location. You can tailor the readout values to the application needs, e.g. report the number of spots and the fraction of spots containing both markers.

Another option to determine spot colocalisation makes use of the **Select Region** building block's method "Restrict by Mask":

➤ Detect spots in first channel using **Find Spots**.

➤ Detect spots in second channel using **Find Spots**.

➤ Restrict the spot region found in the first channel by the spot region found in the second channel.

As a result, you will get a new region containing the overlapping spot regions of the two channels which can be used for further quantification, e.g. overlap area, intensity, location inside the cell.

1.19   Stitching Multiple Images

The well montage view can be used to stitch tiled images for visualization. Please refer to the Harmony Help for information on creating a well montage. This section provides some useful hints for using the stitching feature.

Please note that stitching is used for visualization only. Due to speed and computer memory optimization, analysis is always done on individual image fields. The numerical per-field results are automatically combined to per-well results by the **Define Results** building block.

➤ Due to slight differences in the magnification in the full optical setup from the nominal magnification of the objective (e.g. 20x), a tiled image may show slightly incorrect matching at the junctions between the tiles. See *Figure 1–144*, left part.

➤ This inaccuracy can be corrected by setting a magnification correction factor, see *Figure 1–144*, right part.

➤ For Operetta images, the magnification is the only significant error which affects the image matching; table positioning errors and optical distortions are so low that they usually cannot be seen in the image.

➤ For Opera Phenix images, no correction is needed. The skewcrop correction which maps the images of different cameras onto each other automatically includes the magnification correction.

➤ The intensity of the image fields usually varies slightly for adjacent fields due to the re-focusing at each of the image fields in order to correct for plate bottom height tolerances. The small focus z-height error of about < ±1 μm causes a change in intensity of a certain percentage, which is seen as an intensity jump at the interface of two fields. For an example, see the horizontal line in the image in *Figure 1–144*.
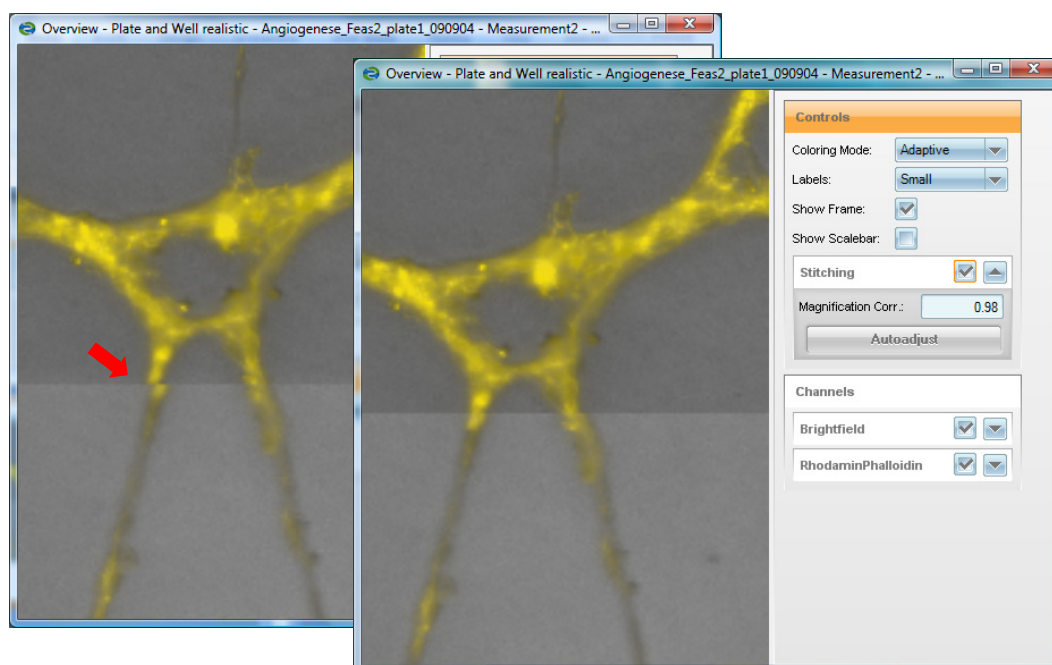

Figure 1–144: Example of a well montage of Operetta images showing the inaccuracy at the border between two image fields. Left: Without magnification correction. Right: Magnification correction applied by manually setting it to a value of 0.98.

➤ For overlapping images, the correction factor can be automatically adjusted using the "Autoadjust" button. See ***Figure 1–145*** for an example. There must be at least 5% overlap of the image fields, otherwise the "Autoadjust" button is disabled.

➤ For non-overlapping images, the factor can be set manually:

- Start at 0.98 which is good for most situations.

- Turn the stitching on and off and see if the correction is ok.

- Increase or decrease the correction factor in small steps (e.g. ±0.01) until the images match the well.

➤ The magnification correction only has to be determined once for an optical setup (objective, instrument, plate) and can be used for all fields / wells.
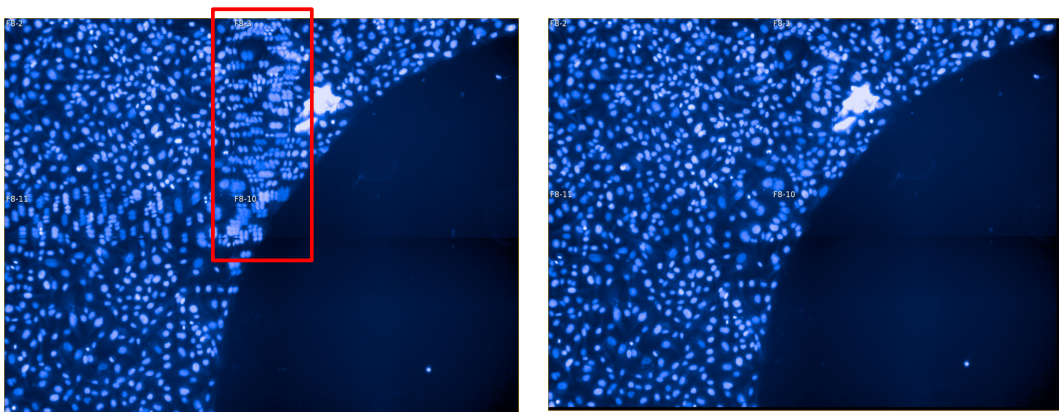

Figure 1–145: Stitching of an overlapping image. Four image fields with 20 % overlap are shown in the illustration (montage image). Left: Without stitching – the nuclei in the overlapping area do not match perfectly (see the example in the red box). Right: With stitching applied – the magnification correction was adjusted to 0.9802 by pressing the "Autoadjust" button.

1.20 Analyzing FRET using Harmony and Tibco Spotfire

1.20.1 FRET Principle

Förster resonance energy transfer (FRET) is a non-radiative transfer of energy from a donor molecule (D) to an acceptor molecule (A) [Förster, 1948]. A prerequisite is that the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor, as shown for the commonly used FRET pair CFP and YFP (***Figure 1–146***). The energy transfer occurs via dipole-dipole interaction of the donor and acceptor and is dependent on close proximity and dipole orientation. If the two molecules are too far away (typically more than 10nm) FRET can no longer be observed.

> A negative FRET result does not necessarily mean no interaction. It could be negative due to wrong dipole-dipole orientation.
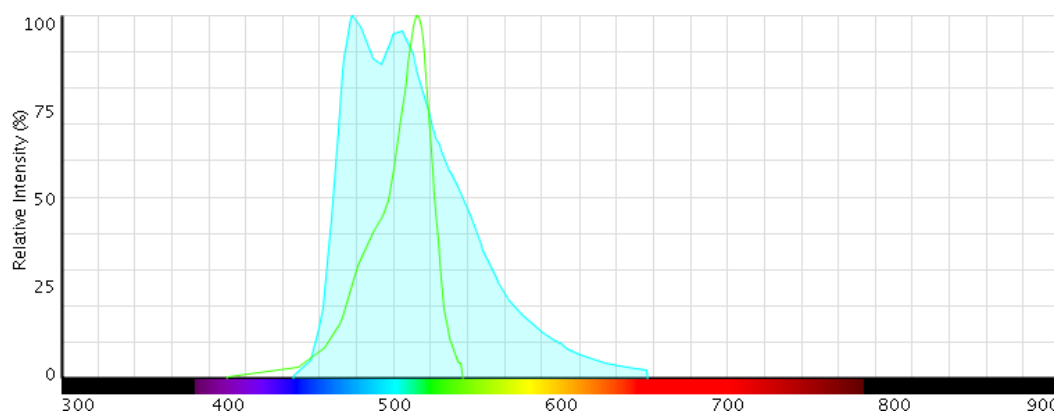

Figure 1–146: Spectral overlap of CFP emission and YFP excitation spectrum
The CFP emission spectrum (blue) strongly overlaps with the excitation spectrum of YFP, making this fluorescent protein combination an ideal FRET pair. x axis: Wavelength in nm, y axis: % relative intensity. (Spectrum taken from http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html)

There are different ways to measure FRET. One way is to measure the fluorescence lifetime of the donor which is a non-intensity based method. Another way is spectrally resolved intensity measurements like acceptor photobleaching or sensitized emission. In this guide we will only address sensitized emission, as this method is applicable to live cells and can easily be set up on Perkin Elmer instruments. For sensitized emission, the donor is excited and the acceptor fluorescence is monitored (exD/emA). This approach requires a donor only, acceptor only and donor-acceptor sample (FRET sample).

In total three images are needed:

➤ **Donor image:** Excitation Donor (exD) – Emission Donor (emD): Shows the level of donor fluorophore

➤ **FRET image:** Excitation Donor (exD) – Emission Acceptor (emA): Shows the level of Förster resonance energy transfer

➤ **Acceptor image:** Excitation Acceptor (exA) – Emission Acceptor (emA): Shows the level of acceptor fluorophore

Looking at the emission and excitation spectra will help to explain why (***Figure 1–147*** a and b).
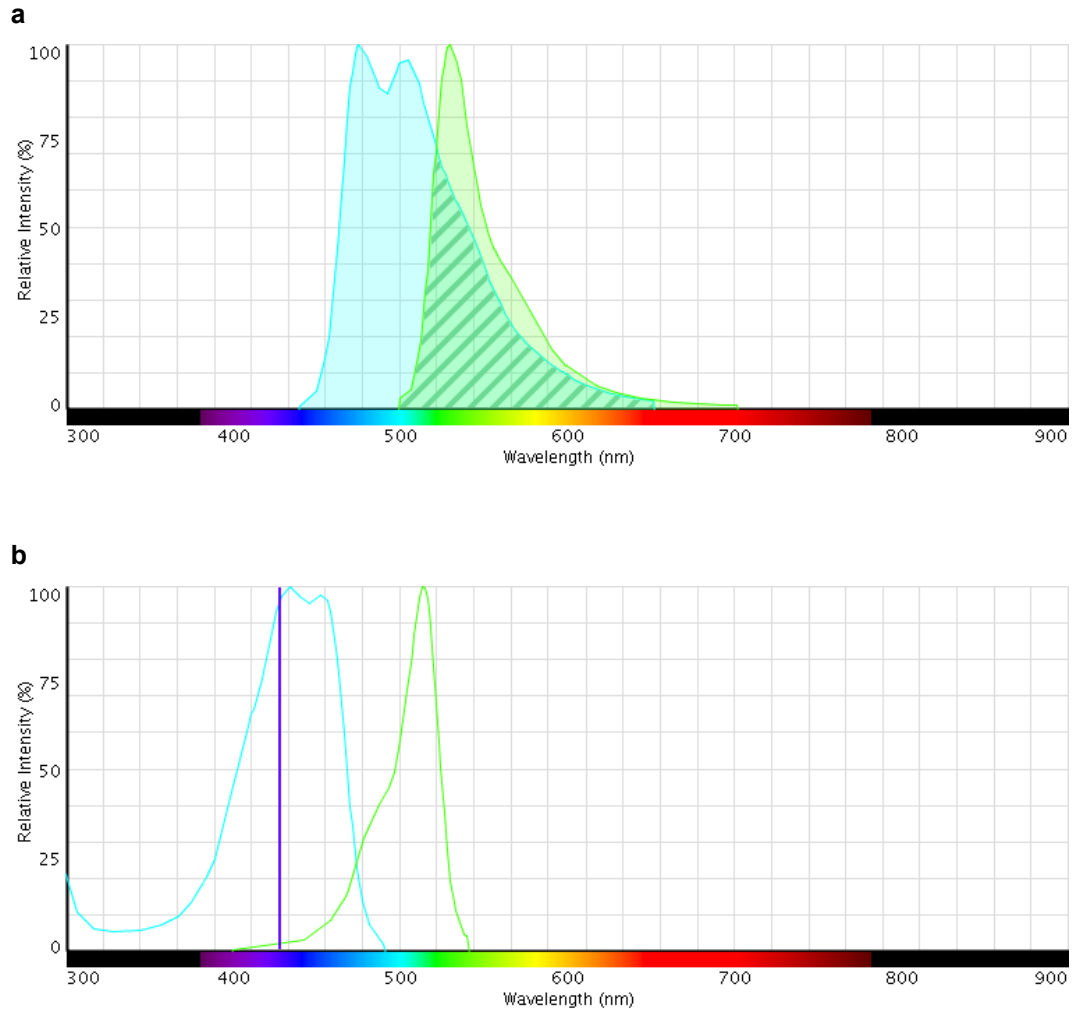
a

b

Figure 1–147: CFP and YFP emission and excitation spectra
(a) The CFP emission spectrum (blue) overlaps with the emission spectrum of YFP (yellow) leading to spectral bleed through (hatched area). **(b)** The excitation spectra also overlap to some extent which will lead to cross excitation of YFP by the CFP excitation light (violet line). x axis: Wavlength in nm; y axis: % emission. (http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html)

Owing to the strongly overlapping emission spectra, the donor will spectrally bleed into the acceptor detection channel (***Figure 1–147a***). Additionally, the excitation spectra also overlap which will lead to cross-excitation of YFP by CFP excitation light (***Figure 1–147b***). Both phenomena will cause contamination of the FRET signal. Therefore, the FRET signal needs to be corrected for this cross talk. To correct for the CFP cross-emission the donor-only sample is excited and the acceptor channel is recorded (exD/emA and exD/emD). For the cross excitation correction the acceptor-only sample is excited with donor excitation light and the acceptor emission recorded (exD/emA and exA/emA). From the donor-acceptor sample (FRET sample) which is used for the actual FRET analysis three images are acquired, exD/emA (I_{FRET}), exD/emD (I_D) and exA/emA (I_A).

To optimize image acquisition the controls are imaged with the same settings as the FRET sample so they can be imaged simultaneously.

To quantify the FRET efficiency, numerous formulae have been developed that include crosstalk correction [Gordon et al., 1998; Xia and Liu, 2001]. The NFRET value as described by Xia and Lui [2001] worked well in our hands, as this method normalizes heterogeneous fluorescent protein expression levels and we recommend using this.

$$N_{FRET} = \frac{I_{FRET} - I_A \times \alpha - I_D \times \beta}{\sqrt{I_A \times I_D}}$$

I_{FRET} = Intensity in FRET channel

I_A = Intensity in Acceptor channel

I_D = Intensity in Donor channel

α = Cross-excitation coefficient describing the amount of acceptor excitation by the donor excitation light

β = Cross-emission describing percentage of donor fluorescence bleeding into the FRET image

On the same instrument, the crosstalk coefficients can be re-used as long as the optical set-up remains unchanged.

The correction coefficients can be neglected if intramolecular FRET is observed. Intramolecular FRET means donor and acceptor are part of the same molecule/protein (for comparison see **Figure 1–148**). Intra-molecular FRET is, for example, measured using FRET-based biosensor like the Ca2+ sensor Cameleon. Donor and acceptor are present at a fixed stoichiometry of 1:1 (**Figure 1–148**). Hence, it is sufficient to perform a two-channel ratiometric experiment (exD/emA and exD / emD) and calculate the relative FRET efficiency as I_{FRET}/I_{Donor}. As the crosstalk signal in this case is proportional to the FRET signal, crosstalk correction is not required [Sun et al., 2012].
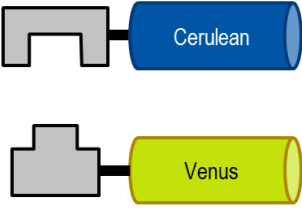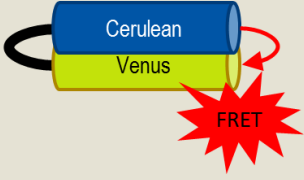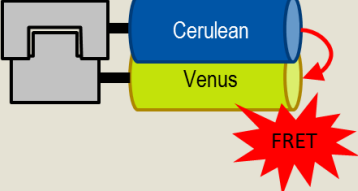
Intra-molecular FRET (FRET sensors)	Inter-molecular FRET (Protein-Protein interaction)
Fixed stoichiometry (1:1)	Varying stoichiometry
Two-channel ratiometric approach (exD / emA, exD / emD)	Three-channel ratiometric approach (exD / emA , exD / emD, exA / emA)
Crosstalk correction not required	Crosstalk correction is required
$E_{FRET} = \dfrac{I_{FRET}}{I_D}$ or $E_{FRET} = \dfrac{I_{FRET} - I_{FRET\,background}}{I_D - I_{D\,background}}$	$N_{FRET} = \dfrac{I_{FRET} - I_A \times \alpha - I_D \times \beta}{\sqrt{I_A \times I_D}}$ or other

Figure 1–148: Comparison between intra-molecular and inter-molecular FRET
exD/emA: excitation of donor and detection of acceptor emission
exD/emD: excitation of donor and detection of donor
exA/emA: excitation of acceptor and detection of acceptor
I_D: Intensity of donor (exD/emD)
I_A: Intensity of acceptor (exA/emA)
I_{FRET}: Intensity of FRET image (exD/emA)

Table 1-9 summarizes the images that need to be acquired.

Filter	Sample	Donor only	Acceptor only	Donor-Acceptor (FRET sample)
exD/emA (FRET: Cerulean / YFP acceptor see example below)		Cross emission Correction	Cross excitation Correction	FRET image (I_{FRET})
exD/emD (FRET: Cerulean / YFP donor see example below)		Cross emission Correction	Recorded but not used*	Intensity Donor (I_D)
exA/emA (YFP see example below)		Recorded but not used*	Cross excitation Correction	Intensity Acceptor (I_A)

Table 1-9: Summary of required samples and acquired images
exD/emA: excitation of donor and detection of acceptor emission
exD/emD: excitation of donor and detection of donor
exA/emA: excitation of acceptor and detection of acceptor
* : These images are recorded to be able to acquire the whole plate in one go but are not used
     for further analyses

1.20.2 FRET Image Analysis

The following analysis is for inter-molecular FRET. For intra-molecular FRET omit the calculation of crosstalk coefficients using TIBCO Spotfire®.

Calculation of crosstalk coefficients

The first part of the FRET analysis is to determine the crosstalk coefficients α and β of the control wells. The cross-excitation coefficient α is determined by analyzing wells containing cells expressing the acceptor only while the crosstalk correction coefficient β is determined by analyzing wells containing cells expressing the donor only. To enable nuclei detection, a reference dye labeling the nuclei is required (e.g. DRAQ5).

In Harmony, use the following building blocks:

➤ Find Nuclei (DRAQ5)

➤ Find Cytoplasm (Acceptor channel)

➤ Calculate Intensity Properties (exD/emA)

➤ Calculate Intensity Properties (exD/emD)

➤ Calculate Intensity Properties (exA/emA)

➤ Define Results

Sample analysis protocol

Input Image	Stack Processing : Individual Planes Flatfield Correction : None		
Find Nuclei	Channel : DRAQ5 ROI : None	Method : B Common Threshold : 0.55 Area : > 70 µm² Split Factor : 7 Individual Threshold : 0.45 Contrast : > 0.1	Output Population : Nuclei
Find Cytoplasm	Channel : YFP Nuclei : Nuclei	Method : C Common Threshold : 0.45 Individual Threshold : 0.05	
Calculate Intensity Properties	Channel : FRET: Cerulean / YFP acceptor Population : Cytoplasm Region : Cytoplasm	Method : Standard Mean Maximum	Output Properties : Intensity Cytoplasm FRET : Cerulean / YFP acceptor
Calculate Intensity Properties (2)	Channel : FRET: Cerulean / YFP donor Population : Cytoplasm Region : Cytoplasm	Method : Standard Mean Maximum	Output Properties : Intensity Cytoplasm FRET : Cerulean / YFP donor
Calculate Intensity Properties (3)	Channel : YFP Population : Cytoplasm Region : Cytoplasm	Method : Standard Mean Maximum	Output Properties : Intensity Cytoplasm YFP
Define Results	Method : List of Outputs Population : Nuclei Number of Objects Population : Cytoplasm Intensity Cytoplasm FRET : Cerulean / YFP acceptor Mean : Mean Intensity Cytoplasm FRET : Cerulean / YFP acceptor Maximum : Mean Intensity Cytoplasm FRET : Cerulean / YFP donor Mean : Mean Intensity Cytoplasm FRET : Cerulean / YFP donor Maximum : Mean Intensity Cytoplasm YFP Mean : Mean Intensity Cytoplasm YFP Maximum : Mean Single Cell Results : Selected		

Table 1-10: Sample analysis protocol
The table contains a sample analysis protocol that can be used to determine the crosstalk correction coefficients

For more information and help with the building blocks used above, please refer to the relevant chapters of this guide.

It might be necessary to include a building block such as *Calculate Morphology Properties* and use the calculated properties in a *Select Population* building block to exclude, for example, fragmented cells.

To finally obtain the crosstalk coefficients:

➤ export single cell results

➤ import results into TIBCO Spotfire®

➤ for donor-only sample, plot mean FRET intensity (exD/emA) versus mean intensity of donor (exD/emD) to obtain cross emission coefficient β

➤ for acceptor-only sample, plot mean FRET intensity (exD/emA) versus mean intensity of acceptor (exA/emA) to obtain cross excitation coefficient α

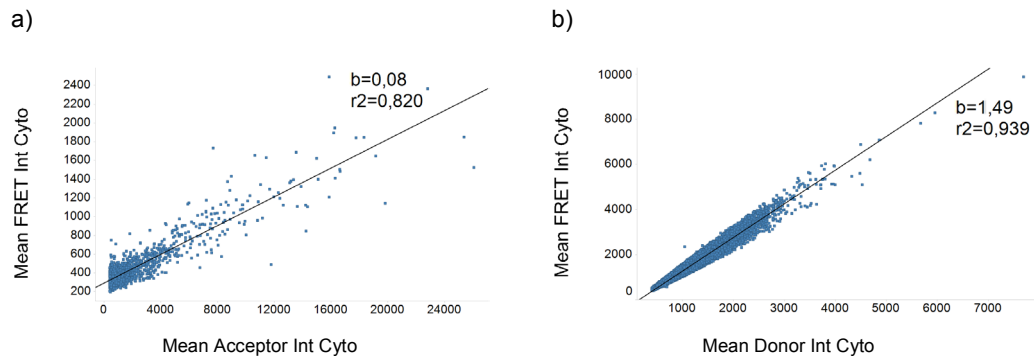➤ fit straight line and slope corresponds to the correction coefficients (α from acceptor and β from donor plot)

a)

b)

Figure 1–149: Cross emission and cross excitation coefficients
(a) Mean intensities from the FRET images (exD/emA) of the acceptor-only sample are plotted against mean intensities from the acceptor images (exA/emA). A straight line is fitted and the slope corresponds to the correction factor α. In this example it's 0.08.
(b) Mean intensities from the FRET images (exD/emA) of the donor-only sample are plotted against mean intensities from the donor images (exD/emD). A straight line is fitted and the slope corresponds to the correction factor β. In this example it's 1.49.

Analyzing Protein-Protein-Interactions (PPI) by calculating NFRET

To analyze PPI, the FRET samples (donor-acceptor samples) are segmented as described above. Additionally, to determine the NFRET value, a new ratiometric image is calculated by using the *Calculate Image* building block. The NFRET formula [Xia & Liu, 2001] is used as the formula input with the previously calculated crosstalk coefficients inserted. The resulting image represents the NFRET value on a pixel-by-pixel basis and also allows the quantification of protein-protein interactions in relation to their localization in the cell. Before defining results, one should include a *Select Population* building block to exclude objects with too low or too high intensity values to exclude non-expressing cells and saturated pixels. Threshold values need to be estimated from actual images.

➤ Find Nuclei (DRAQ5)

➤ Find Cytoplasm (acceptor channel)

➤ Calculate Image

➤ Calculate Intensity Properties (exD/emA), mean and max

➤ Calculate Intensity Properties (exD/emD), mean and max

➤ Calculate Intensity Properties (exA/emA), mean and max

➤ Select Population, Intensity based e.g. mean > 400, max < 63000

➤ Define Results

The above method will analyse FRET in the cytoplasm but it could be applied to any other cellular compartment that has been segmented.
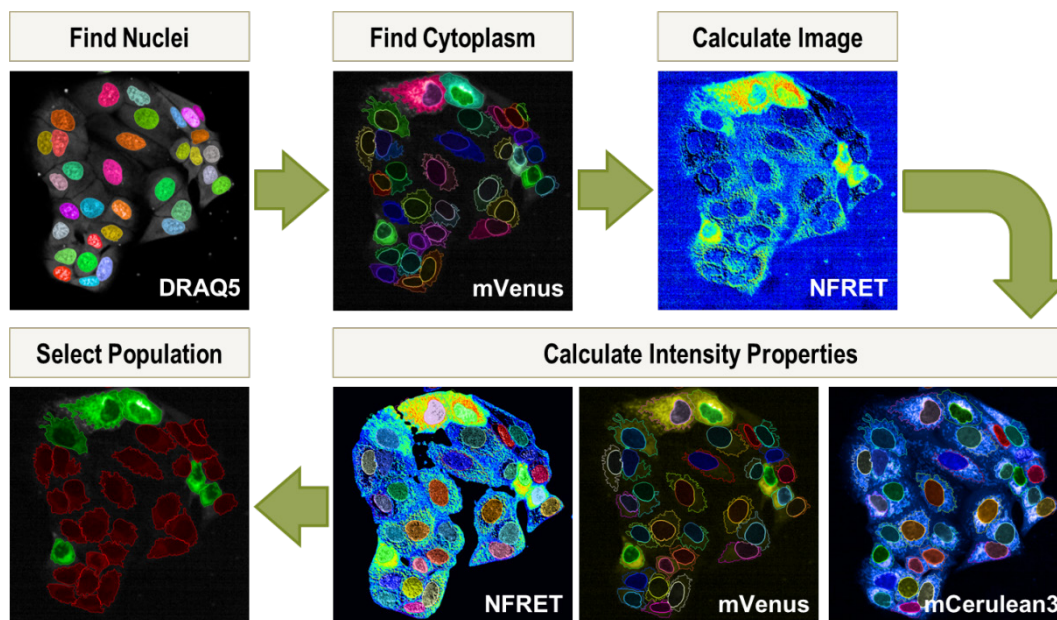

Figure 1–150: Image analysis strategy for a FRET-based protein-protein interaction assay
FRET is quantified by calculating a ratiometric image using the *Calculate Image* building block with the NFRET formula as formula input. Subsequently, the mean intensity in the cytoplasm region of the NFRET, donor and acceptor channels are calculated and the desired population is selected. In this particular example, mVenus is the acceptor and mCerulean3 the donor.
Into the *Calculate Image* building block enter: (F-A*0.08-D*1.49)/sqrt(A*D) for inter-molecular FRET and F/I_D for intramolecular FRET
$F = I_{FRET}$, $A = I_A$, $D = I_D$, α and β, enter the values calculated as described above

1.20.3 References

Förster, T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. Annalen Der Physik, 437(1-2), 55–75.

Gordon, G. W., Berry, G., Liang, X. H., Levine, B., & Herman, B. (1998). Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. Biophysical Journal, 74(May), 2702–2713.

Sun, Y., Wallrabe, H., Seo, S.-A., & Periasamy, A. (2012). FRET microscopy in 2010: The legacy of Theodor Förster on the 100th anniversary of his birth. Chemphyschem., 12(3), 462–474. doi:10.1002/cphc.201000664.FRET

Xia, Z., & Liu, Y. (2001). Reliable and Global Measurement of Fluorescence Resonance Energy Transfer Using Fluorescence Microscopes. Biophysical Journal, 81(October), 2395–2402.

1.21 Considerations Regarding Image Analysis Speed

During the design of an experiment the calculation time of the analysis needs to be considered, especially if an online analysis in parallel to the data acquisition is desired.

1.21.1 How Do I Check the Analysis Time?

➤ Set up the analysis sequence, do not worry about speed.

➤ Determine the speed by analyzing some (e.g. five) wells on the Harmony Evaluation Screen.

➤ Estimate the analysis time for the whole plate.

If the analysis time is below the measurement time of the plate...

This is a convenient situation. You can use the analysis "online", i.e. in parallel to your measurement. The analysis results are available when the measurement is finished.

If the analysis takes longer than the measurement...

➤ You can use online analysis but you have to wait for the analysis to finish after your measurement is completed.

➤ You can try to optimize the analysis sequence for speed by avoiding or replacing time-consuming steps. You can trade "speed" for accuracy if you do not need all readouts or can tolerate a slightly higher noise level. See the sections below.

➤ You can choose to first acquire the data and then run a batch analysis at a later, convenient time after the measurement, e.g. overnight.

➤ You might want to split the analysis: (i) run a basic fast analysis for QC during the measurement (e.g. cell counting, degree of confluency, monitoring of marker intensities) as an online analysis; (ii) perform the full analysis later by batch (re-) evaluation, e.g. overnight.

1.21.2 General Hints to Speed up the Analysis

Calculation time always depends on the actual image contents, analysis methods and parameters chosen. Any recommendations and listed times only give a general hint – actual calculation times may vary strongly. *Table 1-11* gives an overview of the calculation times of most building blocks and methods.

In *Figure 1–151*, the fractions of time that the individual building blocks need are shown for a typical analysis sequence (Ready Made Solution: Texture Analysis – Mitochondrial Classification), using an example dataset consisting of 1350 images.

Absolute calculation times depend on the Harmony software version and computer hardware. See table *Table 1-12* for an example of running the benchmark analysis described above on different configurations. You can use this information for a rough estimation of the calculation time for your analysis and to estimate potential speed gain by using a different computer configuration.

The shown benchmark calculates a single SER texture feature. To estimate the calculation times for other texture and morphology properties, you may refer to *Figure 1–152*. E.g. the calculation time of a set of SER texture properties does not significantly depend on the number of properties. Calculation of the default set of

STAR morphology properties takes roughly the same time as calculation of the SER texture features.

If the Operetta database (ODA) is located on another computer, e.g. when running Harmony on your office computer, the analysis speed may also be limited by the speed of the network connection. In most cases, a 1 Gbit/s network does not significantly influence the speed, while 100 Mbit/s causes slowdown.

Options for Analysis Speed improvement

➤ Don't take higher magnification images than you need in order to identify the desired details in your images.

➤ Don't aquire images with "Binning" set to one on Opera Phenix if not needed for your specific application. Compared to binning 2, the image analysis speed will be slowed down by a factor or four.

➤ Avoid calculating "width", "length" or "width to length" properties of very large objects (e.g. covering the whole image).

➤ Avoid relative regions (percentage) in **Select Region** and **Select Cell Region** building blocks, if possible use resizing by µm (preferred) or pixels instead.

➤ Use a ring region around the nucleus as a first approximation of the cytoplasm, instead of the **Find Cytoplasm** building block**,** to trade speed for accuracy (if appropriate for your application).

Building Block	Method	Comments / Details	Calculation Time
Find Nuclei, Find Cells[1]	A		⏳⏳⏳
	B		⏳⏳⏳
	C		⏳⏳⏳⏳
	M	Slower for unbinned/large images	⏳⏳
Find Cells	P		⏳⏳⏳
Find Cytoplasm	All Methods		⏳⏳
Find Spots	A		⏳⏳
	B		⏳⏳
	C		⏳
	D		⏳
Find Micronuclei	A		⏳⏳
	B		⏳⏳
Find Image Region	All Methods		<⏳
Find Neurites	CSIRO 2		⏳⏳⏳⏳
Find Texture Regions	Find a Class		⏳⏳⏳
	Split into Classes		⏳
Find Surrounding Regions	All Methods		⏳⏳

Select Region, Select Cell Region	Resize [μm or pixel]		<⏳
	Resize Region [%]		⏳
Select Region	Restrict by Mask		<⏳
Calculate Texture Properties	SER		⏳
	Haralick		⏳
	Gabor		⏳⏳
Calculate Morphology Properties	Standard	Area, Roundness	<⏳
		Length and width of typical cells	⏳⏳
		Length and width of "Whole Image Region"	⏳⏳⏳⏳⏳⏳>>
	STAR	Default set of properties	⏳
		Extended set of properties as suggested in *Figure 1–79*	⏳⏳⏳⏳⏳
		All properties	⏳⏳⏳⏳⏳⏳>>
Calculate Intensity Properties	Standard		<⏳
Calculate Properties	All Methods		<⏳
Select Population, Modify Population	All Methods		<⏳
Filter Image, Calculate Image	All Methods		<⏳

Table 1-11: Overview of the time consumption of different building blocks (column 1), their different methods (column 2) and other conditions influencing the speed, e.g. size of objects or parameter settings (column 3). The hourglass symbols (⏳) give a rough indication of calculation times. The exact calculation times depend on the image contents, the computer hardware used, and the setup. Notes: 1) Nuclei detection times may strongly depend on image resolution (unbinned, binned or global image), object size, object density and parameter settings. Algorithms A-C use temporal binning to speed up processing of high resolution images.
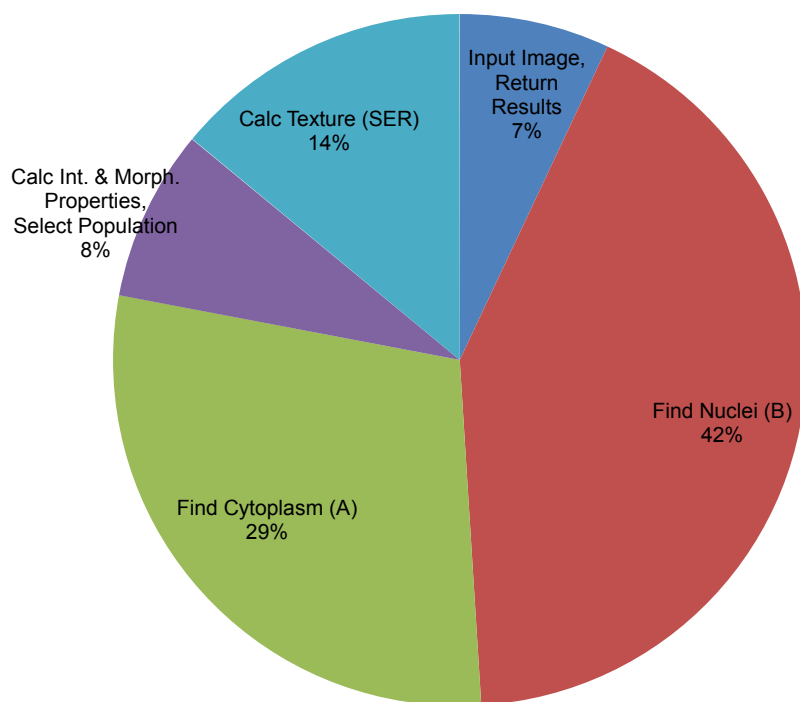
Figure 1–151: Example of the fractions of analysis time used by the individual building blocks of the analysis sequence "RMS Texture Analysis – Mitochondria Classification". The analysis needed approximately 16 % of the measurement time for this specific example dataset (consisting of 1350 images acquired with Operetta, i.e. using a single camera with image size of 1360x1024 pixels). This means that this analysis can be run very well as online analysis in parallel to the measurement. The **Find Nuclei** building block is the most time-consuming step here. The analysis was done with Harmony 4.6 on a Dell T5600 computer with 12 cores and 32 GB memory.

Computer Hardware	Software Version			
	Harmony 3.1	Harmony 3.5.2	Harmony 4.0	Harmony 4.6
4 cores, 4 GB Memory	201 min	--	--	--
4 cores, 12 GB Memory	--	137 min	102 min	83 min
12 cores, 32 GB Memory	--	90 min	21 min	15 min (18 min)*

Table 1-12: Examples of total analysis times needed for 384 wells with 5 fields and 3 channels of the benchmark described in *Figure 1–151*. The analysis time depends on the computer hardware and software version. Harmony software speedup is caused by (i) improvement of algorithms, i.e. the software runs faster on the same hardware platform and (ii) more efficient use of bigger memories and multiple processors in parallel, i.e. additional speedup especially on more powerful hardware. As example for a 4 core machine a Dell T3400 was used, as an example for a 12 core machine a Dell T5600 was used. *Starting with Harmony 4.6 not all available computer resources are automatically used for the analysis any more. This makes the computer more responsive for parallel tasks, but prolongs the analysis time by 3 minutes.
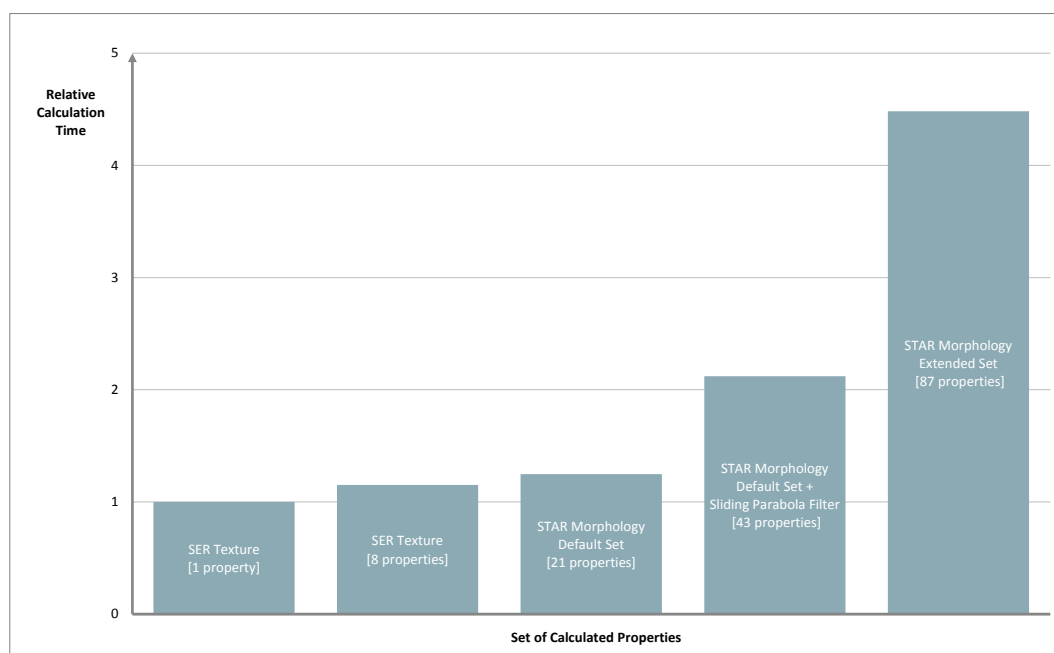
Figure 1–152: Comparison of calculation times of different sets of texture and morphology properties. The bars show times relative to the calculation of a single texture property as used in the "RMS Texture Analysis" (left bar). For a description of the SER texture features see section 1.7, for a description of the STAR morphology features see section 1.8, for the recommended extended set of STAR morphology properties see ***Figure 1–79***.

Chapter 2 – Ready-Made Solutions

This chapter describes our Ready-Made Solutions (RMSs) for image analysis, which cover the most commonly used image analysis methods and their customization for everyday work.

2.1   Overview of RMSs – How to find the best RMS

To find the best matching RMS for your application:

➤ First, use the list of RMSs described on page 171 to identify RMSs that match your application by their title. Each RMS section starts with a brief description of what the RMS is suitable for.

➤ A list of common applications, with a reference to the best RMS to use, is shown in *Table 2-1*.

➤ If you are looking at specific organelles or compartments, refer to *Figure 2–1*.

➤ For translocation assays, refer to section 2.1.1 for an overview of the different types of RMS available for the analysis of translocation.

For an overview of the workflow involved in setting up, optimizing and validating the analysis, see the Appendix section "Setting up the Image Analysis: Workflow" (*Table 3-1*, page 312).
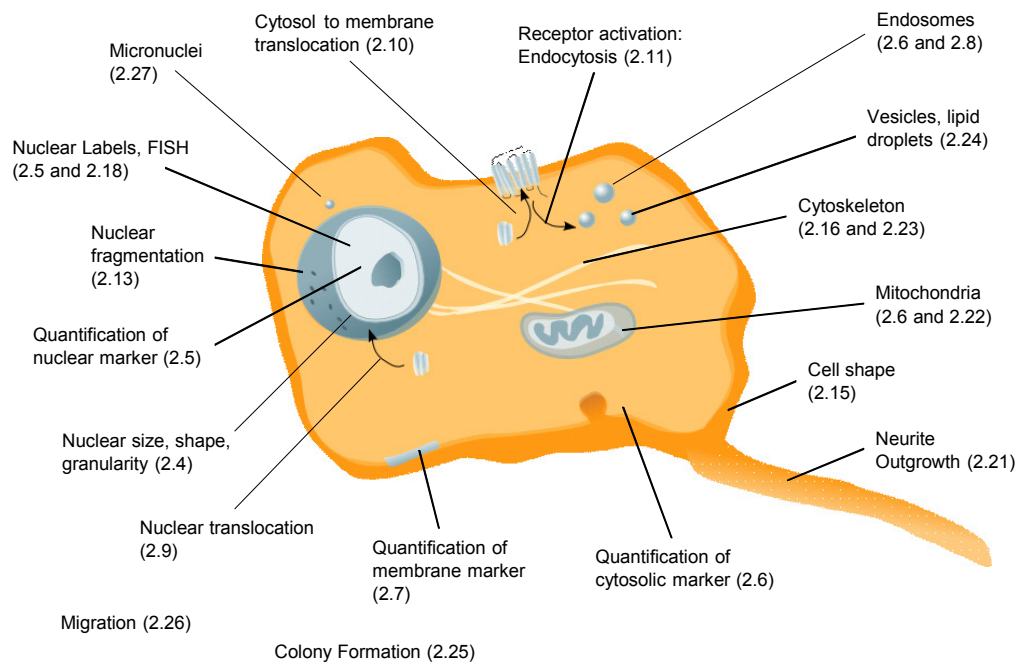
Figure 2–1: Overview of the RMSs available for the cellular component(s) under investigation. The section number of the most suitable RMS is given in brackets.

Application	Ready-Made Solution
Proliferation	Cell counting or Nuclei counting
Cell cycle	
Transition of G2 to M-phase (Cyclin B1)	Quantification of cytoplasmic marker (modified to capture the whole cell fluorescence, including the nucleus)
Mitotic index (phosphohistone H3)	Mitotic index
S-phase (BrdU, EdU)	Quantification of marker in nucleus
DNA content	Nuclear classification – DNA content
Simultaneous phosphohistone H3, EdU, DNA content and nuclear morphology	Cell cycle classification
Cytotoxicity	
Cell count	Cell counting or Nuclei counting
Cell viability: live / dead cell count	Live / dead cell counting
Nuclear swelling and shrinking	Nuclear analysis – nuclear shrinking
Loss of mitochondrial membrane potential	Quantification of cytoplasmic marker
Mitochondrial mass	Quantification of cytoplasmic marker
Calcium homeostasis	Quantification of cytoplasmic marker
Membrane integrity	Live / dead cell counting
Phospholipidosis	Quantification of cytoplasmic marker
Hepatosteatosis	Quantification of cytoplasmic marker
Peroxisome quantification	Spot analysis or Quantification of cytoplasmic marker
Apoptosis	
Nuclear fragmentation	Nuclear fragmentation
Activation of caspase-3	Quantification of marker in nucleus (using an enlarged nuclear region to capture fluorescence in the area of cytoplasm adjacent to the nucleus as well)
Mitochondrial mass	Quantification of cytoplasmic marker
Loss of mitochondrial membrane potential	Quantification of cytoplasmic marker
Protein expression	
In nucleus	Quantification of marker in nucleus
In cytosol	Quantification of cytoplasmic marker
In plasma membrane	Quantification of membrane marker

Table 2-1 (continued over page): Which Ready-Made Solution (RMS) to use for which application.

Receptor activation	
GPCR activation: endocytosis	Receptor internalization
GPCR activation: arrestin recruitment (e.g. Transfluor®)	Spot analysis
Internalization of labeled ligand	Receptor internalization
pH-sensitive dye-mediated internalization	Receptor internalization
Signaling pathways	
Transcription factor activation	Cytosol to nucleus translocation
Cytoplasm to nucleus translocation	Cytosol to nucleus translocation
Nucleus to cytoplasm translocation	Cytosol to nucleus translocation
Cytoplasm to membrane translocation	Cytosol to membrane translocation
Membrane to cytoplasm translocation	Cytosol to membrane translocation
Membrane ruffling	Quantification of membrane marker (starting point for adaptation)
Vesicle formation	Spot analysis
FISH	Nuclear spots
Cytoskeletal re-arrangement	Fluorescence redistribution – cytoskeleton
Cell spreading	Cell shape – cell rounding
Cell detachment, cell rounding	Cell shape – cell rounding

Table 2–1 (continued): Which Ready-Made Solution (RMS) to use for which application.

2.1.1 RMS Selection for Translocation Assays

Four different RMSs are provided for the analysis of different types of fluorescence translocations (shown in *Figure 2–2*).

| Cytosol to Nucleus Translocation (2.9) | Cytosol to Membrane Translocation (2.10) | Receptor Internalization (2.11) Membrane to intracellular compartment | Fluorescence Redistribution (2.16) inside one specific compartment |

Cell regions used to quantify the translocation:

| Nucleus / Whole cell | Membrane / Whole cell or Cytoplasm | Region where internalized receptors mainly accumulate / Whole cell | Region where relocated dye mainly accumulates / Whole cell |

Figure 2–2: Overview of four different translocation assays and their recommended RMSs (the corresponding section numbers are given in brackets). The bottom part of the figure shows the intensity ratios that are used for analyzing the translocation.

Hints and Tips

➤ In addition to the "signal stain" which labels the protein of interest, e.g. a membrane bound receptor, a "reference stain" which labels the nucleus and cytoplasm is also needed to detect the relevant cellular compartments.

➤ The basic principle is to measure the intensity in the area of the cell where the fluorescent molecule accumulates as a result of the translocation: In this area the intensity will be low if no translocation occurs and high if a translocation event does occur.

➤ The intensity is normalized (divided) by the whole cell intensity to remove the dependence of the result on the absolute staining intensity, which may vary from cell to cell or due to variations in plate preparation.

➤ The proposed intensity ratios (shown in *Figure 2–2*) provide good results at a very high robustness for a range of different assays. By using adapted readouts, e.g. intensity differences of two specific regions, the results can be further improved for specific assays. See the "Hints and Tips" section of the corresponding RMS sections for hints on the specific translocation types.

2.2 Cell Counting

This RMS allows for the counting of cells labeled with a fluorescent dye. It works with cytoplasmic or nuclei staining.

2.2.1 Introduction and Assay Principle

Counting cells is useful in many High Content Screening (HCS) applications and is a key readout in cytotoxicity and apoptosis applications. Determining the count of a cell population is a very sensitive indicator of cell stress since cell proliferation requires intact cell structures and function. In our example, the treatment of cells with a specific compound results in a decrease in the cell count as the concentration of the compound increases, see *Figure 2–3*.

This RMS is fundamental to all cellular assays and is therefore a part of almost all of the RMSs provided.

Several basic options for cell counting are available:

➤ Counting fluorescently stained nuclei (mononucleated cells only).

➤ Using a whole cell stain (e.g. Calcein) and counting whole cells.

➤ For manual visual inspection, bright field images can also be used to estimate cell numbers.

Usually, counting of nuclei is used since stained nuclei can be easily and reliably identified from the image analysis (high contrast to the background, defined shape, and a gap between neighboring nuclei even at high degrees of confluency).
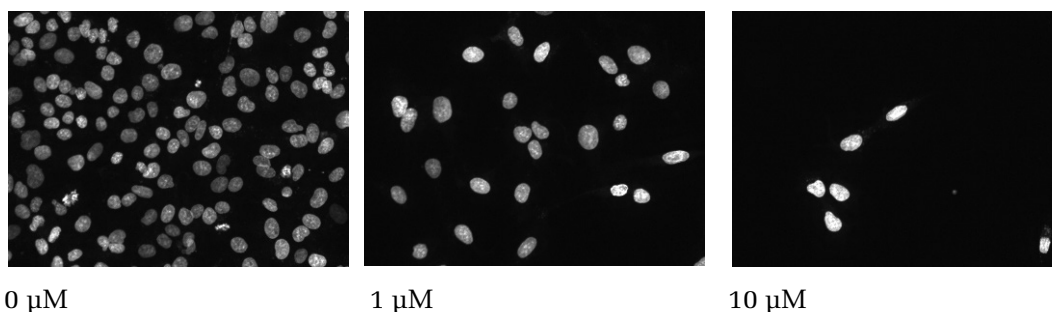
0 µM   1 µM   10 µM

Figure 2–3: Example images from a dataset which shows a decreasing cell count due to treatment with a specific compound (increasing compound concentration from left to right). Details of the example dataset ("Plate P003-CT: Live Tox") can be found in the Appendix on page 313.

2.2.2 How to use the Cell Counting RMS

➤ Load the analysis sequence "RMS Cell Counting" from the Harmony database **OR**

➤ Create a new analysis sequence and add a **Find Nuclei** building block. You can refer to the "Harmony Operation Manual" for creating and modifying analysis sequences.

➤ Select the channel for nuclear stain in the **Find Nuclei** building block.

➤ Option: adjust the nuclei detection parameters (following the instructions in the **Find Nuclei** building block) to optimize the detection results.

Image Analysis Sequence: Cell Counting

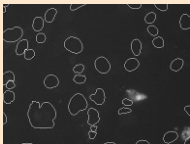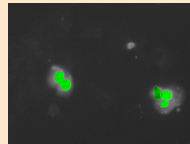| Input Image Channel 1: Nuclear stain image	Untreated control		Treated cells	
Image Segmentation				
Find Nuclei / Find Cells Finds nuclear outlines using the nuclear stain image	Nuclear region overlaid on the nuclear stain image (untreated cells)		Resulting detection of treated cells	
Calculate Readout Values				
Define Results Select values to return as the results per well	Number of Nuclei 1658		Number of Nuclei 330	
Readout Values				
Total Number of Cells	Equivalent to the number of nuclei			

2.2.3   Image Analysis in Detail

➤ This is one of the simplest possible image analysis sequences, consisting of just one building block: **Find Nuclei.**

➤ For whole cell stained images, use the **Find Cells** building block. This building block provides the same basic object detection methods as **Find Nuclei** but has optimized default parameter settings for whole cell detection, e.g. assumes larger objects.

2.2.4   Hints and Tips

➤ For multinucleated cells, use a whole cell stain.

➤ Fragmented nuclei (e.g. due to apoptosis) may lead to artifacts since nuclear fragments are counted as individual nuclei. See the RMS for "Nuclear Fragmentation" (section 2.13) for hints on counting fragmented nuclei.

➤ The **Find Nuclei** building block can also be used to find and count arbitrary bright objects on a dark background, e.g. fluorescent beads or fluorescently stained organelles.

➤ Artifacts (e.g. very weakly stained cells) can be filtered out depending on their properties, by adding the **Calculate Intensity Properties** and **Select Population** building blocks. See, for example, the RMS "Live / Dead Cell Counting" (section 2.3).

2.3　Live / Dead Cell Counting

This RMS allows the counting of two populations of cells marked with two different fluorescent labels.

2.3.1　Introduction and Assay Principle

We describe a live/ dead cell count assay involving a combination of a plasma membrane-permeant and a plasma membrane-impermeant nuclear dye. This assay can be widely applied for profiling the cytotoxic potential of compounds. The membrane-permeant dye labels the nuclei of all cells in a population whereas the plasma membrane-impermeant dye is specific for dying and dead cells, which suffer from a disrupted cell membrane. This RMS is mainly tailored to the evaluation of typical live / dead cell assays, providing a cell count of two subpopulations, live and dead cells.

0 µM　　　　　　　　　　　3 µM　　　　　　　　　　30 µM

Figure 2–4: False color overlay showing Hoechst 33342 (blue, labeling all cells) and BOBO™ 3 (amber, labeling cells with disrupted cell membrane) stained nuclei. The fraction of BOBO™ 3 stained nuclei increases with an increasing concentration of the compound (left to right). Details of the example dataset ("Plate P003-CT: Live Tox") can be found in the Appendix on page 313.

2.3.2　How to use the Live / Dead Cell Counting RMS

➤ Load the analysis sequence "RMS Live / Dead Cell Counting" from the Harmony database.

➤ Select the channel for the nuclear stain, which labels all cells, in the **Find Nuclei** building block.

➤ Select the channel for the dead cell stain in the **Calculate Intensity Properties** building block.

➤ Verify the **Select Population** building block illustrations for the correct identification of dead stain marked cells. Adjust the intensity threshold if needed.

Image Analysis Sequence: Live / Dead Cell Counting
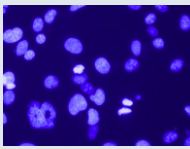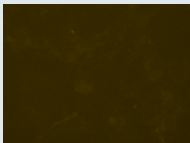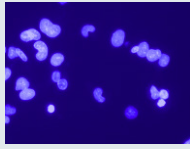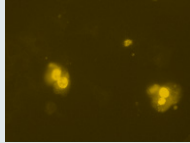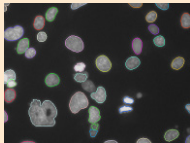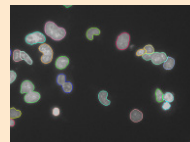
Input Image

Channel 1:
Nuclear stain image (all nuclei labeled)

Untreated control

Treated cells

Channel 2:
Dead cell stained image

Example: BOBO™ 3

Image Segmentation

Find Nuclei

Finds nuclear outlines using the channel 1 nuclear stain image

Nuclear region overlaid on the nuclear stain image (untreated cells)

Resulting detection of treated cells

Quantify Properties in Regions

Calculate Intensity Properties

Mean intensity of dead cell stain in the nuclear region

Resulting intensities for untreated cells. Intensity levels are around 600 units.

Object No	Dead Stain Intensity Mean
40	586,18
41	592,676
42	593,625
43	445,645
44	1038,68
45	555,448
46	459,131
47	537,02

Resulting intensities for treated cells. Cells numbered 84 and 85 have high intensity levels.

Object No	Dead Stain Intensity Mean
81	679,759
82	641,492
83	629,954
84	13536
85	6045,24
86	579,17
87	582,134
88	660,224

Identify Subpopulations

Select Population

Identify cells with high marker intensity of the dead stain

Selected objects with marker intensity > 2000 units

Two cells selected (marked green)

Calculate Readout Values

Define Results

Calculate the percentage of dead cells

Field	Total Number of Cells	Percentage of Dead Cells [%]
1	310	0,322581

Field	Total Number of Cells	Percentage of Dead Cells [%]
1	102	29,4118

Readout Values

Total Number of Cells	Equivalent to the number of stained nuclei in both living and dead cells
Percentage of Dead Cells	Measure of the health of the cell population in each well

2.3.3   Image Analysis in Detail

Image Segmentation

➤ A **Find Nuclei** building block is used to identify all nuclei using a nuclear stain which stains all nuclei (Hoechst 33342 in this example).

➤ As a result, a list of all nuclei and an overlay showing the position of each nucleus in the image is generated.

Quantification of Properties

➤ In each of the detected nuclear regions the mean intensity in the dead cell marker channel (here: BOBO™ 3) is measured using a **Calculate Intensity Properties** building block.

➤ The resulting intensity values are added to the list of nuclei in a new column "Dead Stain Intensity Mean".

Identify Cells with significant Dead Cell Marker Intensity

➤ A **Select Population** building block is used to detect the population of cells with significant dead cell marker fluorescence, which represents the fraction of cells with disrupted membranes ("dead cells").

➤ The criterion used is "Dead Stain Intensity Mean > threshold". The threshold is needed to filter out cells at dead stain background level and below. Since the signal window (the intensity difference between the background and staining intensity of dead cells) is very large, the adjustment is not critical. A value at the middle of the signal window is appropriate.

➤ The threshold is adjusted by visual inspection of both the illustration and the object property table provided by the **Select Population** building block. The table can be sorted by dead cell marker intensity by clicking on the column header.

➤ The object property table can also be exported by copying and pasting into excel for further visualization of individual cell intensities.

Calculate Readout Values

➤ The main readout value for this assay is the percentage of dead cells in the cell population.

➤ The **Define Results** building block is used to report the total number of cells and calculate and report the fraction of dead cells.

2.3.4   Hints and Tips

➤ Live / dead cell quantifications can be added to other assays as an add-on. A nuclear stain is already included in most assay setups, therefore only one additional channel for a dead cell marker is needed.

➤ If fragmentation of nuclei occurs in the dead cells, the **Find Nuclei** building block parameters might have to be adapted for correct identification of clusters of fragments as one nucleus. See the RMS "Nuclear Fragmentation" (section 2.13).

➤ Alternative approaches may use a combination of a live cell stain (instead of a stain for all cells) in combination with a dead cell stain. In this case, two **Find Nuclei** building blocks have to be used for sequential identification of the "live" and "dead" cell populations. See the RMS "Live Dead Cell Counting-2" in the Harmony database.

2.4 Nuclear Analysis – Nuclear Shrinking

This RMS allows for the quantification of different nuclear fluorescence parameters, such as staining intensity and area, as well as geometric parameters, based on a single nuclear stain.

2.4.1 Introduction and Assay Principle

Toxicity results in cell death which is accompanied by characteristic morphological changes in the nucleus. Nuclear shrinking and condensation are commonly observed during apoptosis, whereas cells undergoing necrosis initially show nuclear swelling prior to shrinkage.

In addition to measuring the nuclear area and staining intensity, this RMS also provides other nuclei-relevant and more general read-outs that are used to measure toxic-effects.

In the example shown below, the size of the nuclei in the compound-treated cells is determined based on nuclear staining.

0 µM   10 µM   100 µM
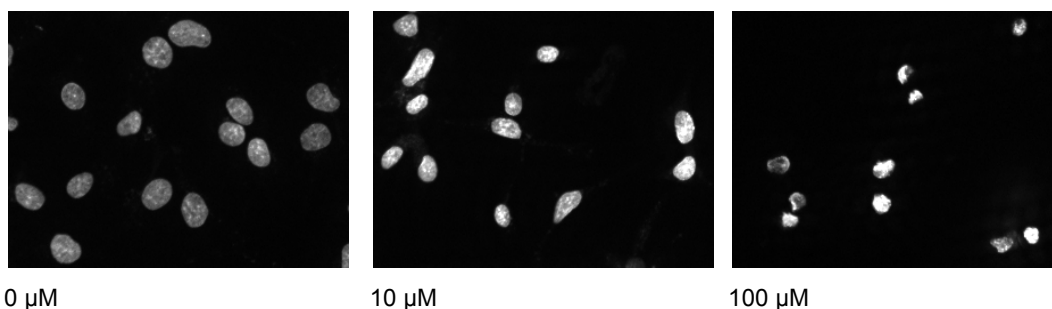
Figure 2–5: Images showing nuclear staining (Hoechst 33342) at different concentrations of a toxic compound (FCCP). As the concentration of the compound is increased (from left to right), there is a notable decrease in the size of the nuclei, due to nuclear shrinking. The cell line used in this experiment was the human hepatocellular liver carcinoma cell line (HepG2). Details of the example dataset ("Plate P003-CT: Live Tox") can be found in the Appendix on page 313.

2.4.2 How to use the Nuclear Analysis – Nuclear Shrinking RMS

➤ Load the analysis sequence "RMS Nuclear Analysis" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Check the nuclei detection results and adjust the parameters if needed.

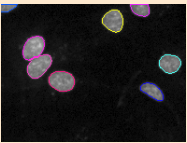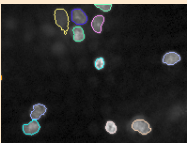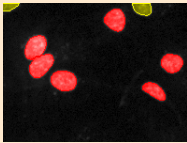## Image Analysis Sequence: Nuclear Analysis – Nuclear Shrinking
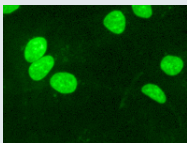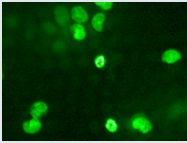
Input Image

Channel 1:
Nuclear stained image

Untreated control

Treated cells showing nuclear shrinkage

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Select Population

Only keep the nuclei that are completely visible in the image

Nuclear region overlaid on the nuclear stain image (untreated cells)

Resulting detection of nuclear shrinkage

Removed nuclei (yellow)

Quantify Properties in Regions

Calculate Intensity Properties

Nuclear dye intensity, standard deviation, integrated intensity (sum)

Intensity Mean	Intensity StdDev	Intensity Sum
587.919	122.314	303366
439.221	113.036	187108
520.243	121.705	220583
530.49	102.916	335800
289.563	68.6085	154337

Intensity Mean	Intensity StdDev	Intensity Sum
628.777	209.201	138331
683.432	284.18	110716
832.771	286.212	130745
472.896	185.653	72826
936.13	361.193	144164

Calculate Morphology Properties

Area, roundness, width to length ratio

Area [µm²]	Roundness	Ratio Width to Length
215.268	0.93365	0.558156
177.722	0.981565	0.681499
176.887	0.945272	0.616817
264.079	0.965008	0.720969
222.361	0.966603	0.751774

Area [µm²]	Roundness	Ratio Width to Length
91.7811	0.981357	0.669983
67.5843	0.901968	0.664364
65.4983	0.859389	0.58722
64.2468	0.898736	0.685994
64.2468	0.925619	0.685994

Calculate Readout Values

Define Results

Averaged values of all the cells in the well (all fields)

NF - Number of Objects	NF - Intensity Mean - Mean
132	456.496

NF - Intensity StdDev - Mean	NF - Intensity Sum - Mean
116.228	189946

NF - Area [µm²] - Mean	NF - Roundness - Mean
175.49	0.945326

NF - Ratio Width to Length - Mean
0.676617

NF - Number of Objects	NF - Intensity Mean - Mean
26	702.163

NF - Intensity StdDev - Mean	NF - Intensity Sum - Mean
272.102	130482

NF - Area [µm²] - Mean	NF - Roundness - Mean
80.6294	0.90192

NF - Ratio Width to Length - Mean
0.62862

Readout Values

Number of Nuclei	Equivalent to the number of cells. Very sensitive indicator of toxic effects
Mean Nuclear Intensity	Brightness of the nuclear staining (per area)
StdDev of Nuclear Intensity	Homogeneity of nuclear staining in each individual cell
Sum Intensity	Total amount of nuclear stain, e.g. measurement of DNA content
Mean Nuclear Area	Measure nuclear swelling or nuclear shrinking
Roundness of the Nucleus	Measure nuclear shape, a value of 1.0 means a perfect circle
Width to Length Ratio	Measure nuclear shape, e.g. indicator of late M-phase nuclei

2.4.3   Image Analysis in Detail

Image Segmentation

In the first step, a **Find Nuclei** building block is used for image segmentation. A nuclear region mask is automatically generated as a default result which will be used in the subsequent steps to calculate all nuclei properties.

In the second step, a **Select Population** building block is used to remove all nuclei which are not completely visible in the image. These nuclei would introduce artifacts, e.g. the measured area of the nuclei would be smaller than the actual area.

Quantifying Intensity and Morphology

The **Calculate Intensity Properties** and **Calculate Morphology Properties** building blocks are used to determine the most relevant properties for characterization of the nuclei:

➤ The **mean intensity** of the DNA / RNA staining provides information about the DNA / RNA concentration in the nucleus. Condensation of nuclei leads to higher concentrations of DNA / RNA, hence brighter fluorescence. See section 1.4 for hints on nuclear stains.

➤ The **standard deviation of the intensity** in the nuclear region provides information about the homogeneity of the dye distribution inside the nuclear area, allowing, for example, conclusions to be made about the fragmentation of the nucleus. See the RMS "Nuclear Fragmentation" (section 2.13).

➤ The **integrated intensity**, i.e. the summed intensity values of all the pixels in the nuclear region, provides information about the total amount of dye in the nucleus, allowing determination of the DNA content. The doubling of the chromosome set during S-phase can be monitored using the integrated intensity. The staining has to be quantitative for exact results. See section 1.4 and the RMS "Nuclear Classification – DNA content" (section 2.12).

➤ The **area** is used to measure nuclear swelling and shrinking. The area can be calculated in either μm^2 or the number of pixels included.

➤ The **roundness** and **width to length ratio** are common measurements for quantifying the nuclear shape, see *Figure 2–6*.

Demo shape			
Width to length ratio	1.0	0.33	0.59
Roundness	1.0	0.77	0.78
Area	2000	1695	2113

Figure 2–6: Three example shapes are shown, demonstrating the readout-values of different morphology parameters. The width to length ratio and roundness give a value of 1.0 for a perfect circle, and decrease in value for elongated objects (especially the width to length ratio), and objects with irregular borders (especially roundness). The roundness is proportional to the square root of the area divided by the circumference. It is normalized to give 1.0 for a perfect circle.

Calculate Readout Values

The results for each single cell within the well are averaged to generate the result for the whole well. For each value also additional statistical parameters can be reported on demand, e.g. the standard deviation, providing information about the variability of the readout value in the cell population.

Be careful not to get confused by "double mean" values like the "Mean Mean Nuclear Intensity" result for each well.

➤ Firstly, the mean intensity for each individual cell is calculated and is given as an individual cell result: it is the mean intensity value of all the image pixels within the chosen region of interest, e.g. the nucleus (see *Figure 2–7*).

➤ Secondly, the result per well is calculated and is given by the **Define Results** building block. This is the mean value of all the individual cell values, hence the "Mean Mean Nuclear Intensity".
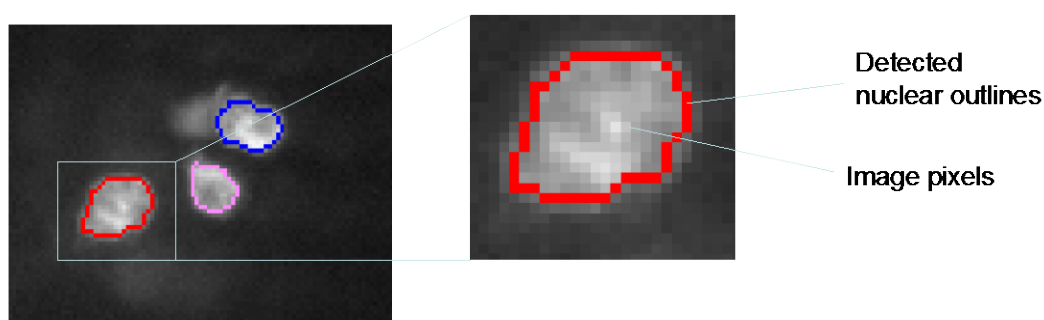
Figure 2–7: Mean intensity versus sum (= integrated) intensity. Left panel: Section of an image showing a nuclear stain channel with three detected nuclear outlines marked. Right panel: One nucleus is enlarged to see the individual pixels. Each pixel can have an intensity value between 0 and 16383 for a 14 bit camera. The mean intensity (mean of all pixels in the region marked by the red line) is 2,918.4 units, the area includes 209 pixels and the sum intensity is 609,980.0 units.

2.4.4   Hints and Tips

➤ To calculate more properties: open the **Calculate Intensity Properties** or **Calculate Morphology Properties** building block options and select more parameters. See the "Harmony Help Function" for a detailed description of all parameters.

➤ To quantify additional markers recorded in other channels, add additional calculate properties building blocks that work on different channels, see for example, the RMS "Quantification of Marker in Nucleus" (section 2.5).

➤ Single cell results are generated by **Define Results** and are also included in the individual results of each building block (in interactive mode). They can be used to analyze nuclear size distributions across the cell population, e.g. the occurrence of a subpopulation of cells with a smaller nucleus. For more uses of single cell results, see the RMS "Cell Cycle Classification" (section 2.14).

Be aware that the morphology parameters become inaccurate for small objects since they suffer due to the digital image resolution. For example, if the area of a round object includes 50 pixels the radius is about 4 pixels. An inaccuracy of one pixel corresponds to a 25 % error, leading to increased rounding errors and sensitivity to noise. Though the Harmony software uses the most robust algorithms available to compensate for quantification artifacts, the analysis sequence has to be carefully validated if width or roundness is calculated for objects with an area below 100 square pixels.

2.5 Quantification of Marker in Nucleus

This is a RMS for measurement of marker intensity in an adjustable region of interest inside the nucleus, and identification of a cell population carrying the marker. In addition, morphological or textural properties of the marker can be evaluated on demand.

2.5.1 Introduction and Assay Principle

Many cell assays can be quantified by measuring the intensity of a fluorescent marker in the nucleus, e.g. the uptake of a membrane-impermeable nuclear stain in cell viability assays, quantifying DNA content in cell cycle assays, Fluorescence *In Situ* Hybridization (FISH) assays, and expression of a nuclear protein.

➤ This RMS allows for two common observations: (a) continuous increase of the marker intensity or (b) "yes-no" characteristics.

➤ For (a), the per cell or mean intensity values are of interest, whilst for (b), the number or fraction of cells showing the marker ("positive cells") are of interest.

➤ The example dataset in *Figure 2–8* uses EdU as a marker for detection of S-phase cells.

➤ EdU accumulates in the nucleus during S-phase. A compound that blocks the cell cycle leads to varying numbers of S-phase cells. See Application Guide, section 3.3 "Ready-Made Application: Cell Cycle-1".
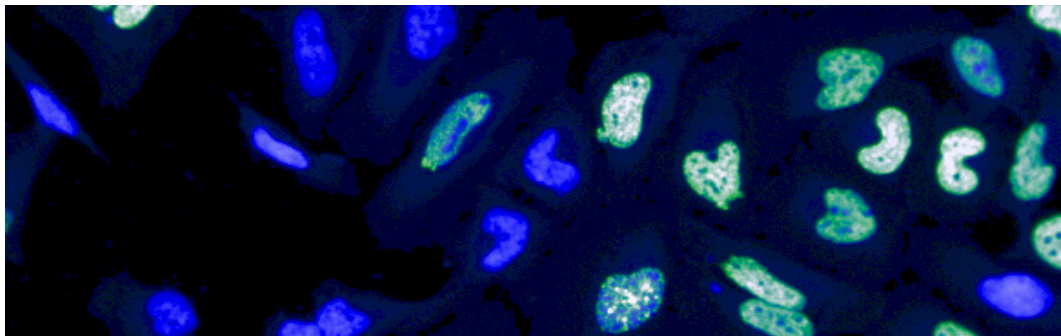
Figure 2–8: Color overlay of a DNA stained channel (blue) and a marker (EdU) stained channel (green). The DNA / RNA stain (combined Hoechst 33342 + CellMask™ Blue) also marks the cytoplasm at a lower intensity. The marker stain (EdU) accumulates in some of the nuclei at different intensity levels and with different textures (homogeneous / granular). See the example dataset "P004-CC: EdU-pHH3" on page 314 in the Appendix for details.

2.5.2 How to use the Quantification of Marker in Nucleus RMS

➤ Load the analysis sequence "RMS Quantification of Marker in Nucleus" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the specific marker channel in the **Calculate Intensity Properties** building block.

➤ Visually check the building block illustrations for representative wells.

➤ Adjust the intensity threshold for determination of "positive" cells in the **Select Population** building block if needed.

Image Analysis Sequence: Quantification of Marker in Nucleus

Input Images

Channel 1:
Nuclear stain

Channel 2:
Marker

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Quantify Properties in Regions

Calculate Intensity Properties

Measure marker intensity in the nuclear region

Object No	Marker Intensity Mean
45	30,9444
46	269,925
47	875,573
48	36,5076
49	1339,47
50	31,7619

Identify Subpopulations

Select Population

Identify cells with high marker intensity ("Marker positive" or "POS")

Selected objects (red) with marker intensity > 200 units.
Discarded objects (blue) with marker intensity < 200 units

Calculate Readout Values

Define Results

Select the average values to give the results per well.

Calculate fraction of marker positive cells.

Full table of results per well

Number of Objects	Marker Intensity - Mean	Stddev of Marker Intensity
843	362,052	540,406

Number of POS Cells	Fraction of POS Cells [%]	Marker Intensity of POS Cells - Mean
246	29,1815	1119,01

Readout Values

Number of Cells	Corresponds to the number of nuclei divided by the number of objects
Mean Marker Intensity	Mean marker intensity over all cells. Higher values indicate that more cells contain the marker and / or have higher marker intensities
StdDev of Marker Intensity	Measure how uniform the marker intensity is within the cell population
Number of POS Cells	Number of cells in the "Marker positive" population
Fraction of POS Cells	Fraction of cells in the "Marker positive" population
Mean Marker Intensity of POS Cells	Mean marker intensity in the "Marker positive" population

2.5.3   Image Analysis in Detail

Image Segmentation

A single **Find Nuclei** building block is used for image segmentation since the nucleus is the only region of interest.

Defining Regions of Interest

A nuclear region mask is automatically provided as a result of the **Find Nuclei** building block. Explicit building blocks for region selection can be optionally added if the nuclear region needs to be modified, e.g. expanded or shrunk to cover only part of the detected nuclear area.

Quantifying the Intensity

A **Calculate Intensity Properties** building block is used to measure the marker intensities in the nuclear regions. Additional measurements can be added here as needed (more channels, further intensity or morphological properties). See section 3.3.3 for more details.
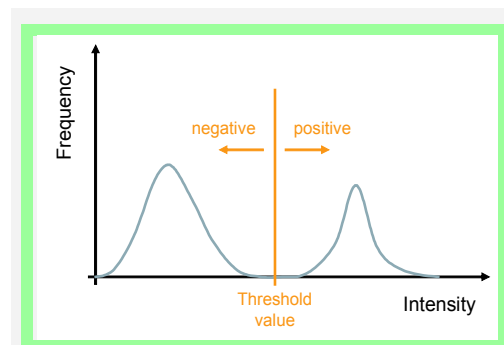
Determination of a Subpopulation

To divide the cells into two groups – cells that show the marker ("positive") and cells that do not show the marker ("negative"), a criterion is needed. A common criterion is to use an intensity threshold: if the marker intensity exceeds the given threshold then a cell is marked "positive'". A **Select Population** building block is used for this purpose.

The threshold selection needs some consideration:

Scenario A – For well separated populations (see upper part of *Figure 2–9*), select a threshold value half way between the low and high values. You will also need to take into account the fact that the low and high values may fluctuate from well to well, or plate to plate: use random wells at different positions to determine a generic threshold.

Scenario B – The marker intensities are continuously distributed (see lower part of *Figure 2–9*). Selecting an intensity threshold arbitrarily defines the "splitting point" between the two populations. The results of the analysis depend on the selected threshold value. The result is more sensitive to well-to-well fluctuations and noise. The marker is not suited for

Scenario A – Two populations are well separated by the measured intensity. Select a threshold value half way between the two populations.
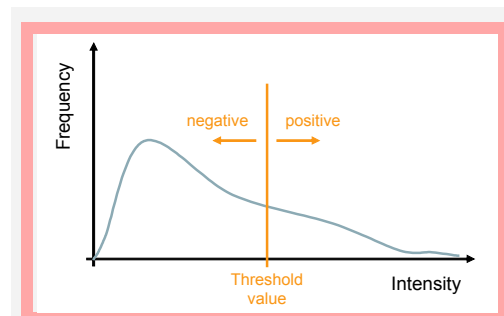
Scenario B – The threshold is arbitrarily defining the population of "positive" cells ("high" marker intensity).

Figure 2–9: Threshold selection for sub-population determination using a fluorescent marker.

completely unambiguous identification of the population, though its accuracy may be sufficient in many cases to define a threshold value.

➤ Browse through different wells using the visual feedback illustrations of the **Select Population** building block, and the object list display included in the individual results of the building block, to find a value.

➤ You may also want to export the object list in order to create an intensity histogram as shown in *Figure 2–10* (see section 3.5.6 for information on exporting the result tables).

➤ To assess the robustness of the analysis try thresholds ± 20 %. The conclusions of the experiment must not be affected.
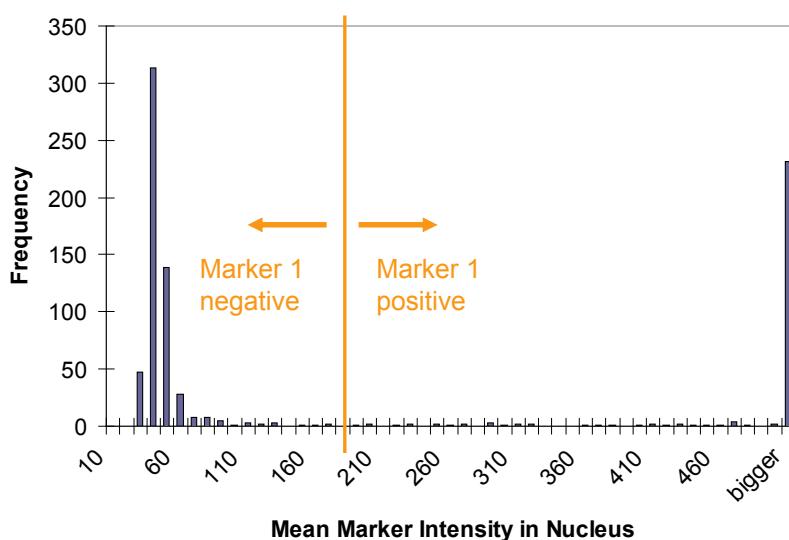

Figure 2–10: An example intensity histogram for one well of the example dataset with the lower intensity values expanded (Excel diagram of the property "Mean Marker 1 Intensity in Nucleus"). The baseline intensity of the marker is about 60 units. Positive cells have a flat distribution over a broad range of intensities from 110 to 2000 (values > 500 are pooled into the "bigger" column in the diagram). The position of the selected threshold is marked orange – it has a high security margin from the baseline intensity – even background fluctuations of 100 intensity units (i.e. more than 100 %) would not create artificially "positive" rated cells.

2.5.4   Hints and Tips

➤ Additional marker channels can be added by inserting additional **Calculate Intensity Properties** and **Select Population** building blocks.

➤ The region for marker quantification can be adjusted by inserting a **Select Region** building block and using the "extending / shrinking" method.

➤ Nuclei which are only partially visible at the image borders can be removed from the analysis by inserting a Select Population building block to avoid artifacts.

➤ Further cell properties can be quantified using an additional **Calculate Morphology Properties** building block, or by selecting more parameters in the **Calculate Intensity Properties** building block.

2.6 Quantification of Cytoplasmic Marker

This RMS is tailored to quantify any marker located in the cytoplasm by fluorescence intensity measurement, regardless of whether the marker is ubiquitously distributed in the cytoplasm or accumulates in defined regions within the cytosol.

2.6.1 Introduction and Assay Principle

Cytoplasmic marker quantification is used in a variety of HCS assays. It can quantify any marker targeted to organelles in the cytoplasm of a cell such as mitochondria, endosomes, endocytotic vesicles, peroxisomes, etc. It can also quantify proteins expressed in the cytosol such as cyclins (in a certain stage of the cell cycle) or enzymes labeled with fluorescent proteins or antibodies. Cytoplasmic proteins and cellular organelles are often affected by macromolecules, resulting in varying intensities or redistribution of the target molecule. In the example shown in *Figure 2–11*, caspase-3 was activated by treatment with staurosporine and was labeled using a fluorescently tagged antibody.
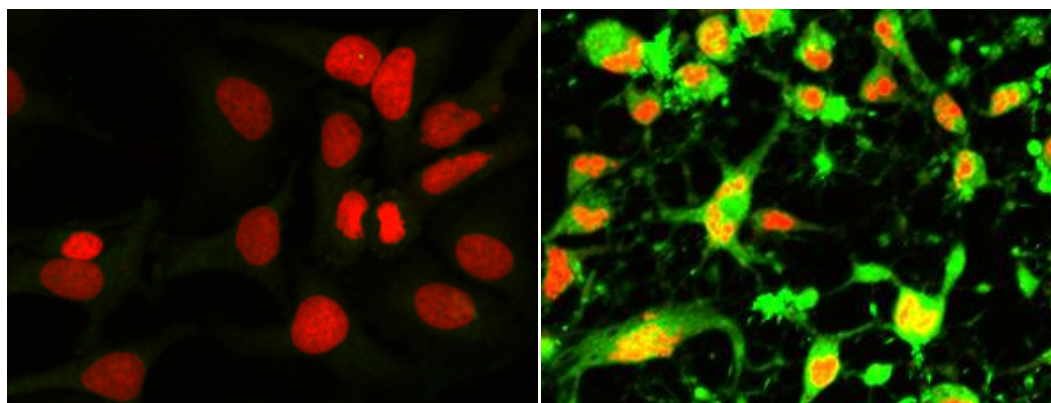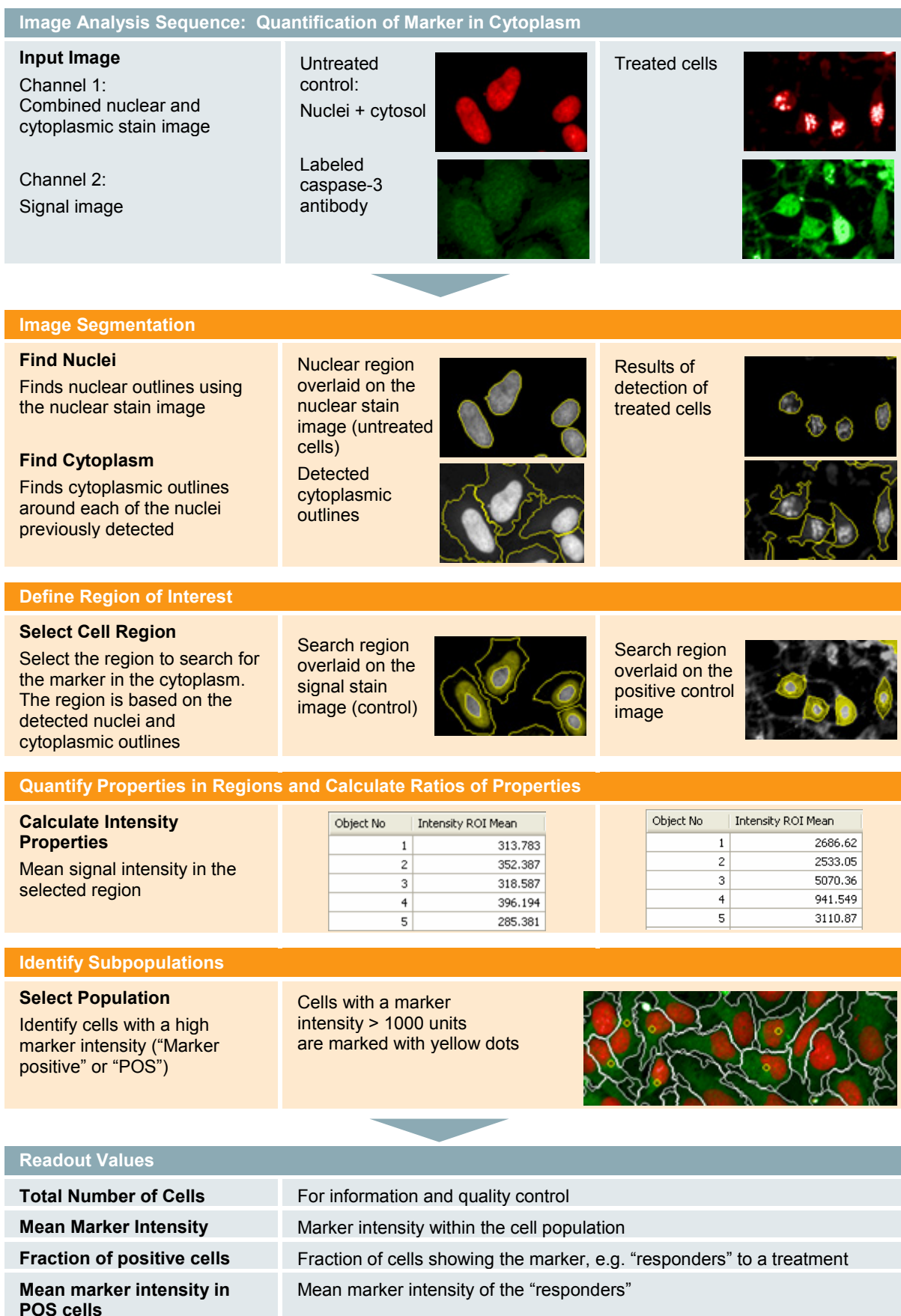

0 µM   10 µM

Figure 2–11: Example of a two channel false color overlay. Nuclei were stained with DRAQ5™ (red) and active caspase-3 was immunofluorescently labeled with anti-cleaved-caspase-3 antibody-Alexa Fluor® 488 conjugate. Left: Untreated cells without activated caspase-3. Right: Cells incubated for 4 h with 10 µM staurosporine show a significant increase in activated caspase-3. Details of the example dataset ("Plate P005-A: Caspase3 Apoptosis") can be found in the Appendix on page 315.

2.6.2 How to use the Quantification of Cytoplasmic Marker RMS

➤ Load the analysis sequence "RMS Cytoplasmic Marker" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic (or combined nuclear / cytoplasmic) dye channel in the **Find Cytoplasm** building block.

➤ Adjust the cytoplasmic ROI in the **Select Cell Region** building block.

➤ Select the specific marker channel in the **Calculate Intensity Properties** building block.

➤ Visually check the building block illustrations for representative wells.

➤ Adjust the intensity threshold for the determination of "positive" cells in the **Select Population** building block if needed.

Image Analysis Sequence: Quantification of Marker in Cytoplasm

Input Image

Channel 1:
Combined nuclear and cytoplasmic stain image

Channel 2:
Signal image

Untreated control:

Nuclei + cytosol

Labeled caspase-3 antibody

Treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around each of the nuclei previously detected

Nuclear region overlaid on the nuclear stain image (untreated cells)

Detected cytoplasmic outlines

Results of detection of treated cells

Define Region of Interest

Select Cell Region

Select the region to search for the marker in the cytoplasm. The region is based on the detected nuclei and cytoplasmic outlines

Search region overlaid on the signal stain image (control)

Search region overlaid on the positive control image

Quantify Properties in Regions and Calculate Ratios of Properties

Calculate Intensity Properties

Mean signal intensity in the selected region

Object No	Intensity ROI Mean
1	313.783
2	352.387
3	318.587
4	396.194
5	285.381

Object No	Intensity ROI Mean
1	2686.62
2	2533.05
3	5070.36
4	941.549
5	3110.87

Identify Subpopulations

Select Population

Identify cells with a high marker intensity ("Marker positive" or "POS")

Cells with a marker intensity > 1000 units are marked with yellow dots

Readout Values

Total Number of Cells	For information and quality control
Mean Marker Intensity	Marker intensity within the cell population
Fraction of positive cells	Fraction of cells showing the marker, e.g. "responders" to a treatment
Mean marker intensity in POS cells	Mean marker intensity of the "responders"

2.6.3   Image Analysis in Detail

Image Segmentation

➤ The first step of the analysis is always to detect nuclei, which is done by applying the **Find Nuclei** building block. In the example shown in *Figure 2–11*, untreated cells show regular and well defined nuclei whereas treated cells are apoptotic and show karyorrhectic nuclei. Choosing the right method and adjusting the parameters for nuclei detection is therefore crucial for image analysis.

➤ Correct nuclei detection is very important since any errors will be "inherited" by the cytoplasmic detection!

➤ In the second step the cytoplasm is detected **around** the previously detected nuclei, using the **Find Cytoplasm** building block. The cytoplasms are "secondary objects".

Defining Regions of Interest

➤ The selection of an appropriate region is crucial for a robust read-out, therefore you should restrict the region used for signal measurement to an area where the desired effect is maximal and noise is minimal. For example, if a cytoplasmic signal occurs in a ring around the nucleus, restrict the region of interest to a ring region around the nucleus. This avoids capturing fluorescence at the cell borders. See *Figure 2–12* for examples of different regions.

➤ The whole cytoplasmic region is automatically selected by the **Find Cytoplasm** building block. More specific regions can be defined by the **Select Region** and **Select Cell Region** building blocks (see section 1.3.2).

➤ The **Select Region** building block allows modification of the previously created regions, e.g. expanding a nuclear region to create a ring around the nucleus. Restrict the ring to the cytoplasm of the cell; otherwise fluorescence from neighboring cells might be captured as well (see *Figure 2–12*).

➤ **The Select Cell Region** building block allows you to define a relative region inside the cytoplasm based on the previously detected nuclear and cytoplasmic outlines (see *Figure 2–23*).
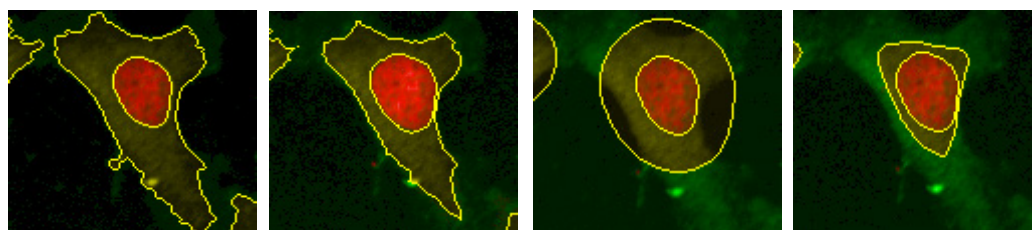
A – Whole cytoplasm region

B – Membrane and nuclear border excluded to avoid noise

C – Fixed ring region around nucleus

D – Ring region around nucleus (restricted to cytoplasm)

Figure 2–12: Examples of the selection of different regions inside the cytoplasm. A – Whole cytoplasm region. B – 20 – 80 % region of cytoplasm, excluding the membrane and nuclear borders. C – Ring region around nucleus. D – Restricted ring region around the nucleus, avoiding the capture of background signal or fluorescence from neighboring cells.

Calculate Readout Values

➤ Two different readouts are available:

- "Number of positive cells", e.g. "Responders" to treatment with a compound. This readout gives the best results if each individual cell switches from one state to another. Absolute marker intensities do not matter, each cell is either positive or negative.

- Mean intensity of fluorescence of the whole cell population. This readout is best suited for measuring the amplitude of an effect on a continuous scale, i.e. the mean level of a marker in a cell population.

➤ See the RMS "Quantification of Marker in Nucleus" (section 2.5) for more information.

2.6.4   Hints and Tips

➤ For more information on the **Select Cell Region** and **Select Region** building block features and their differences, see section 3.3.2, "Step 2 – How to Define Regions of Interest inside the Objects".

➤ Removal of cells which are not completely visible in the image can avoid artifacts: see the analysis sequence variant RMS "Cytoplasmic Marker – Remove Border Cells", and also the RMS "Quantification of Marker in Nucleus" (section 2.5).

➤ Combine with quantification of markers in multiple regions, e.g. nucleus and cytoplasm to determine the location of the marker inside the cell. Also see the RMS "Cytosol to Nucleus Translocation" (section 2.9) and "Cytosol to Membrane Translocation" (section 2.10).

➤ Note that regions of different objects never overlap.

2.7   Quantification of Membrane Marker

This RMS allows for the accurate quantification of fluorescent markers located in the cell membrane. Populations of cells can be identified based on their membrane fluorescence.

2.7.1   Introduction and Assay Principle

Binding of fluorescently labeled ligands or antibodies to plasma membrane receptors or other cell surface molecules would typically be analyzed with this RMS. The expression of receptors carrying a fluorescent protein tag is another application.

Since membrane ruffling (when using fluorescently tagged receptors) is typically associated with an increase in fluorescence in the outer regions of the cell membrane of adherent cells in an image, this RMS can be the starting point for this application as well. An increase in intensity in these outer areas of the cell will appear as increased membrane fluorescence here.

➤   The quantification of a marker located in the cell membrane is very similar to the quantification of a marker in the cytoplasm, except that a different region of interest is used.

➤   Here, we explain what is specific to the analysis of markers in the plasma membrane. Please refer to section 4.6, "Quantification of Cytoplasmic Marker" for the general approach to marker quantification and identification of a subpopulation of cells, e.g. cells with increased membrane fluorescence.
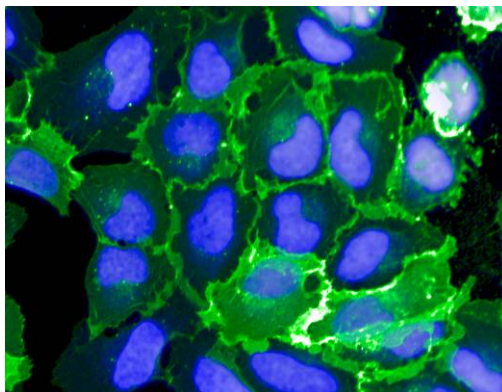
Example for a membrane bound marker. Blue – Hoechst 33342 nuclear dye,
green – membrane bound receptor labeled by a fluorescent protein.
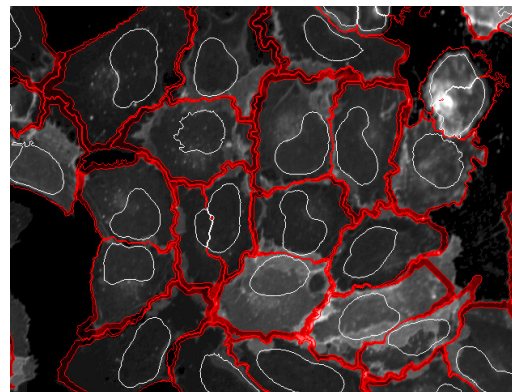
Region of interest for membrane fluorescence quantification overlaid on the membrane channel gray scale image.

Figure 2–13: Example of membrane fluorescence detection. Details of the example dataset ("Plate P010-R: ETAR") illustrating membrane fluorescence can be found in the Appendix on page 316.

2.7.2 How to use the Quantification of Cytoplasmic Marker RMS to quantify Membrane Fluorescence

➤ Load the the analysis sequence "RMS Cytoplasmic Marker" from the Harmony database.

➤ Select the nuclear dye channel in the Find Nuclei building block.

➤ Select the cytoplasmic (or combined nuclear / cytoplasmic) dye channel in the **Find Cytoplasm** building block.

➤ Select the region type "Membrane Region" in the **Select Cell Region** building block.

➤ Select the specific marker channel in the Calculate Intensity Properties building block.

➤ Visually check the building block illustrations for a representative well and adjust the membrane region size in the **Select Cell Region** building block.

➤ Adjust the intensity threshold for the determination of "positive" cells in the **Select Population** building block if needed.

2.7.3 Image Analysis in Detail

The same analysis sequence as described in the "Quantification of Cytoplasmic Marker" section (page 191) is used, but with a different region of interest. (Please refer to the descriptions given on page 193).

Specific to Membrane Quantification:

➤ Determination of the membrane position is more difficult than detection of the nuclei or cytoplasm → higher noise due to inaccuracies in positioning.

➤ The visibility of the membrane strongly depends on imaging conditions (e.g. focal plane, numerical aperture, confocal imaging), cells (e.g. adherent vs. suspension cells, size) and assay conditions (e.g. staining, confluency). Please pay particular attention when setting up the image acquisition for these assays (see chapters 1 and 2).

➤ To optimally capture the membrane fluorescence, adjust the membrane region settings in the **Select Cell Region** building block. See *Figure 2–14* for an example of the two different options:

- The region can be defined relative to the dimensions of the cell on a percentage scale. *Figure 2–23* gives an explanation of the relative percentage scale where the membrane is located at 0 %. Negative percentages extend the region border outside the cell whereas positive percentages move the region border inside the cell. See *Figure 2–14* (left panel) for an example of a region extending from – 5 % (outer border) to + 5 % (inner border).

- The region can also be specified using absolute values, either in μm or image pixels around the detected cell outlines. See the right panel of *Figure 2–14*.

➤ The optimal settings can most easily be determined interactively using one representative example image. Adjust the region type and inner and outer border locations to capture most of the membrane fluorescence of interest while avoiding capturing background or unwanted fluorescence. Check the settings on at least five more images.
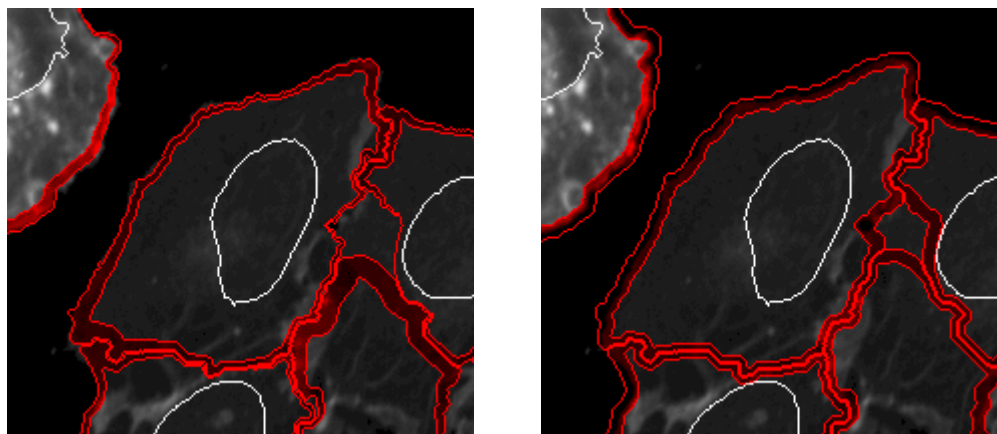
Dynamic membrane region of ± 5 % around the detected cytoplasmic outlines.

Fixed region of ± 4 image pixels (px) around the detected cytoplasmic outlines.

Figure 2–14: Examples of the different options for specifying a membrane region in the **Select Cell Region** building block. To find the most suitable region for your specific application, try different settings and choose the one which best matches the location of the membrane fluorescence. Use an image with high membrane fluorescence for adjustment.

2.7.4   Hints and Tips

➤ Precise detection of the cellular outlines using the **Find Cytoplasm** building block is absolutely essential in this application since the membrane area is very small and can easily be "missed"!

➤ The membrane fluorescence detection can be combined with detection of fluorescence in any other compartments and / or channels by further adding the **Select Cell Region** and **Calculate Intensity Properties** building blocks.

➤ Cells which are not completely visible in the image can introduce artifacts. These cells can be removed with an additional **Select Population** building block. Please refer to the RMS "Quantification of Marker in Nucleus" (section 2.5) for an example.

➤ You can define multiple membrane regions by using multiple **Select Cell Region** building blocks and checking which region gives the best results for your assay. The quality of the results can be judged by the Z' value, see section 1.5.1.

➤ You can also create a weighting of fluorescence by using multiple regions and summing the fluorescence intensities using different factors with a **Calculate Properties** building block:

• Near to cell border (= membrane) → high weight, e.g. 1.0.

• Near to nucleus → low weight, e.g. 0.1.

➤ This kind of weighting or "scoring" approach is less prone to imprecise detection of the membrane position.

➤ Alternatively, texture analysis can be used to detect membranes and quantify membrane fluorescence. Please see section 1.7.1 and the Application Note "Image-based Quantification of PKC Activation using Operetta" on the PerkinElmer website:
http://www.cellularimaging.com/application_notes/operetta/

2.8 Spot Analysis

This RMS quantifies all spot-like features of homogeneous size (endosomes, round shaped organelles, molecular clusters, etc.) in any sort of cellular compartment. For various sized spots (lipid droplets, organelles, protein agglomerates), please see the RMS "Lipid Droplet Analysis – Analysis of various sized spots" (section 2.24).

2.8.1 Introduction and Assay Principle

Many small cellular organelles or molecular clusters appear as "spots" in an image. Spot Analysis can be used for functional receptor activation assays, analyzing receptor internalization into endosomes upon ligand stimulation. Spot analysis can also identify clathrin-coated pits and clathrin-coated vesicles using fluorescently labeled arrestin, which binds to activated receptor complexes as in the Transfluor® assay.

Other spot fluorescence assays include endocytosis and peroxisome analysis.

➤ The RMS is tailored to find spots in a selectable area of the cell: Nucleus, Cytoplasm, Membrane, Whole Cell region.

➤ Readouts give information on the number of spots, and spot intensity related values.
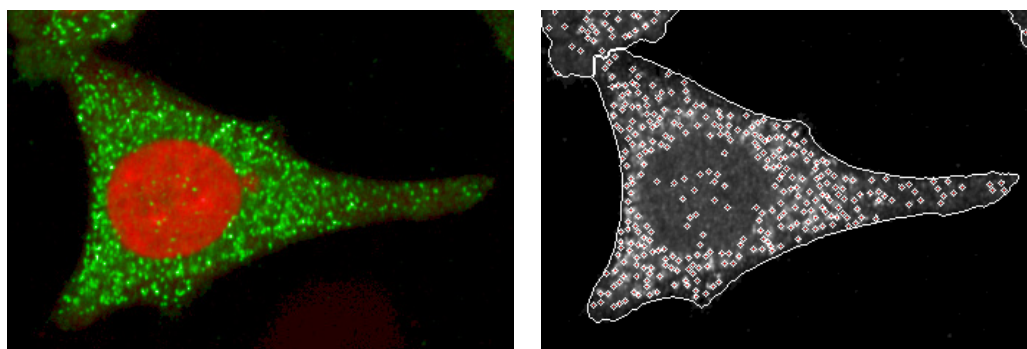
Color overlay of the DRAQ5™ nuclear and cytoplasmic stain channel (red), and the peroxisome marker channel (green).

Spots detected on peroxisome marker channel are overlaid on the gray scale image of the marker channel. Cellular outlines, as detected on the DRAQ5™ stain image, are marked white.

Figure 2–15: Example of a spot analysis. Details of the example dataset ("Plate P001-CT: Peroxisomes" with 20x objective) showing spot features can be found in the Appendix on page 318.

2.8.2 How to use the Spot Analysis RMS
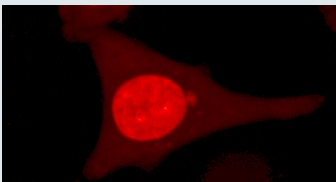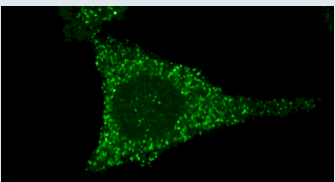
➤ Load the analysis sequence "RMS Spot Analysis" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic (or combined nuclear / cytoplasmic) dye channel in the **Find Cytoplasm** building block.

➤ Select the specific marker channel in the **Find Spots** building block.

➤ Adjust the search region and spot radius in the **Find Spots** building block.

Image Analysis Sequence: Spot Analysis

Input Images

Channel 1:
Combined nuclear and cytoplasmic stain image

Channel 2:
Spot signal image

DRAQ5™ stain image

Peroxisome specific dye

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Nuclear region overlaid on the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around each of the previously detected nuclei

Detected cytoplasmic outlines
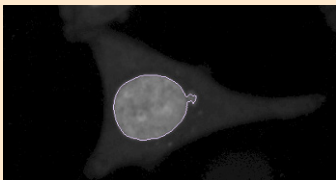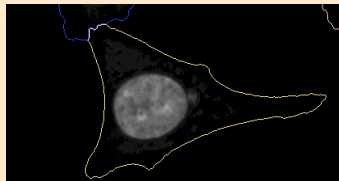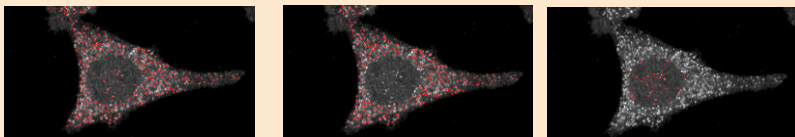
Image Segmentation 2 – Find Spots and Quantify Spot Properties

Find Spots

Find spots on the spot signal image in a selected region

Detected spots in the cytoplasmic region are overlaid on the spot signal image in three different example regions

Calculate Readout Values

Define Results

Create average results for the cell population in the well

Object No	IntensityCell Relative Spot Signal	Relative Spot Signal on Cell	Number of Spots on Cell	Number of Spots per Area of Cell	Field
1	0.224726	0.0128763	461	0.0128466	1
2	0.194796	0.00964787	178	0.00963516	1
3	0.280802	0.0178768	455	0.0127059	1
4	0.272909	0.0141668	550	0.0135178	1

Individual spot analysis results for each cell

Total Number of Cells	Mean Number of Spots per Cell	Mean Relative Spot Intensity	Mean Number of Spots per Area
9	303	0.0119018	0.0112921

Averaged values to give the results per well

Readout Values

Total Number of Cells	For information and quality control
Mean Number of Spots per Cell	For information and quality control. Tip: this is not a good readout parameter due to its nonlinearity and dependence on parameter settings
Mean Relative Spot Intensity	Robust quantifier for biological effects that affect the spot intensity
Mean Number of Spots per Area	Robust quantifier for biological effects that affect the number of spots

2.8.3 Image Analysis in Detail

Image Segmentation

The location of the cells and the nuclear and cytoplasmic compartments of each cell are detected using the **Find Nuclei** and **Find Cytoplasm** building blocks. Please refer to the more detailed description in the RMS "Quantification of Cytoplasmic Marker" (section 2.6) for more information. A set of standard regions (whole cell, nucleus, cytoplasm and membrane) are generated by these building blocks for display and subsequent spot detection.

Find Spots and Quantify Spot Properties

Spots are detected in the spot signal channel using the **Find Spots** building block's method "C", which is optimized to detect round spots of homogeneous size. To specify which kind of spots should be detected, several parameters can be adjusted:

- ➤ Specify a search "**Region"** for the spots (see *Figure 2–16*).

- ➤ Specify the desired spot "**Radius"** by zooming into a part of the image that contains typical spots and adjusting the radius according to instructions given in *Figure 2–17*.

- ➤ Option: specify the filter criteria to select spots by their intensity. Two parameters are available:

  - The "**Contrast**" is the spot intensity compared to its immediate surroundings (neighboring image pixels). High contrast spots are bright in a dark surrounding. Even if the background intensity inside the search region varies, the spots can still be qualified by their contrast.

  - The "**Spot to Region Intensity**" parameter specifies how bright a spot has to be compared to the mean intensity in the whole search region. See *Figure 2–18* for an example and instructions on how to adjust this parameter.

For a detailed description of all the available Find Spots parameters, please refer to the built in "Harmony Help Function", which is accessible by clicking the right mouse button in the parameter input fields in the **Find Spots** building block.

Whole cell   Cytoplasm   Nucleus   Membrane
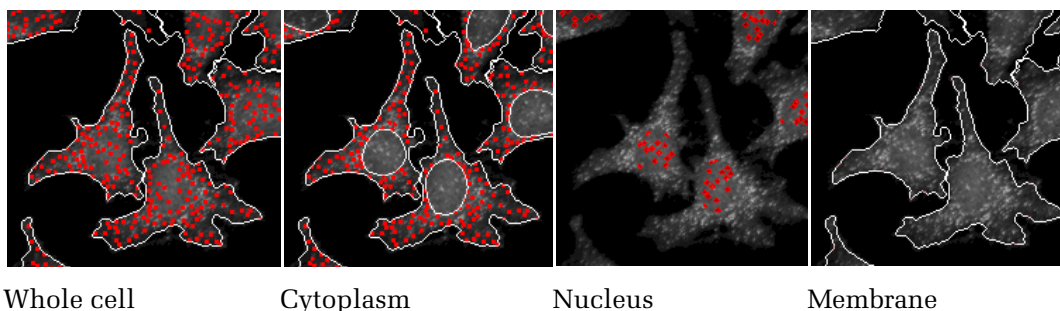
Figure 2–16: The figure shows several search region options that are available in the **Find Spots** building block. There are no spots located on the cell membrane.
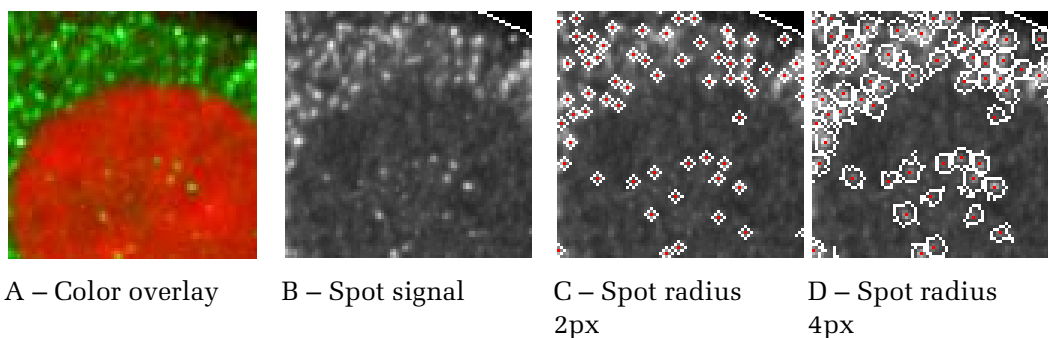
A – Color overlay B – Spot signal C – Spot radius 2px D – Spot radius 4px

Figure 2–17: Examples of adjustments of "Spot Radius". From left to right: A – Color overlay of the reference stain (red, nucleus) and signal stain (green, peroxisomes). B – Gray scale image of spot signal. C – Detected spots with the **Find Spots** building block radius parameter set to 2 pixels (px). D – Detected spots with the radius parameter set to 4px. For the spot size shown here, a radius of 2px is more suitable than 4px. Generally: adjust the spot radius parameter to match the spots of interest.

A – Spot to region intensity = 1 B – Spot to region intensity = 1.5 C – Spot to region intensity = 2 D – Yellow – discarded spots
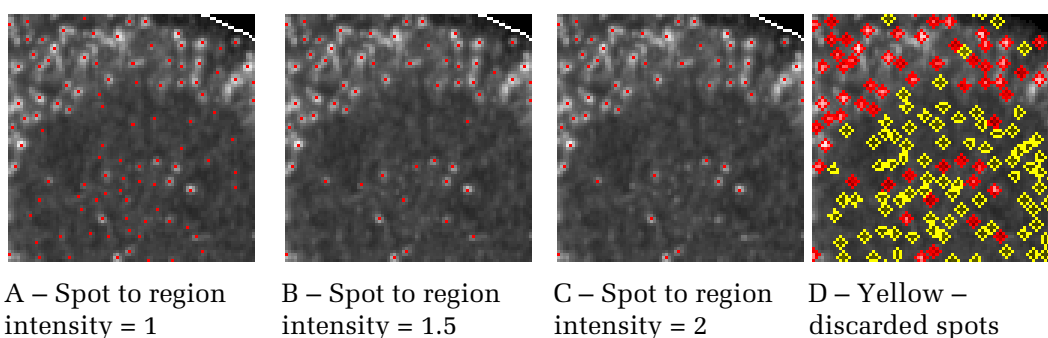
Figure 2–18: Examples of parameter adjustments of "Spot to Region Intensity". Filter criteria are chosen to select spots based on their intensity relative to the mean background of the cell. From left to right: A – Detected spot centers are marked with a red dot on the spot signal image. B – Same illustration as in A, but with a higher spot to region intensity threshold set. Only the brighter spots are selected. C – The threshold is further increased and only the brightest spots are selected. D – Illustration of all the detected spots categorized into "discarded spots" (yellow) and "qualified spots" (red), the latter of which passed the intensity threshold criteria. Illustrations such as these are shown when selecting the Find Spots building block on the Harmony Image Analysis GUI (see *Figure 1–1*, panel 4). Adjust the "Spot to Region Intensity" to the desired sensitivity: low values to incorporate more spots, higher values to select only the brighter spots.

Calculate Readout Values

The following set of readout values, which are applicable to spot signal related assays, are automatically generated by the **Find Spots** building block. These readout values can be selected and modified in the Define Results building block:

➤ The "Mean Number of Spots per Cell" is useful for information and quality control. Although this value may appear to be a good readout for characterizing a biological effect, try using an alternative readout value and compare the robustness and signal to noise ratio (Z' value). The "Mean Number of Spots per Cell" is highly dependent upon the parameter settings (e.g. the selected spot radius).

➤ The "Mean Relative Spot Intensity" is a robust quantifier of biological effects that affect the spot intensity, e.g. to measure the agglomeration of fluorescent labels in endosomal compartments, visible as "pits" or "vesicles".

➤ The "Mean Number of Spots per Area" is a robust quantifier of biological effects that alter the number of spots, e.g. spot generation.

2.8.4   Hints and Tips

➤ To add multiple spot marker channels add more **Find Spots** building blocks.

➤ Try combining with non-spot marker quantification, e.g. the RMS "Quantification of Marker in Nucleus" (section 2.5).

➤ Use a tailored spot detection region: add a **Select Region** or **Select Cell Region** building block to define customized regions, e.g. parts of the cytoplasm. See the RMS "Receptor Internalization" (section 2.11) for a detailed description of a region selection.

➤ The individual spot parameters such as spot size or the contrast for each spot can be seen in the Object List Table of the **Find Spots** building block. This table can be easily transferred (by copy and paste) to other software for further investigation of spot properties (correlation of parameters or histograms).

➤ Texture analysis can be used for quantification of fluorescence intensity concentrated in spots as well, e.g. by using the "SER Spots" property of the **Calculate Texture Properties** building block. See the RMS "Texture Analysis – Mitochondria Classification" (section 2.22). In contrast to using the Find Spots building block, the spots are not identified as individual objects when using this method, i.e. counting spots is not possible.

➤ Spot co-localization with other markers can be analyzed by using the "Spot" region created by the **Find Spots** building block to detect fluorescent intensity in another channel using a **Calculate Intensity Properties** building block.

➤ The spot population can be filtered using the **Select Population** building block, e.g. to only select specific spots like bright ones. See RMS "Lipid Droplets – Analysis of various sized spots" (section 2.24).

2.9 Cytosol to Nucleus Translocation

This RMS is designed to accurately quantify a fluorescence translocation from the cytosol to the nucleus in a variety of different assays. The example below shows its use with the Cellomics® NFκB HitKit™.

2.9.1 Introduction and Assay Principle

The activation of transcription factors such as NFκB or Forkhead (FKHR) is typically monitored with nuclear translocation assays using either fluorescently labeled antibodies or transcription factors with a fluorescent protein tag. Transcription factors function as key regulators in receptor signaling. Inactive factors are typically cytoplasmic but are rapidly imported into the nucleus upon receptor activation.

➤ Our example shows the use of the RMS with the Cellomics® NFκB HitKit™ (# K01-0001-1).

➤ The Cellomics® NFκB HitKit™ (# K01-0001-1) secondary antibody was labeled with Alexa Fluor® 488.
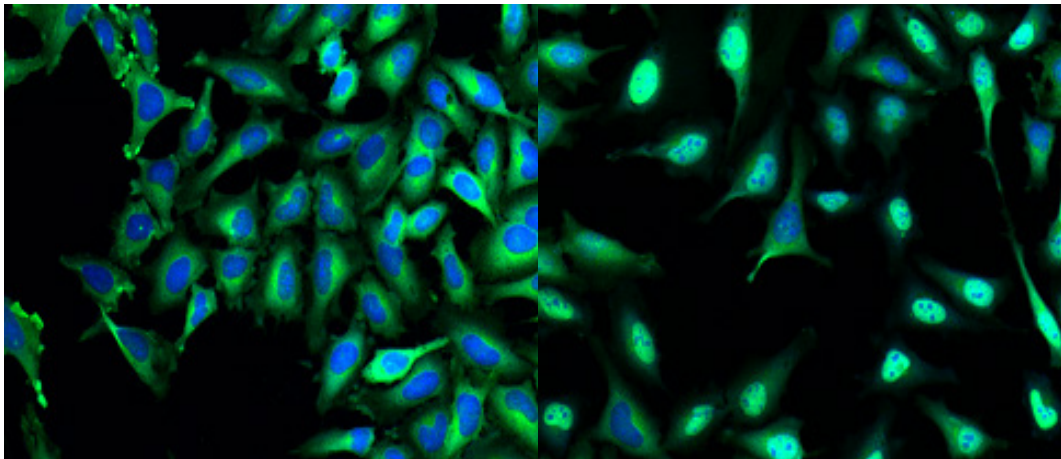
Unstimulated cells. Color overlay of the nuclear stain image (Hoechst 33342, blue channel) and labeled $NF_\kappa B$ antibody (green channel).

Stimulated cells. Labeled $NF_\kappa B$ translocates to the nucleus. Cells respond differently to the stimulation.

Figure 2–19: Examples of an $NF_\kappa B$ translocation assay. Labeled $NF_\kappa B$ moves from the cytoplasm to the nucleus. Details of the example dataset ("Plate P009-T: $NF_\kappa B$") can be found in the Appendix on page 315.

2.9.2 How to use the Cytosol to Nucleus Translocation RMS

➤ Load the analysis sequence "RMS Cytosol to Nucleus Translocation" from the Harmony database.

➤ Select the nuclear dye channel in the Find Nuclei building block.

➤ Select the cytoplasmic (or combined nuclear / cytoplasmic) dye channel in the **Find Cytoplasm** building block.

➤ Select the specific marker channel in the **Calculate Intensity Properties** building block.

➤ Visually check the building block illustrations for representative wells.
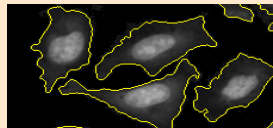
Image Analysis Sequence: Cytosol to Nucleus Translocation
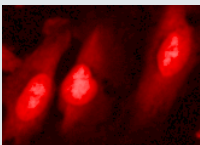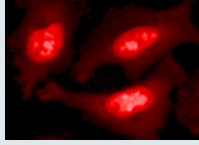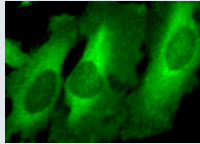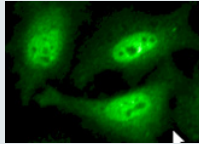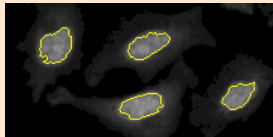
Input Image

Channel 1:
Combined nuclear and cytoplasmic stain image

Channel 2:
Signal image

Untreated control:
Nuclei + cytosol

Labeled NF$_K$B antibody

Treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Nuclear regions overlaid on nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around each of the previously detected nuclei

Detected cytoplasmic outlines

Define Region of Interest

Select Cell Region

Select the region to measure the cytoplasmic fluorescence based on the detected nuclear and cytoplasmic outlines.

Search the region overlaid on the signal stain image (control)

Quantify Properties in Regions

Calculate Intensity Properties

in the nuclear region

Object No	Intensity Nucleus Mean
10	346.552
11	405.603

Calculate Intensity Properties

in the cytoplasmic region

Object No	Intensity Cytoplasm Mean
10	446.409
11	547.204

Object No	Intensity Nucleus Mean
22	850.549
23	720.187

Object No	Intensity Cytoplasm Mean
22	280.082
23	264.559

Calculate Ratios of Properties

Calculate Properties

Determine the fraction of fluorescence that is located in the nucleus

Define Results

Select readout values to report

Nuclei - Number of Objects
446

Nuclei - Fraction of Nuclear Fluorescence - Mean
0.412433

Nuclei - Fraction of Nuclear Fluorescence - StdDev
0.0816361

➢ Marker is mainly located in the cytoplasm

Nuclei - Number of Objects
494

Nuclei - Fraction of Nuclear Fluorescence - Mean
0.643923

Nuclei - Fraction of Nuclear Fluorescence - StdDev
0.103853

➢ Marker is mainly located in the nucleus

Readout Values

|	
Total Number of Cells	For information and quality control
Mean Fraction of Nuclear Fluorescence	Indicates the location of the marker: 0.0 – marker is only located in the cytoplasm; 1.0 – marker is only located in the nucleus
StdDev Fraction of Nuclear Fluorescence	Homogeneity of the marker distribution in the cell population. A low value indicates that all cells show a similar translocation

Image Analysis Guide

## 2.9.3   Image Analysis in Detail

Image Segmentation

➤ The translocation analysis is based on measuring the marker intensity in both of the cellular compartments between which the translocation event occurs (the cytosol and the nucleus).

➤ For details on measuring the nuclear fluorescence, please refer to the RMS "Quantification of Marker in Nucleus" (section 2.5).

➤ For details on measuring the cytosolic fluorescence, please see the RMS "Quantification of Cytoplasmic Marker" (section 2.6).

Define Region of Interest

➤ For quantification of the nuclear fluorescence the default nuclear region that is automatically returned by the **Find Nuclei** building block is used. In most cases, the fluorescence is homogenously distributed in the nuclear area and the outlines of the nuclei can be reliably detected.

➤ For the quantification of the cytoplasmic fluorescence intensity, a new region is explicitly defined by the **Select Cell Region** building block.

➤ In most cases, the region defined in the RMS works well. The adjustment of this region has a big impact on the assay performance. Individually adjusting it for each specific assay is the most efficient way to improve the performance:

- Open the **Select Cell Region** building block to view the illustration of the cell region and to see the parameters for region adjustment.

- Make sure that you cover a region which shows a big difference between the positive control images and the negative control images.

- Please refer to the RMS "Receptor Internalization" (section 2.11) for a detailed description of the region selection options.

- The ultimate criterion for region adjustment is to look at the Z' value. Please see section 3.5.1.
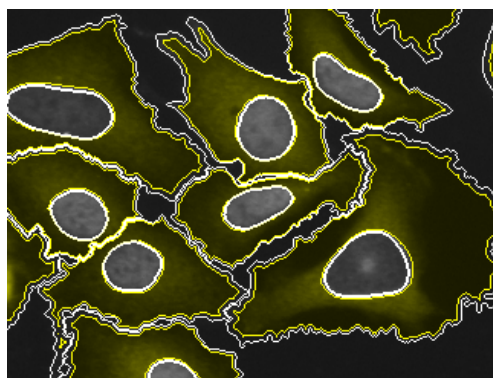
Standard choice: Membrane and nuclear border excluded to avoid noise.
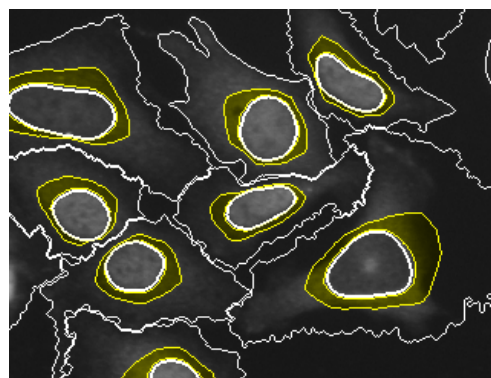
Special adjustment: Only the inner part of the cytoplasm is covered since the intensity fluctuations increase when getting closer to the membrane.

Figure 2–20: Examples of two different regions of interest chosen for the cytoplasmic intensity measurement in the NF$_k$B example dataset (*Figure 2–19*). The standard choice (left panel) gives a Z' value of 0.47, whilst the special adjustment gives a Z' value of 0.61 (right panel).

Quantification of Properties

➤ To quantify the location of fluorescence for each cell, the "Fraction of Nuclear Fluorescence" is defined as follows:

$$Fraction\ of\ Nuclear\ Fluorescence \equiv \frac{Intensity\ in\ Nucleus}{(Intensity\ in\ Nucleus + Intensity\ in\ Cytoplasm)}$$

➤ The value covers a range from 0 indicating that all the fluorescence is located in the cytoplasm (no translocation) to 1.0 indicating that all the fluorescence is located in the nucleus (complete translocation).

➤ The use of a ratio makes the translocation analysis independent from the absolute intensity of the maker, which may vary from cell to cell.

Calculate Readout Values

To create a readout value for each well, the "Fraction of Nuclear Fluorescence" of all the cells in that well is averaged. In a homogeneous cell population this value quantifies the location of the marker and can be used to screen for compounds that induce or inhibit translocation.

In an inhomogeneous cell population the value is also dependent on the fraction of cells which actually respond to the stimulation and show a translocation of the marker. This is indicated by an increased standard deviation of the "Fraction of Nuclear Fluorescence". Export the single cell results to further analyze the individual cell responses (create histograms or investigate the correlations between marker location and other parameters, such as cell size).

2.9.4 Hints and Tips

➤ See the RMS "Cytosol to Membrane Translocation" (section 2.10) and the RMS "Receptor Internalization" (section 2.11) for further information on translocation analysis.

➤ For inhomogeneous populations specific readout values can be generated by adding **Select Population** building blocks and defining more readouts in the **Define Results** building block. Useful readout values include the fraction of respondent cells.

➤ Remove cells at the border that are not completely visible in the image using the **Select Population building block.**

➤ Remove cells based on intensity or geometrical properties, for example cells smaller than a given size, or with a weak staining intensity (e.g. untransfected cells). Use the **Calculate Morphology / Intensity Properties** and **Select Population** building blocks. For an example, please see the RMS "Nuclear Analysis – Nuclear Shrinking" (section 4.4).

➤ It is useful to combine this with the RMSs for quantification of markers in multiple regions and in multiple other channels.
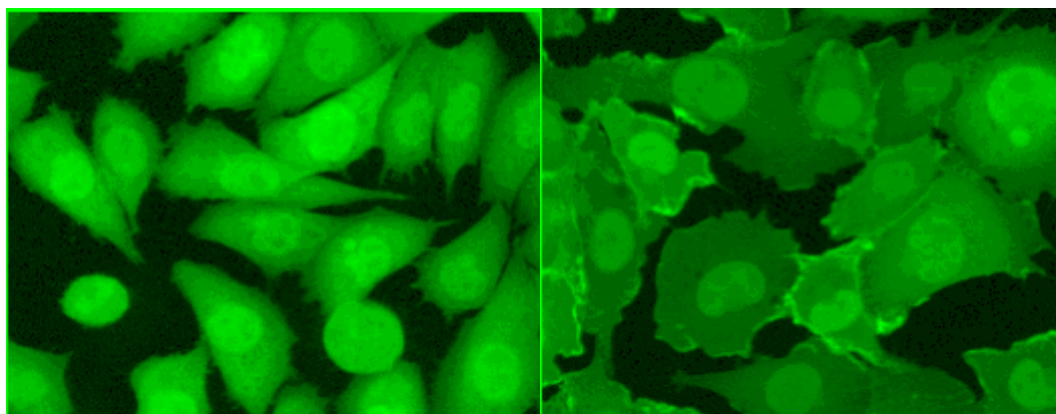
2.10 Cytosol to Membrane Translocation

This RMS allows for the accurate quantification of fluorescent markers located in the cytoplasm and translocated to the plasma membrane.

2.10.1 Introduction and Assay Principle

Plasma membrane receptor activation often results in the recruitment of cytoplasmic signaling molecules to the receptor complex at the plasma membrane. The Akt3/PKB Redistribution® assay (Thermo Scientific) is our example for such an assay type and monitors the translocation of a GFP-human Akt3 fusion protein from the cytoplasm to the plasma membrane. The Akt3/PKB signaling pathway is activated by various growth factors and mediates cell survival signals. Insulin-like growth factor-I (IGF-I) is used as reference agonist, and compounds are assayed for their ability to inhibit the IGF-I-stimulated membrane translocation of Akt3/PKB. In inhibited cells, fluorescence is evenly distributed in the cytoplasmic space. In activated cells, there are regions of high intensity in the plasma membrane area.

The quantification of a marker located in the cell membrane is very similar to the quantification of a marker in the cytoplasm, except that a different region of interest is used.
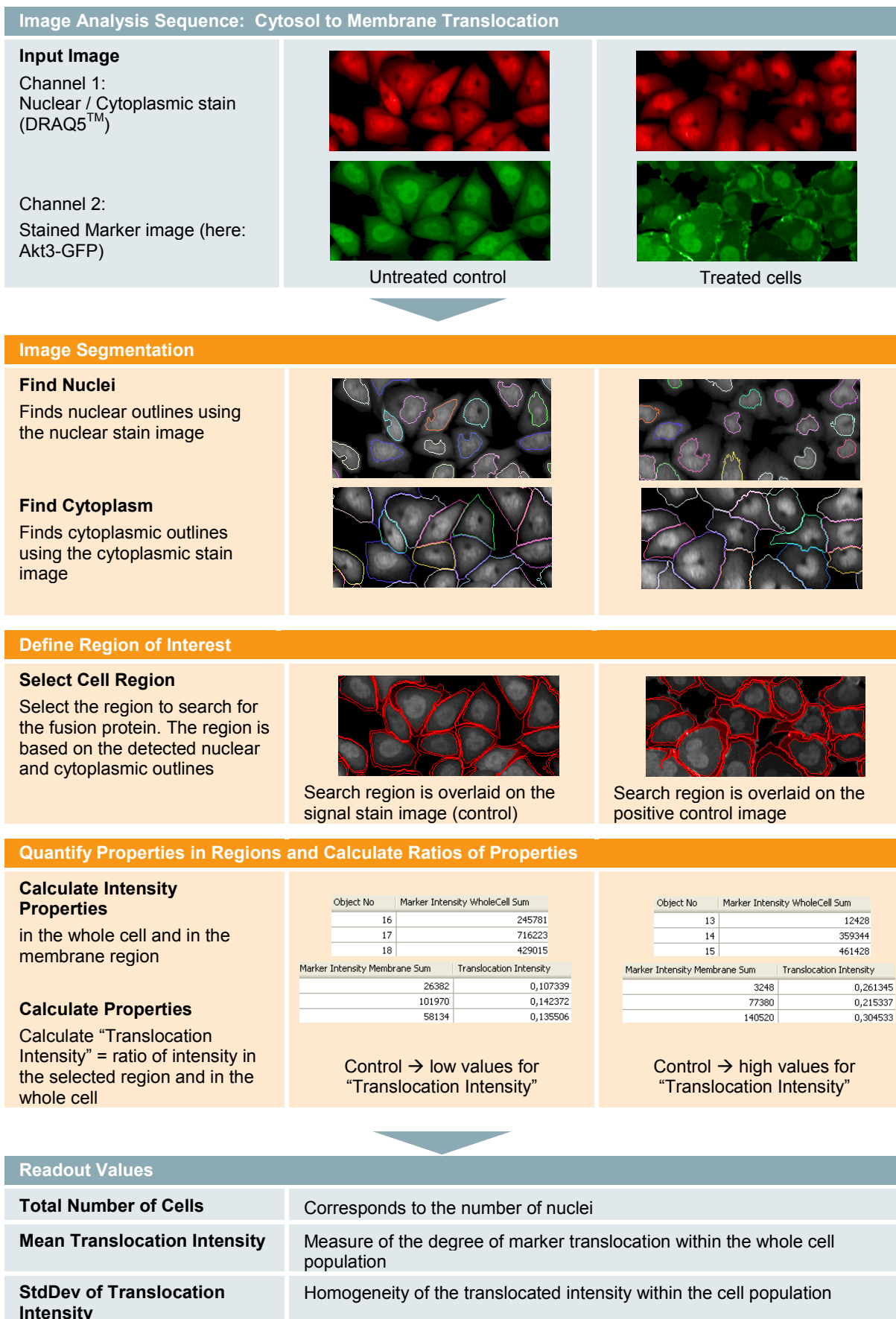

Signal channel – no translocation event.   Signal channel – translocation event.

Figure 2–21: Example images of a cytoplasm to membrane translocation event (Akt3 assay by Fisher BioImage ApS). The left panel shows a typical image of the GFP-human Akt3 fusion protein located in the cytoplasmic space. The right panel image shows a typical image of the fusion protein translocated to the cell membrane.

2.10.2 How to use the Cytosol to Membrane Translocation RMS

➤ Load the analysis sequence "RMS Cytosol to Membrane Translocation" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic (or combined nuclear / cytoplasm) dye channel in the **Find Cytoplasm** building block.

➤ Select the specific marker channel in the **Calculate Intensity Properties** building block.

➤ Visually check the building block illustrations for representative wells.

Image Analysis Sequence: Cytosol to Membrane Translocation

Input Image

Channel 1:
Nuclear / Cytoplasmic stain
(DRAQ5™)

Channel 2:

Stained Marker image (here: Akt3-GFP)

Untreated control

Treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines using the cytoplasmic stain image

Define Region of Interest

Select Cell Region

Select the region to search for the fusion protein. The region is based on the detected nuclear and cytoplasmic outlines

Search region is overlaid on the signal stain image (control)

Search region is overlaid on the positive control image

Quantify Properties in Regions and Calculate Ratios of Properties

Calculate Intensity Properties

in the whole cell and in the membrane region

Calculate Properties

Calculate "Translocation Intensity" = ratio of intensity in the selected region and in the whole cell

Object No	Marker Intensity WholeCell Sum
16	245781
17	716223
18	429015

Marker Intensity Membrane Sum	Translocation Intensity
26382	0,107339
101970	0,142372
58134	0,135506

Control → low values for "Translocation Intensity"

Object No	Marker Intensity WholeCell Sum
13	12428
14	359344
15	461428

Marker Intensity Membrane Sum	Translocation Intensity
3248	0,261345
77380	0,215337
140520	0,304533

Control → high values for "Translocation Intensity"

Readout Values

Total Number of Cells	Corresponds to the number of nuclei
Mean Translocation Intensity	Measure of the degree of marker translocation within the whole cell population
StdDev of Translocation Intensity	Homogeneity of the translocated intensity within the cell population

Image Analysis Guide

2.10.3 Image Analysis in Detail

The analysis sequence is very similar to the RMS "Receptor Internalization" (section 2.11), except that the destination region used to quantify the amount of translocated receptor is the membrane region (instead of a region inside the cytoplasm).

For the determination of the membrane region please refer to the RMS "Quantification of Membrane Marker" (section 2.7).

Image Segmentation

➤ The **Find Nuclei** building block, in combination with the **Find Cytoplasm** building block, is used to determine the outline of the nucleus and the cytoplasm.

Define Region of Interest

➤ To determine the amount of translocated kinase, a region of interest has to be defined to measure the translocated fluorescence intensity.

➤ The **Select Cell Region** building block is used since the kinase is moving from the cytosol to the membrane. The region of interest is defined near the membrane in terms of distance ranges.

➤ The proper selection of a region of interest is crucial for reliable detection of internalization (see ***Figure 2–24***). Make sure to select the region with the:

- Highest intensity difference between the negative and positive controls

- Lowest noise by unwanted fluorescence, e.g. fluorescent artifacts

Quantification of Properties

➤ The labeled kinase sum intensity is measured in both the defined region of interest and in the whole cell region using **Calculate Intensity Properties** building blocks. The whole cell region is available by default; this does not need to be defined by a separate building block.

➤ As a measure of the degree of internalization, the Translocation Intensity is defined and calculated for each individual cell using a **Calculate Properties** building block:

$$Translocation\ Intensity \equiv \frac{Intensity\ in\ Region\ of\ Interest}{Intensity\ in\ Whole\ Cell\ Region}$$

Calculate Readout Values

➤ To measure the degree of translocation for all the cells in a well, the mean translocation intensity value is used. This value increases with the fraction of activated cells.

➤ As a measure of the homogeneity of translocation in the cell population, the standard deviation of the "translocation intensity" is reported. A high standard deviation means a high diversity within the population: some cells are activated and others cells are not, e.g. because individual cells respond differently to an agonistic compound.

2.10.4 Hints and Tips

➤ Quantification of membrane intensity is inherently difficult since the membrane location is difficult to determine. Accurate cytoplasmic staining and detection are of key importance for this type of assay.

➤ Weighted scoring of the fluorescence location as suggested in the hints and tips section of the RMS "Quantification of Membrane Marker" (section 2.7) is useful for improving the performance of membrane translocation assays.

2.11 Receptor Internalization

This RMS quantifies the translocation of a membrane bound fluorescent receptor into cellular compartments.

2.11.1 Introduction and Assay Principle

Receptor internalization assays are mainly used for investigating GPCR activation. Receptors which are internalized upon ligand stimulation mostly end up in a cluster of endosomes in the proximity of the nucleus, which can be identified as a single endosomal compartment per cell in our RMS. This is a very robust and easy way of quantifying receptor activation and is an alternative to the "spot analysis" approach described previously. You can use receptors carrying an autofluorescent protein tag, fluorescently labeled ligands, or pH sensitive dyes which show brighter fluorescence in the acidic environment of the endosomes.

➤ Our RMS quantifies the translocation of a fluorescent marker from the border of the cell (cell membrane) to regions closer to the nucleus.

➤ The application example shown below describes the monitoring of fluorescently labeled Endothelin-A receptor (ET$_A$R). Prior to activation the receptor is membrane bound. By activation with Endothelin-1, the receptor is internalized into compartments near the nucleus (see *Figure 2–22*).
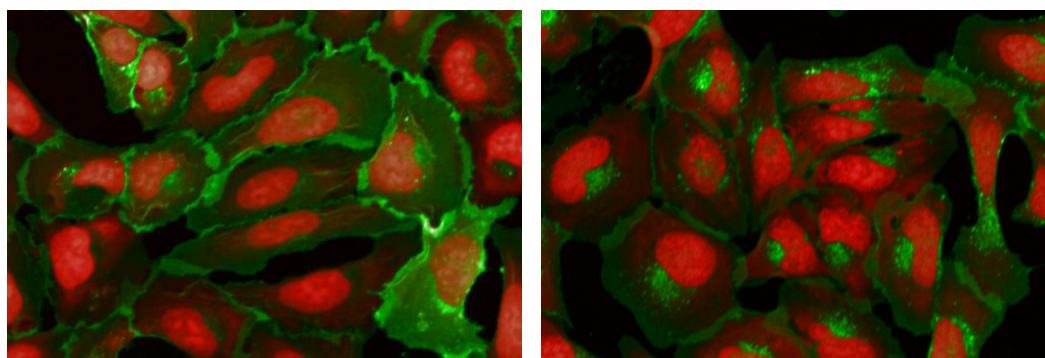
Figure 2–22: Color overlay of a nuclear stain image (red) and a labeled ET$_A$ R image (green). Left panel: Example image in which the receptor is located at the cell membrane. Right panel: Example image showing stimulated cells, in which the receptor has been internalized. Details of the example dataset ("Plate P010-R: ET$_A$R") can be found in the Appendix on page 316.

2.11.2 How to use the Receptor Internalization RMS

➤ Load the analysis sequence "RMS Receptor Internalization" from the Harmony database.

➤ Select the nuclear stain channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic or combined nuclear / cytoplasmic dye channel in the **Find Cytoplasm** building block.

➤ Select the signal stain channel (labeled receptor) in the two **Calculate Intensity Properties** building blocks.

➤ Adjust the signal detection region in the **Select Cell Region** building block to cover the region with the highest signal in the positive control images (the region where the receptor is located after internalization).
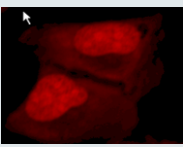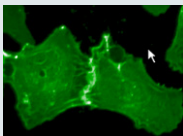
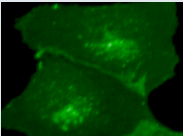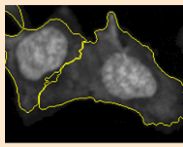## Image Analysis Sequence: Receptor Internalization
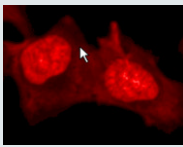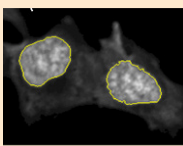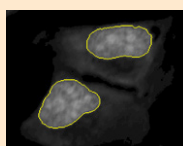
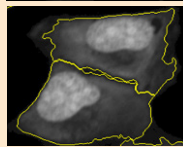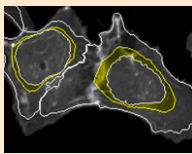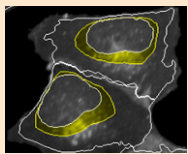**Input Image**

Channel 1:
Combined nuclear and cytoplasmic stain image

Channel 2:
Signal image

Untreated control:
Nuclei and cytosol

Labeled ET$_A$R Receptor

Treated cells

Internalized ET$_A$R Receptor

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around each of the previously detected nuclei

Nuclear region is overlaid on nuclear stain image (untreated cells)

Detected cytoplasmic outlines

Results of detection on treated cells
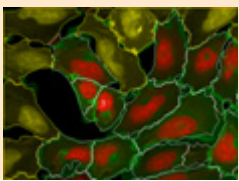
Define Region of Interest

Select Cell Region

Select the region to search for the internalized receptor. The region is based on the detected nuclear and cytoplasmic outlines.

Search region is overlaid on the signal stain image (control)

Search region is overlaid on the positive control image
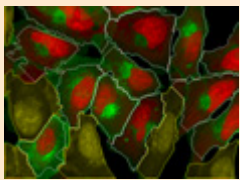
Quantify Properties in Regions and Calculate Ratios of Properties

Calculate Intensity Properties

Sum signal intensity in the selected region and in the whole cell

Calculate Properties

Calculate "Internalized Intensity" = ratio of intensity in the selected region and in the whole cell

Select Population

Only keep the cells that are completely visible in the image

Object No	Intensity Cell Sum	Intensity ROI Sum
14	3995635	890291
15	3948934	793078

Object No	Internalized Intensity
14	0.222816
15	0.200833

Control → low values of "Internalized Intensity"

Untreated cells

Object No	Intensity Cell Sum	Intensity ROI Sum
5	5407775	1467707
6	5210943	1422400

Object No	Internalized Intensity
5	0.271407
6	0.272964

Activated cells → high values of "Internalized Intensity"

Treated cells with internalized ET$_A$ Receptor

Readout Values

Total Number of Cells	For information and quality control
Mean Internalized Intensity	Measure of the mean degree of receptor internalization in the cell population
StdDev of Internalized Intensity	Homogeneity of receptor internalization within the cell population

2.11.3   Image Analysis in Detail

Image Segmentation

➤ The **Find Nuclei** building block, in combination with the **Find Cytoplasm** building block, is used to determine the borders of the nucleus and the cytoplasm. In this application example a single image channel containing a combined nuclear (high intensity) and cytoplasmic (low intensity) stain is used. See Application Guide, section 1.4.5 "Water immersion vs. air objectives" for details on the staining options.

Define Region of Interest

➤ To determine the amount of internalized receptors, a region of interest has to be defined to measure the internalized fluorescence intensity.

➤ Since the receptor is moving from a position at the cell membrane to cellular compartments located closer to the nucleus, the region of interest is defined here in terms of the distance range between the membrane and nucleus (*Figure 2–23*). The **Select Cell Region** building block is used for this.

➤ The proper selection of a region of interest is crucial for reliable detection of internalization (*Figure 2–24*). Make sure to select the region with the:

• Highest intensity difference between negative and positive controls.

• Lowest noise by unwanted fluorescence, e.g. fluorescent artifacts.

➤ Please note that the relative definition of a ring region provides more precise quantification of fluorescence than using a ring around the nucleus when the nucleus of fixed width is not located in the middle of the cell (see *Figure 2–23*, panel C).
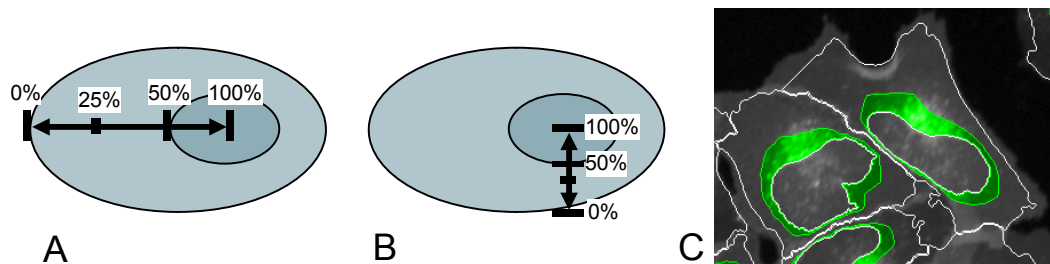

Figure 2–23: Relative definition of the position of fluorescence inside a cell. A – Schematic diagram of a cell. The cytosol is colored light blue; the nucleus is colored dark blue. 0 % corresponds to the membrane, 50 % corresponds to the border of the nucleus and 100 % corresponds to the center of the nucleus. B – Relative measure at a different position in the cell. C – A ring region (green) ranging from 30 % to 50 % is marked in an example image. The outlines of the detected nuclei and cytoplasms are shown in white. The background image shows the signal channel (labeled $ET_A R$) intensity.

Quantification of Properties

➤ The labeled receptor intensity is measured in the defined region of interest and in the whole cell region using **Calculate Intensity Properties** building blocks. The whole cell region is available by default and does not need to be defined by a separate building block.

➤ As a measure of the degree of internalization, the internalized intensity is defined and calculated for each individual cell with the **Calculate Properties** building block:

$$Internalized\ Intensity \equiv \frac{Intensity\ in\ Region\ of\ Interest}{Intensity\ in\ Whole\ Cell\ Region}$$

➤ The normalization of the intensity compensates for fluctuations in the overall staining intensity of the cells. Only the location of the fluorescence contributes to the internalized intensity whilst the absolute intensity does not affect the value. Brightly as well as more weakly stained cells are quantified correctly.
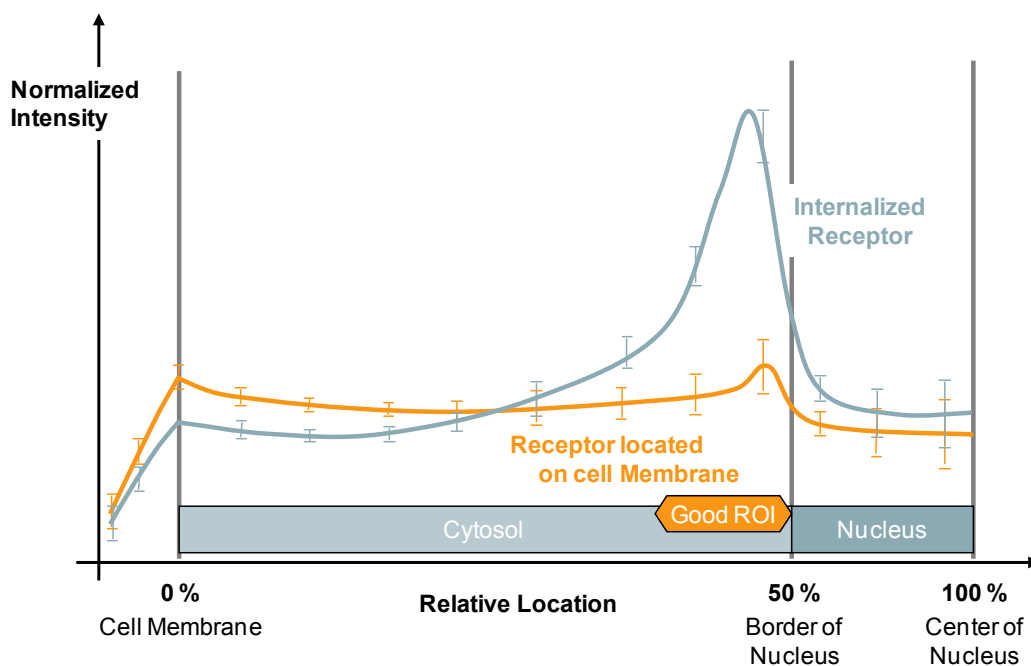

Figure 2–24: Distribution of the labeled receptor intensity at different distances from the nucleus. Orange: Untreated cells. Blue: Activated cells with internalized receptor leading to an increased intensity near the nuclear border. The distance range marked orange is well suited for quantifying internalization since the difference is high and noise is low within this range.

Calculate Readout Values

➤ To measure the mean degree of internalization for all cells in a well, the mean internalized intensity value is used. The value increases:

- With the fraction of cells internalizing the receptor.

- With the degree of internalization (in case of incomplete internalization).

➤ As a measure of the homogeneity of receptor internalization in the cell population the standard deviation of the "internalized intensity" is reported. A high standard deviation means a high diversity within the population: some cells have internalized the receptor and others have not, e.g. because individual cells respond differently to an agonistic compound.

➤ Finally, by applying the **Select Population** building block, cells that are not completely visible in the image are removed. These cells would introduce artifacts due to their reduced area, resulting in a lower sum intensity of the cell.

2.11.4   Hints and Tips

➤ Detection of nuclei and cytoplasm is crucial for the correct determination of the region of interest since it is defined relative to the nuclei and cytoplasm. Make sure that the segmentation gives good results for all wells and re-adjust the parameters if needed. See the "Harmony Help Function" for detailed hints on adjusting the parameters for nuclei and cytoplasm detection.

➤ Depending on your application, alternative regions of interest may be useful to optimally capture the fluorescence signal of the internalized receptor. Please refer to section 1.3.2 (page 29) and the "Harmony Help Function" for a description of the available regions in the **Select Cell Region** and **Select Region** building blocks. Multiple regions may be defined by adding several **Select Region** building blocks.

➤ Depending on your application, other measures may be used to quantify the degree of receptor internalization. Measures to be considered include: the intensity in the region with the greatest change divided by the whole cell intensity; differences in intensity between a region with an increase and a region with a decrease in intensity, divided by the whole cell intensity. However, there are rare cases when dividing by the whole cell intensity does not improve the signal to noise ratio.

➤ For additional guidance on how to find out which measure and / or region is best suited to your application, refer to section 3.5.

➤ Cells that are not completely visible in the image may lead to analysis artifacts for some applications. A **Select Population** building block can be added to the analysis sequence to filter out cells which touch the image borders. Please refer to the pre-configured analysis sequence "RMS Receptor Internalization – Remove Border Cells" in the Harmony database.

2.12 Nuclear Classification – DNA content

This RMS allows you to classify nuclei according to different nuclear fluorescence parameters, such as intensity and area, as well as geometrical parameters. An example is given for the quantification of DNA content.

2.12.1 Introduction and Assay Principle

The DNA content in a cell varies during the cell cycle. The DNA is doubled during S-phase and is condensed and divided during mitosis.

By monitoring the amount of DNA and the morphology of the nucleus, the phases of the cell cycle can be distinguished (see *Figure 2–26*).

The amount of DNA can be determined by quantitative staining of the DNA and measurement of the total amount of dye (summed intensity) in the nuclear area. By using a DNA content threshold, the fraction of cells with "high" or "low" DNA content can be determined and used, for example, for screening for compounds that arrest the cell cycle at certain points.

A more detailed classification can also be achieved by taking the nuclear shape into consideration, e.g. determining the fraction of "small and bright" nuclei with "high" DNA content (corresponding to early M-phase cells). See the blue lines in *Figure 2–26*.
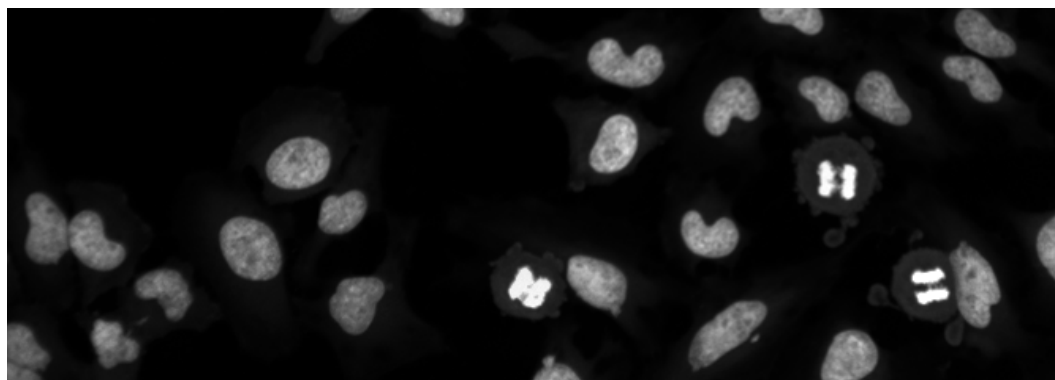

Figure 2–25: The image shows nuclear staining of a mixed cell population with different cell cycle phases. Three M-phase cells are visible (condensed DNA). Cells may contain different amounts of DNA which is not directly visible in the image, but which can be quantified by image analysis. Details of the example dataset ("Plate P004-CC: EdU-pHH3") can be found in the Appendix on page 314.

2.12.2 How to use the Nuclear Classification – DNA content RMS

➤ Load the analysis sequence "RMS Nuclear Classification" from the Harmony database.

➤ Select the nuclear stain channel in the **Find Nuclei** building block and check the nuclei detection results. Adjust the parameters if necessary.

➤ Select the nuclear stain channel in the **Calculate Intensity Properties** building block.

➤ To determine a suitable DNA content threshold for 2N / 4N discrimination, analyze a representative well and create a scatter plot and / or histogram by copy and pasting the single cell result table into Excel®.

➤ Set the DNA content threshold in the **Select Population** (2) building block.

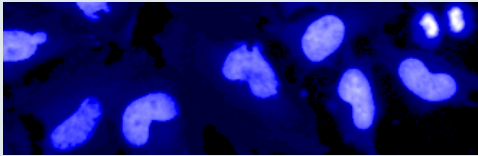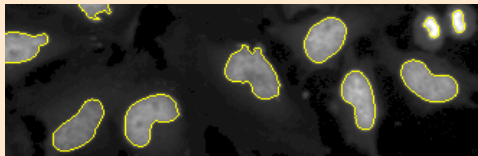## Image Analysis Sequence: Nuclear Classification – DNA Content

Input Image
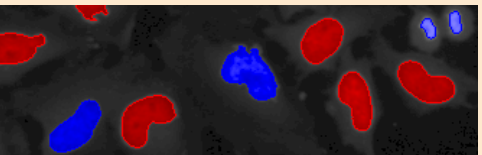
Channel 1:
Nuclear stain

Nuclear stain image

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Select Population

Remove nuclei which are not completely visible in the image

Nuclear detection results (yellow border) on the nuclear stain image

Quantify Properties in Regions

Calculate Intensity Properties

Mean intensity and Sum intensity of DNA stain in the nuclear area

Calculate Morphology Properties

Nuclear Area

Regions for intensity measurements (red) overlaid on the nuclear stain image

Table of DNA stain intensity and nuclear area "per object (cell)", ready to copy and paste into Excel®

Object No	DNA Marker Intensity Mean	DNA Marker Intensity Sum	Nucleus Area [µm²]
40	715,996	499765	291,196
41	1025,96	354983	144,347
42	740,33	490839	276,595
43	772,481	539964	291,614
44	617,234	517242	349,603
45	487,706	175574	150,187
46	384,966	318752	345,431

Identify Subpopulations

Select Population

Identify cells with high DNA content (higher than a specified threshold)

Selected cells with marker DNA content > 2.1 x 10^6 units (red) and discarded cells below this threshold (blue)

Calculate Readout Values

Define Results

Select and calculate values to report back as the results per well

Full table of results per well

Nuclei - Number of Objects	
843	

High DNA content - Number of Objects	Fraction of POS cells [%]
56	6,64294

Nuclei - DNA Marker Intensity Sum - Mean	Nuclei - Nucleus Area [µm²] - Mean
530242	266,5

Readout Values

Number of Cells	Corresponds to the number of nuclei
Fraction of Cells with High DNA content	Corresponds to the fraction of G2- / S-phase and early M-phase cells
Mean Nuclear Area	Average size of the nuclei
Mean Sum Intensity	Corresponds to mean DNA content

Other optional readout values of interest: Mean and standard deviation values of sum intensity, mean intensity, roundness, width, length and width to length ratio.

2.12.3   Image Analysis in Detail

Image Segmentation

➤ The first step is the detection of nuclei using the **Find Nuclei** building block. The building block also returns the nuclear region overlay that indicates the position of each detected nucleus to be used by the following building blocks.

Quantification of Properties

➤ The intensity and morphological properties of each nucleus are determined next using **Calculate Intensity Properties** and **Calculate Morphology Properties** building blocks. For a detailed description of the available properties, see the RMS "Nuclear Analysis – Nuclear Shrinking" (section 4.4).

➤ In this example application, the "sum intensity" and "mean intensity" of the nuclear dye are used for classification of the cell cycle state of each cell. An example of these intensity values can be found in *Figure 2–7*.

➤ The sum intensity directly quantifies the DNA content (Preconditions: A – Quantitative DNA staining, i.e. the staining intensity is proportional to the DNA content. B – Clipping of the signal intensity during image acquisition is avoided. See page 14 for hints on clipping). The mean intensity (intensity per area) of the nucleus is fairly constant, even when the DNA content doubles during S-phase, since the nuclear size also increases in proportion (see *Figure 2–26* for a schematic overview of this).
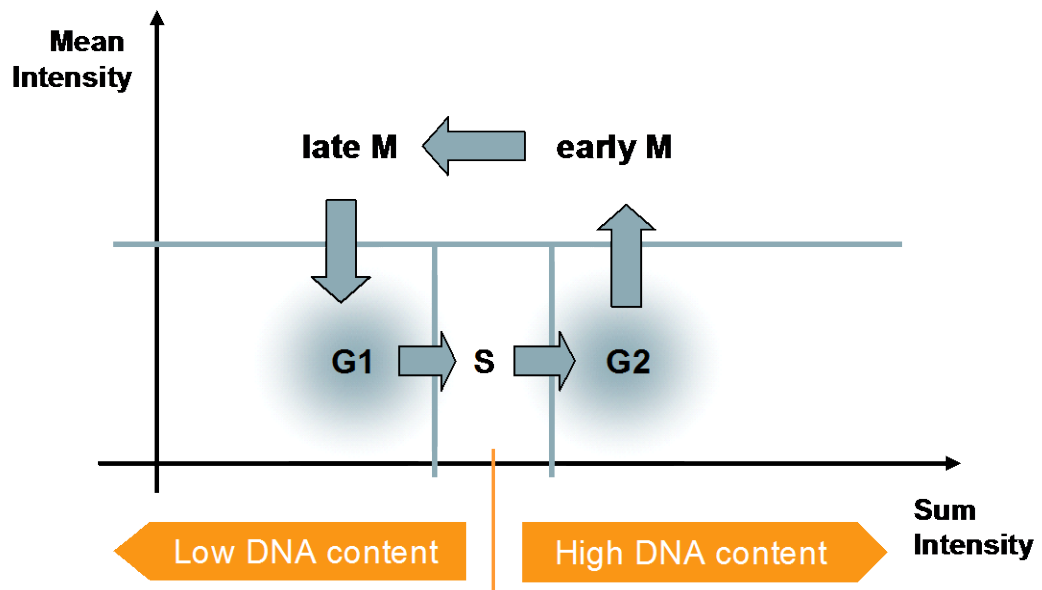
Figure 2–26: Schematic overview of the nuclear DNA content and intensity during different cell cycle phases. The classification of DNA content (by a threshold) into "Low DNA content" and "High DNA content" allows investigation of the effects of a compound on the cell cycle, e.g. an accumulation of cells in one phase could be due to an arrest of the cell cycle. The orange bar marks the threshold for low / high DNA content and the blue bars mark the thresholds for cell cycle stage classification.

Determination of the Subpopulation of Cells with high DNA content

➤ For threshold adjustment (orange bar in *Figure 2–26* and *Figure 2–27*), **scatter plots** or **histograms** of single cell properties such as "sum intensity", "mean intensity" or "area" are most helpful. To create these, the single cell results can be directly transferred from the Object List Table of the Harmony Image Analysis GUI (*Figure 1–1*, panel 5) to your data analysis program of choice by copying and pasting to the clipboard (Control-C and Control-V keyboard shortcuts respectively). *Figure 2–27* and *Figure 2–28* show typical examples created using Excel®.

➤ A suitable threshold value of "2,100,000" intensity units is indicated in orange in the scatter plot (*Figure 2–27)* and histogram (*Figure 2–28*). This value is used in a **Select Population** building block to determine the population of "High DNA content" cells.

Calculation of Readout Values

➤ A suitable readout value for monitoring the effect of a compound on the cell cycle is the "Fraction of High DNA content cells", which corresponds to the fraction of cells containing a duplicated chromosome set (late S- / G2- / early M-phase cells). This is calculated using the **Define Results** building block.
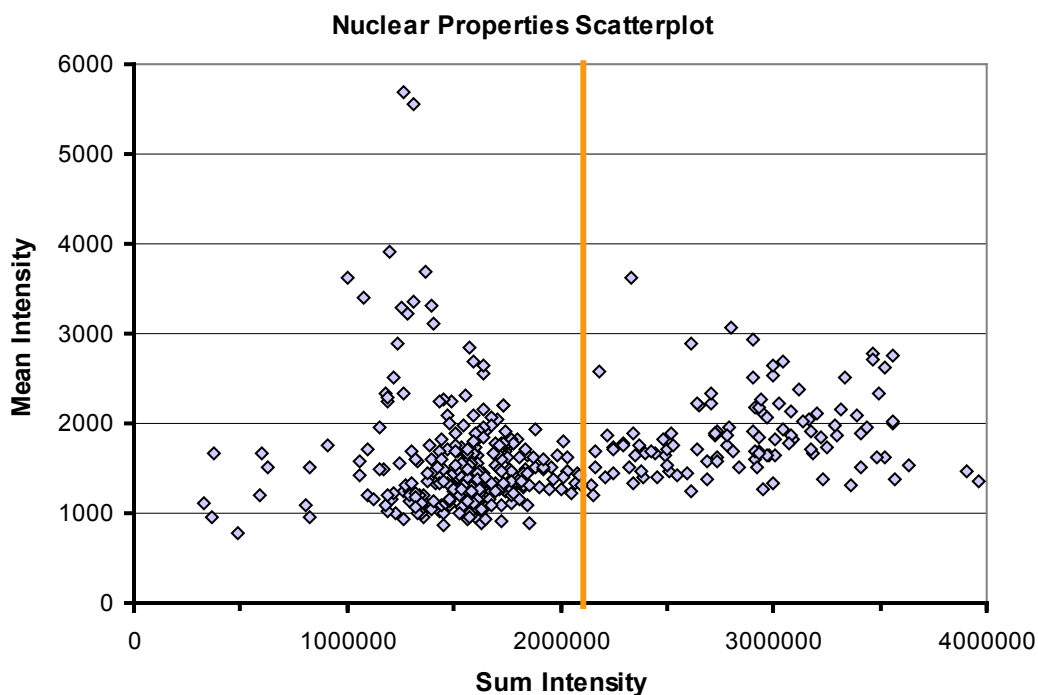

Figure 2–27: Scatter plot showing the nuclear property "Mean Intensity" plotted against the property "Sum Intensity" for one well. The single cell results table was transferred to Excel® (by copy and paste). The scatter plot was then generated using the standard Excel® Chart Wizard. The orange line indicates a Sum Intensity value of 2,100,000 units.
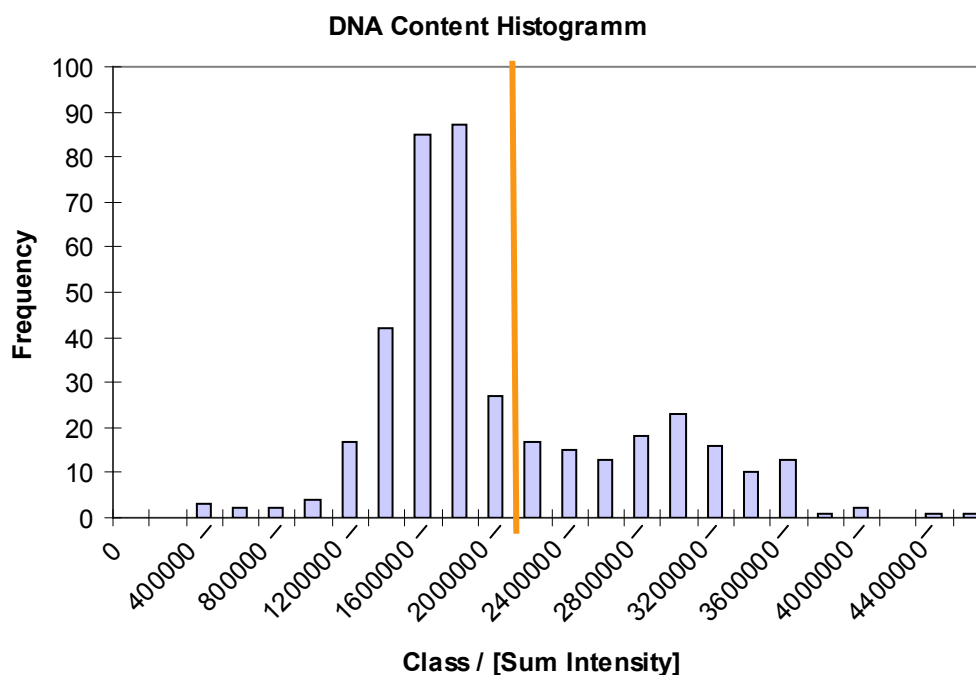
DNA Content Histogramm

Figure 2–28: Histogram showing DNA content. Single cell results were transferred to Excel® (by copy and paste) and the Excel® Histogram Wizard was used to generate the histogram. The intensity classes were set to range from 0 – 4,400,000 with 200,000 units spacing. The orange line indicates a Sum Intensity value of 2,100,000 units.

2.12.4 Hints and Tips

➤ Removal of nuclei which are not completely visible in the image improves the results. Refer to the RMS "Nuclear Analysis – Nuclear Shrinking" (section 4.4) for an example.

➤ In addition to the nuclear intensity and morphology, cell cycle phase-specific markers can also be used for cell cycle classification. See the RMS "Cell Cycle Classification" (section 2.14) for further information.

➤ More **Select Population** building blocks can be added to create further categories for the nuclei, e.g. the fraction of G1-, G2-, S- or early M-phase cells. The blue bars in *Figure 2–26* mark the thresholds for some of these potential categories. Multiple conditions can be combined in a **Select Population** building block to easily create, for example, the category of "small bright nuclei with high DNA content".

➤ For screening applications the classification must be robust, in particular, the threshold settings for the categories must fit for a whole batch of plates. Use several representative wells to determine / verify the threshold.

➤ Toxic effects, apoptosis, and cell cycle effects will all occur when compounds are applied to cells, creating ambiguities. For example, an increased number of small nuclei may be due to an increase in the number of M-phase cells, or because of nuclear shrinking due to apoptosis.

➤ To remove ambiguities, apoptotic cells can be discriminated from M-phase cells by using a dead cell stain such as Propidium iodide in a separate channel.
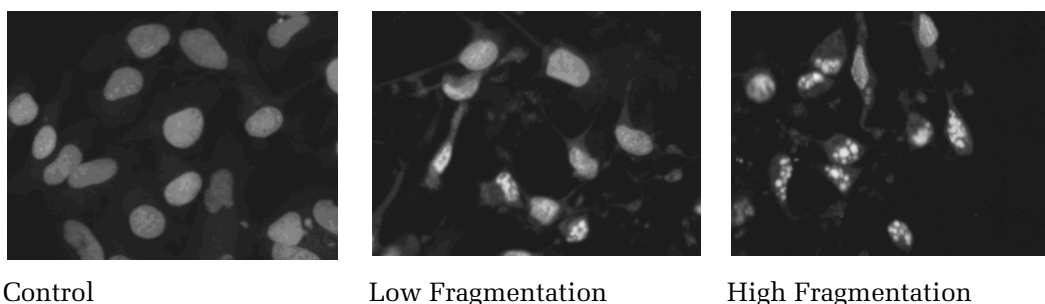
2.13   Nuclear Fragmentation

This RMS allows you to detect the degree of nuclear fragmentation in a cell sample. In addition, nuclear size, number of cells and nuclear intensity are also measured.

2.13.1   Introduction and Assay Principle

Changes in nuclear morphology are an important indicator of cell health. For example, during apoptosis the nucleus breaks apart into smaller fragments which can be analyzed using this RMS.

- ➤ This RMS calculates an index number which characterizes the global degree of nuclear fragmentation in all cells in a well.

- ➤ The mean intensity fluctuations of a nuclear stain in the nuclear region are used for measuring fragmentation.

- ➤ No arbitrary threshold settings are needed since a global "fragmentation index" is calculated.

- ➤ Only a single measurement channel (nuclear stain) is needed.

- ➤ Our example dataset shows DRAQ5™ stained nuclei of HeLa cells which were exposed to increasing concentrations of staurosporine to induce apoptosis (see *Figure 2–29*).

Control   Low Fragmentation   High Fragmentation

Figure 2–29: Example images showing nuclear fragmentation in response to treatment with staurosporine. The image on the left shows intact nuclei of untreated cells. The images in the middle and on the right show fragmentation of nuclei in staurosporine treated cells. The degree of nuclear fragmentation is greater in the image on the right as the concentration of staurosporine is higher. The key features of the example dataset ("Plate P005-A: Caspase3 Apoptosis") can be found in the Appendix on page 315.

2.13.2   How to use the Nuclear Fragmentation RMS

- ➤ Load the analysis sequence "RMS Nuclear Fragmentation" from the Harmony database.

- ➤ Select the nuclear dye channel in the **Find Nuclei** and **Calculate Intensity Properties** building blocks.

- ➤ Make sure to select a method and an appropriate parameter setting in the **Find Nuclei** building block that detects fragmented nuclei as one object.

Image Analysis Sequence: Nuclear Fragmentation

| **Input Image** Channel 1: Nuclear stained image | Untreated cells | | Treated cells with fragmented nuclei | |

Image Segmentation

| **Find Nuclei** Finds all intact nuclei and clusters of fragmented nuclei using the nuclear stain image | Results of detection of intact nuclei | | Results of detection of fragmented nuclei | |

Define Regions of Interest

| **Select Region** Nuclear regions with the outer 2 border pixels excluded | | | | |

Quantify Properties in Regions

| **Calculate Intensity Properties** Use Coefficient of Variance (CV) of the nuclear dye intensity in the region of interest → "Fragmentation Index" (FI) | | | | |

Calculate Readout Values

| **Define Results** Average FI values of all cells | Fragmentation index: **0.14** | | Fragmentation index: **0.21** | |

Readout Values

Number of Nuclei	Number of intact and fragmented cells
Mean Nuclear Area	Measure of nuclear swelling or nuclear shrinking
Mean Integrated Intensity	Measure of DNA content
Fragmentation Index	Measure of the degree of fragmentation of the nuclei. A higher index means more nuclei are fragmented

2.13.3   Image Analysis in Detail

Image Segmentation

The Find Nuclei building block is used to detect nuclei and agglomerates of fragmented nuclei. The detection parameters have to be adjusted for every application / cell type / staining condition in order to identify fragmented nuclei as one object (upper part of *Figure 2–30*). If these parameters are not adjusted, fragments will be considered as individual nuclei, compromising the cell count and fragmentation index.

Capturing portions of the background as part of a nucleus should also be avoided (lower part of *Figure 2–30*). This would lead to an artificially higher fragmentation index since strong intensity differences occur within these areas (background vs. DNA).

The software provides "best guess" default parameters. These can be adjusted automatically or manually through trial and error by adjusting primarily the "Method" and "Individual Threshold" parameters.
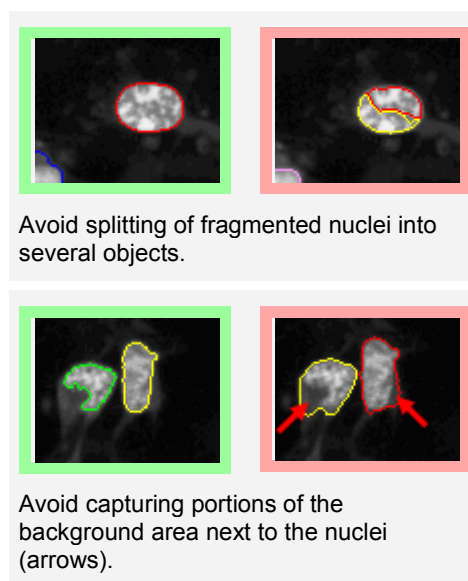
Avoid splitting of fragmented nuclei into several objects.

Avoid capturing portions of the background area next to the nuclei (arrows).

Figure 2–30: Proper adjustment of **Find Nuclei** Parameters.

Definition of Regions of Interest

A Nuclear region, reduced by a two image pixel wide zone to exclude the borders of the nuclei, is recommended here as a region of interest for measuring the degree of fragmentation. In this region the difference between healthy and fragmented nuclei is clearly visible with low ambiguity. Also, small portions of background captured next to the nucleus are excluded by the area of interest.

Quantifying the Degree of Fragmentation

The coefficient of variance (CV) of the nuclear intensity is used as a measure of the degree of nuclear fragmentation. Healthy nuclei have a homogeneous intensity, resulting in a low CV value, whereas fragmented nuclei have an inhomogeneously distributed intensity resulting in a higher CV value. This value has been named the "fragmentation index" here. Please note that the CV gives a more robust value than the standard deviation since the absolute intensities do not influence the result (CV = standard deviation / mean value).

Calculation of Readout Values

In order to calculate a readout value for the whole cell population, the individual fragmentation index values are averaged. The average value increases with the fraction of fragmented cells and the degree of the fragmentation of individual cells. An example result is shown in *Figure 2–31*.
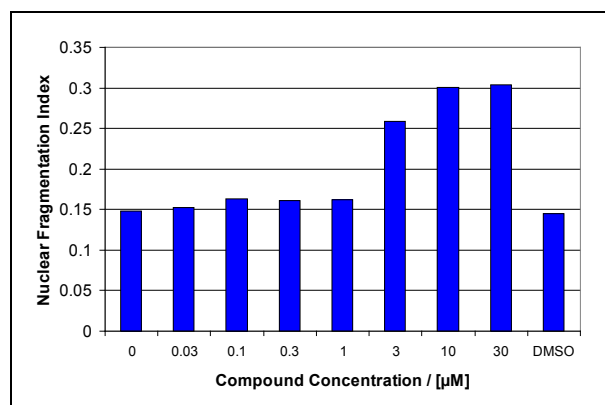

Figure 2–31: Nuclear fragmentation index for an example dataset. The fragmentation index increases as the concentration of the apoptotic inducer increases.

2.13.4 Hints and Tips

➤ The "Number of Nuclei", "Mean Nuclear Area" and "Mean Nuclear Intensity" are also significant readout values for the quantification of apoptosis.

➤ The analysis can be easily added to any other analysis sequence to monitor nuclear fragmentation. No extra marker channel is needed since the analysis is based on the nuclear dye channel which is already available in most cases.

➤ Multiplexing with additional apoptotic markers is useful for apoptosis assays (see Application Guide, section 3.1 "Ready-Made Application: Apoptosis-1").

➤ Alternative analysis: Determine the subpopulation of cells with fragmented nuclei. For example, set an adjustable threshold criterion for the minimum nuclear fragmentation value. The readout value will show the fraction of cells with fragmented nuclei. The drawback is that an additional threshold value has to be adjusted, and therefore the robustness is compromised by the dependency on threshold settings.

2.14   Cell Cycle Classification

This RMS enables marker and nuclear morphology based classification of a cell population according to their different cell cycle phases.

2.14.1   Introduction and Assay Principle

Cell cycle phases can be distiguished based on the expression and localization of specific proteins called "cyclins", the incorporation of labelled nucleotides during DNA replication, and by monitoring changes in the overall DNA content using a quantitative DNA stain. The characteristic staining intensity and morphological changes of the nucleus can also give an indication of the status of the cell. In a typical screening application, compounds are tested for their effect on the cell cycle by monitoring changes in the distribution of cells in different phases (G1 / G2 / S / early M / late M).

➤ For changes in DNA content and morphological changes of the nuclei only, please refer to the RMS "Nuclear Classification – DNA content" (section 2.12).

➤ For the analysis of cell cycle phase-specific marker dyes, see also the RMS "Mitotic Index" (section 2.17).

➤ The "Cell Cycle Classification" RMS combines the information from two marker dyes and also uses the DNA staining intensity and morphology to classify the cells.
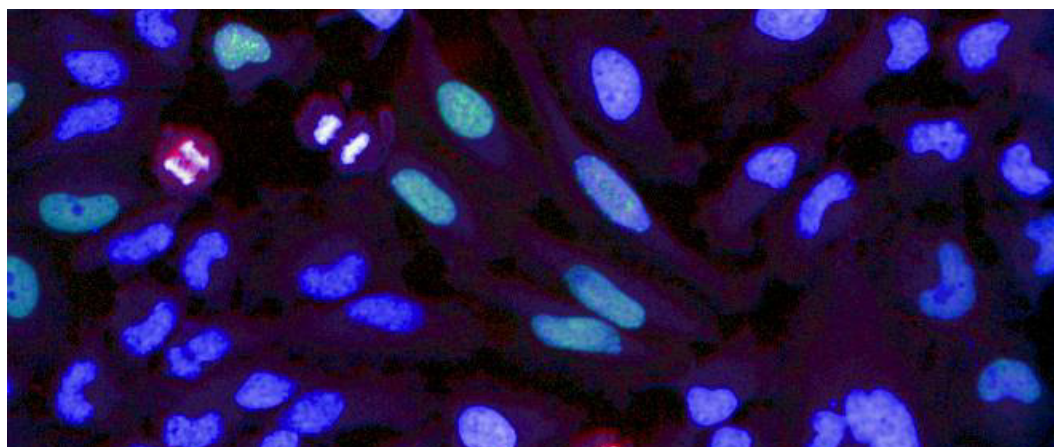

Figure 2–32: Example of marker based cell cycle classification. The cell cycle phase-specific EdU marker is used to detect S-phase cells, and the pHH3 marker is used to detect cells in early M-phase. A color overlay of the nuclear dye (blue), pHH3 marker (red) and EdU marker (green) is shown. Cells in different stages of the cell cycle can be identified according to their DNA staining intensity, shape and the cell cycle phase-specific marker dyes. Details of the example dataset ("Plate P004-CC: EdU-pHH3") can be found in the Appendix on page 314.

2.14.2   How to use the Cell Cycle Classification RMS

➤ Load the analysis sequence "RMS Cell Cycle Classification" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the marker dye channels in the three **Calculate Intensity Properties** building blocks.

➤ Set the thresholds for "pHH3-positive" and "EdU-positive" cell classification in the **Select Population** building blocks using the single cell result tables.

➤ Set the threshold for "High DNA content" classification in the **Select Population** building block: run the analysis for one well then export the single cell results and create a scatter plot (see *Figure 2–34*) to determine the optimal threshold value.
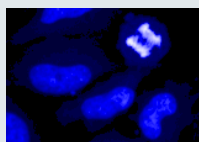
Image Analysis Sequence: Nuclear Analysis – DNA Content
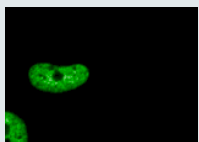
Input Images

Channel 1: DNA stain

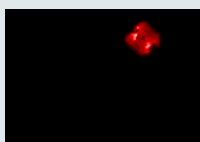**Channel 2**: Marker 1 (here: EdU)

Channel 3: Marker 2 (here: pHH3)

Channel 1   Channel 2   Channel 3

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Nuclear detection results on the nuclear stain image

Quantify Properties in Regions

Calculate Intensity Properties

Mean and Sum DNA intensity

Mean marker 1 intensity

Mean marker 2 intensity

Calculate Morphology Properties

Nuclear Area

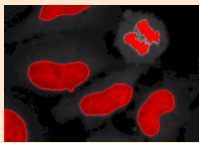Regions for intensity measurement (red) overlaid on the marker image

Table of results "per cell" ready for copying and pasting into Excel®

DNA Intensity Mean	DNA Intensity Sum	Nucleus Area [µm²]
1090,51	717554	274,509
1318,47	1459547	461,826
3235,86	517737	66,7499

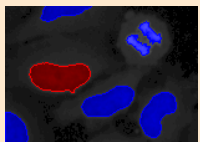| Intensity Marker 1 (EdU Mean)	Intensity Marker 2 (pHH3) Mean
28,3359	11,9286
557,537	13,084
40,55	371,962

Identify Subpopulations

Select Population
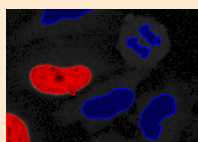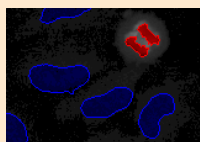
High DNA content (a)

Marker 1 positive (b)

Marker 2 positive (c)

a   b   c

The classified objects are colored red, the discarded objects colored blue

Calculate Readout Values

Define Results

Select and calculate values to report back as the results per well

Full table of results per well

	Nuclei - Number of Objects
	788

Fraction of cells with high DNA content [%]	Fraction of EdU positive cells [%]	Fraction of pHH3 positive cells [%]
56,9797	38,7056	2,03046

Readout Values

Number of Cells	Corresponds to the number of nuclei / objects
Fraction of cells in S-phase	Corresponds to Marker 1 (EdU) positive cells
Fraction of cells in M-phase	Corresponds to Marker 2 (pHH3) positive cells ("Mitotic index")

Fraction of cells with high DNA content	Corresponds to G2- / S- and early M-phase cells

Other optional readout values of interest:
- Percentage of cells in G1-phase (low DNA content, not in S-phase, not in M-phase).
- Percentage of cells in G2-phase (high DNA content, not in S-phase, not in M-phase).

2.14.3 Image Analysis in Detail

➤ The analysis sequence is a combination of the RMS "Quantification of Marker in Nucleus" (section 2.5) for two different markers and the RMS "Nuclear Classification – DNA content" (section 2.12) for the DNA stain channel.

➤ A set of properties is calculated for each cell:

- DNA content (corresponding to the "Sum Intensity" of the DNA stain, see the RMS "Nuclear Classification – DNA content" (section 2.12) for a more detailed description).

- DNA mean intensity (corresponding to the intensity per area. This also corresponds to the visual impression of the "brightness" seen in the image).

- Marker 1 intensity value (here: EdU).

- Marker 2 intensity value (here: pHH3).

➤ The individual properties are used to classify the cells using **Select Population** building blocks. One building block for each class ("EdU positive", "pHH3 positive", "High DNA content") is used to flag all the cells that belong to that class:

- An "EdU positive" flag indicates whether the intensity exceeds the given EdU intensity threshold.

- A "pHH3 positive" flag indicates whether the intensity exceeds the given pHH3 intensity threshold.

- A "High DNA content" flag indicates whether the DNA sum intensity exceeds the specified threshold.

➤ Please refer to *Figure 2–33* for an illustration of how thresholds define the potential classes.
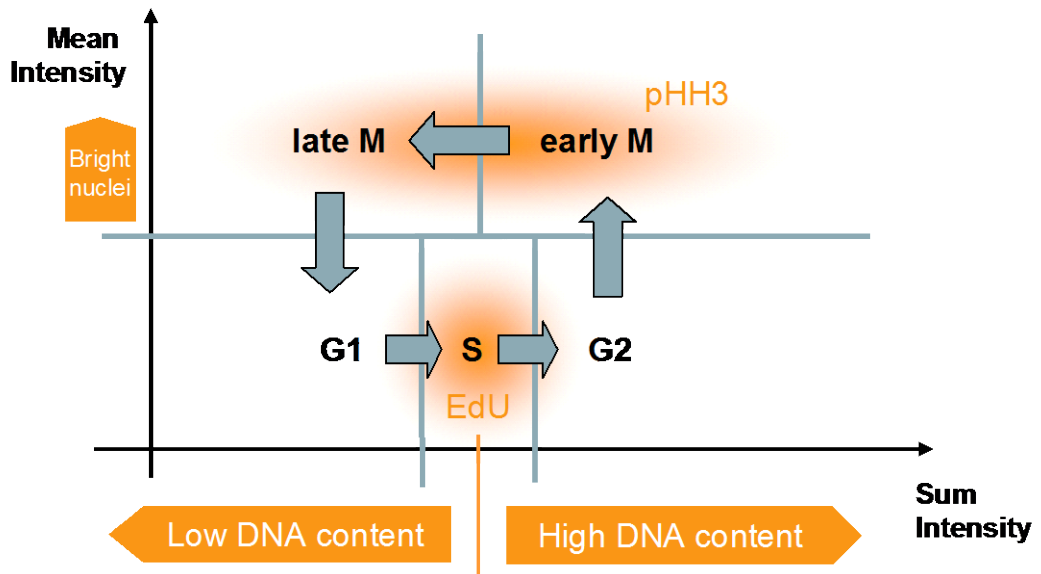
Figure 2–33: Schematic overview of the marker based classification of cells into different cell cycle phases. The orange bar marks the threshold for low / high DNA content and the blue bars mark the thresholds for cell cycle stage classification.
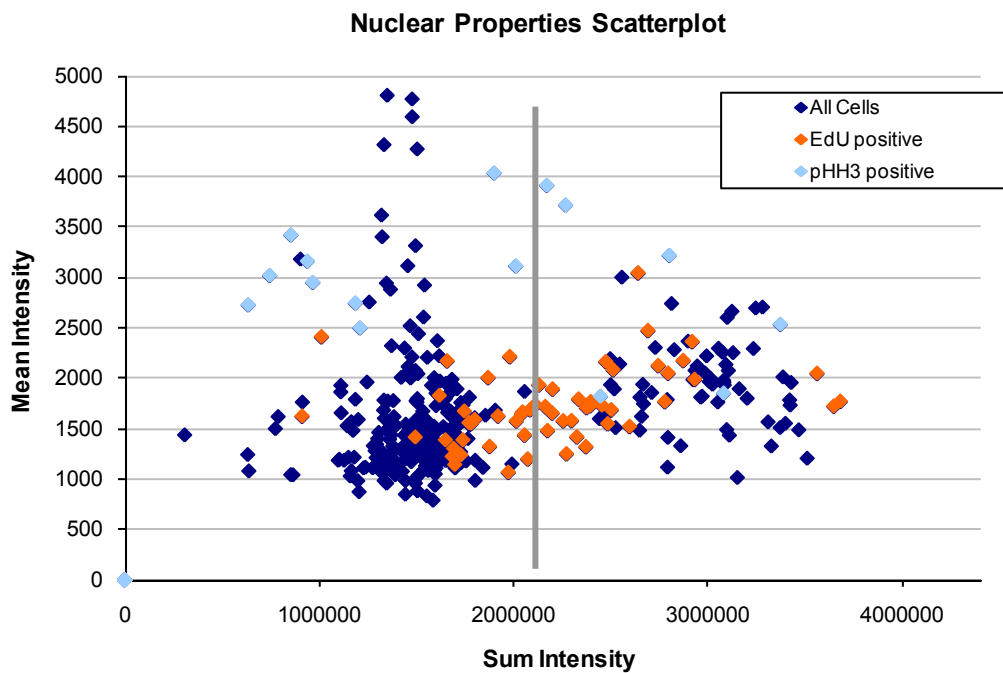

Figure 2–34: Example of cell cycle classification using fluorescent phase-specific markers in combination with a DNA stain. The scatter plot shows the properties of single cells, with each cell represented by one dot. The Y-axis shows DNA intensity per area ("Mean Intensity") and the X-axis shows DNA content (corresponding to "Sum Intensity"). The grey line indicates a Sum Intensity value of 2,100,000 units. Dark blue dots – all cells. Light blue dots – pHH3 positive cells (mean pHH3 intensity of the cell > 500). Orange dots – EdU positive cells (mean EdU intensity of the cell > 2000). The data shown is for well E15 of the example dataset "Plate P004-CC: EdU-pHH3", details of which can be found in the Appendix on page 311.

2.14.4 Hints and Tips

➤ Also see the RMS "Nuclear Classification – DNA content" (section 2.12) and the RMS "Mitotic Index" (section 2.17).

➤ Different classes can be easily created using different combinations of **Calculate Intensity** / **Morphology Properties** and **Select Population** building blocks. Different conditions can be linked by AND / OR logical operations to create readout parameters such as:

- Percentage of cells in G1-phase (low DNA content, not in S-phase, not in M-phase).

- Percentage of cells in G2-phase (high DNA content, not in S-phase, not in M-phase).

➤ Also see Application Guide, section 3.3 "Ready-Made Application: Cell Cycle-1".

2.15 Cell Shape – Cell Rounding

This RMS allows you to classify cells by morphological parameters. An example for cell rounding is given.

2.15.1 Introduction and Assay Principle

The rounding up of cells from an attached and spread-out state on a substrate can be observed when cells are dividing or when they experience toxic effects. It can also be observed when examining cell adherence to a substrate. This RMS can also be used to analyze cell spreading, e.g. to screen for inhibitors of cell spreading.
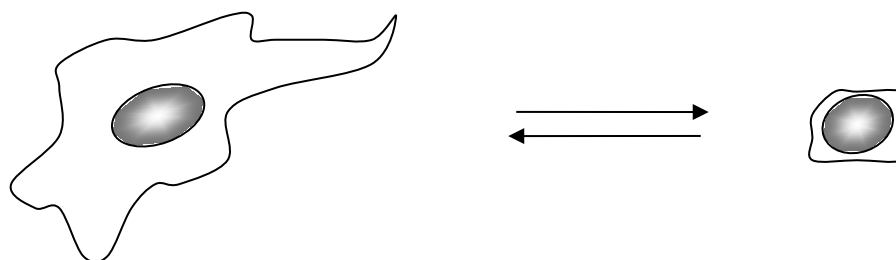

Figure 2–35: Illustration of cell rounding. For an example dataset showing cell rounding, please see the description of "Plate P013-CC: Cytoskeleton" (with compound Cytochalasin) in the Appendix, on page 317.

2.15.2 How to use the Cell Shape – Cell Rounding RMS
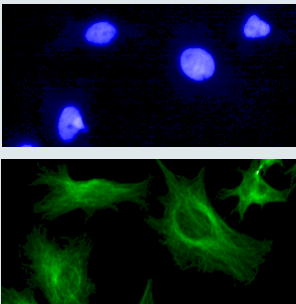
➤ Load the analysis sequence "RMS Cell Shape" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic or combined nuclear / cytoplasmic dye channel in the **Find Cytoplasm** building block.

➤ When using the "Percentage of rounded cells" readout, set the roundness threshold in the **Select Population** building block so as it classifies cells as being rounded.

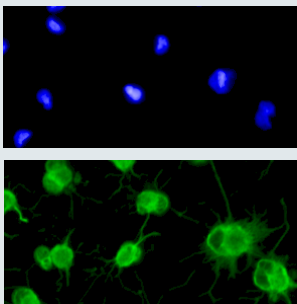## Image Analysis Sequence: Cell Shape – Cell Rounding

Input Images

Channel 1:
Nuclear stain

Channel 2:
Cytoplasmic stain (here: tubulin)
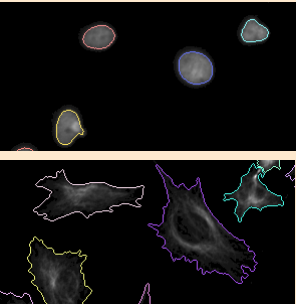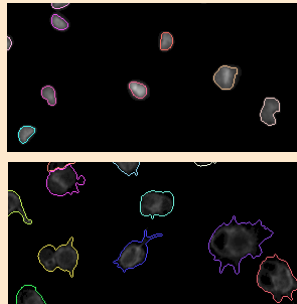

Untreated control

Treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines using the cytoplasmic stain image

Untreated cells

Rounded cells

Quantify Properties

Calculate Morphology Properties

Area, Roundness, Width, Length, Width to Length Ratio

Object No	Cell Area [µm²]	Cell Roundness
10	957,027	0,956448
11	332,915	0,913721
12	778,888	0,898203
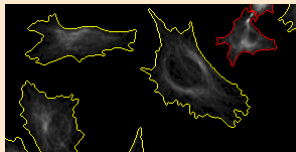
Cell Width [µm]	Cell Length [µm]	Cell Ratio Width to Length
25,8683	43,7547	0,591212
14,2098	31,3311	0,453536
15,6089	55,2311	0,282611

Object No	Cell Area [µm²]	Cell Roundness
7	159,783	0,943471
8	297,037	0,934295
9	338,756	0,973026

Cell Width [µm]	Cell Length [µm]	Cell Ratio Width to Length
8,66566	20,6789	0,419058
15,1201	24,722	0,611606
15,9787	24,9572	0,640244

Identify Subpopulations

Select Population

Find rounded cells (red) (roundness above threshold)

Calculate Readout Values

Define Results

Calculate the percentage of rounded cells

Mean Area	Mean Width to Length Ratio
1249,67	0,41523

Mean Roundness	Percentage of round Cells
0,945919	9,52381

Mean Area	Mean Width to Length ratio
404,681	0,526459

Mean Roundness	Percentage of rounded cells
0,914656	86,0465

Readout Values

Total Number of Cells	Equivalent to the number of detected nuclei
Mean Cell Area	Size of the cells
Mean Width to Length Ratio	The Width to Length Ratio characterizes the cell shape
Mean Roundness	A roundness value of 1.0 corresponds to a perfect circular cell shape
Percentage of rounded cells	Here: cells with modified cytoskeletal structure

Image Analysis Guide

2.15.3 Image Analysis in Detail

Image Segmentation

➤ In the first step, the nuclei are detected, then the cytoplasmic area around each nucleus is detected.

➤ Either a combined nuclear and cytoplasmic stain can be used in the same channel, or a cytoplasmic stain can be used in a separate channel. For the advantages and disadvantages of this staining, see Application Guide, section 1.4.5 "Water immersion vs. air objectives".

➤ In the example shown below, a tubulin stain is used for detection of the cytoplasm. The tubulin stain is also used as a marker to determine the cytoskeletal structure, and is affected by treatment with the experimental compound. In this example, the tubulin signal always covered the whole cytoplasmic area. This was ensured through the use of a separate cytoplasmic stain during assay development, and by comparison of the areas marked by the tubulin and cytoplasmic stains.

➤ Generally speaking, the stain used for identification of cellular compartments must be carefully checked to ensure that it is not affected by the compound under investigation; otherwise artificial cross-talk between channels can occur. Consider the following example:

• The size of the detected cytoplasmic region is dependent on a compound

  AND

• The amount of marker dye is measured in that detected region

In this example, the **measured amount** of marker will be indirectly dependent upon the compound, even though the marker itself is not affected in any way; only the location where it is measured is affected.

Quantification of Properties

➤ The morphological properties of the detected cytoplasmic outlines are measured using a **Calculate Morphology Properties** building block.

➤ Common measurements of the cell shape are **Area**, **Roundness** and **Width to Length Ratio**. See *Figure 2–6* in the RMS "Nuclear Analysis – Nuclear Shrinking" (section 4.4) for example shapes that demonstrate the read-out values of these morphology parameters. Refer to *Figure 2–36* for the definition, and an example, of the width and length of cells.
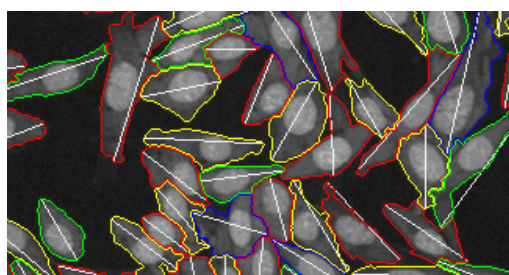
Example of cells with "Length" property indicated.
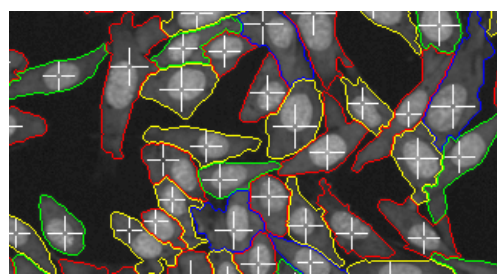
Example of cells with "Width" property indicated.

Figure 2–36: Example of the "Width" and "Length" properties of cells. The length is defined as the longest straight line that fits into the cell (parts of the line may also be outside the cell if it has a concave shape). The width is defined as the largest circle that completely fits into the cell; therefore it is the "Width" of the cell at the widest point.

Determination of the Subpopulation of Cells

➤ To determine a population of cells with specific properties (e.g. rounded cells), a **Select Population** building block is used. To define a criterion, first identify suitable properties, or combinations of properties, of the group of cells you are interested in. For example, for counting round-looking cells, the "**roundness**" may be best suited, perhaps in combination with a size threshold to prevent small round debris from being counted.

➤ You can sort the Object List Table (see *Figure 1–1*, panel 5) by specific properties by clicking on the column headers in the table. You can then easily select the 10 largest or smallest entries in the table and see the corresponding cells highlighted in the image. You may also select cells in the image and see the corresponding row in the table. For advanced visualization, e.g. scatter plots or histograms, the list can be transferred to external programs by copying and pasting (see the example in the RMS "Nuclear Classification – DNA content", section 2.12).

➤ Work iteratively to identify a proper threshold. Identify all, or most, of the cells of interest and then set the identified criterion, e.g. "roundness > 0.85" in the **Select Population** building block. You can combine multiple criteria using AND conditions. More conditions are available in the secondary level options of the building blocks; see the "Harmony Help Function" for more details.

➤ Be aware that you never identify all cells; but do not worry too much about outliers at this point. In many cases, 80 % accuracy is sufficient for a robust solution. Proceed with the validation of the analysis using High / Low controls or dose-response curves to check the overall performance, e.g. determination of the Z' value. Return to optimization of the thresholds or criteria only if necessary.

Readout Calculation

➤ The "Percentage of rounded cells" calculation formula is specified in the **Define Results** building block.

2.15.4 Hints and Tips

➤ Rounded and other poorly adhered cells are likely to be washed away during the washing steps. Be sure to consider this when interpreting the Harmony analysis results. Rounded cells accumulate at the well borders, therefore image fields located at a well border might show high random fluctuations due to accumulated washed away cells.

➤ More subpopulations can be identified by adding further **Select Population** building blocks, e.g. to determine the percentage of "long" cells.

➤ Marker quantifications in an arbitrary number of channels can be determined by adding **Calculate Intensity Properties** building blocks.

➤ Combinations of intensity & morphology object parameters can be created using the **Calculate Properties** building block.

➤ Dendritic structures can be quantified using the "Skeletonisation" method in the **Select Region** building block, by calculating the area to determine the length of the dendritic structures.

2.16 Fluorescence Redistribution – Cytoskeleton

This section describes the use of the RMSs "Receptor Internalization" (section 2.11) and "Quantification of Cytoplasmic Marker" (section 2.6) to measure cytoskeletal rearrangements. For detailed descriptions about each individual RMS, please refer to the appropriate sections.

2.16.1 Introduction and Assay Principle

Cytoskeletal re-arrangements due to treatment with a compound can be monitored by using a cytoskeleton stain, and:

➤ Measuring and comparing the cytoskeletal stain intensity in different regions of the cytoplasm.

➤ Monitoring the structure of the cytoskeletal stain, e.g. change from a granular to a smooth distribution.

The example shown here demonstrates how to modify the "Receptor Internalization" RMS (section 2.11) in order to quantify the cytoskeletal re-arrangement in tubulin stained HeLa cells.
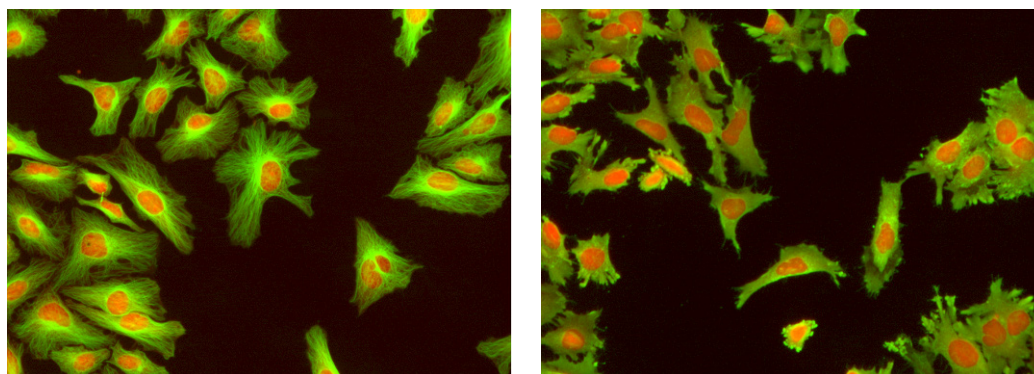
Untreated cells – structured distribution of tubulin with high staining intensity near the nucleus and a decreasing intensity towards the plasma membrane.

Re-arrangement of tubulin due to treatment with Demecolcin (2 ng / ml) – a homogeneous distribution is observed.

Figure 2–37: Example of a cytoskeletal rearrangement. Details of the example dataset ("P013-CC: Cytoskeleton") can be found in the Appendix on page 317.

2.16.2 How to use the "Receptor Internalization" RMS to quantify Cytoskeletal re-arrangement

➤ Load the analysis sequence "RMS Receptor Internalization" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the tubulin stain channel in the **Find Cytoplasm** and **Calculate Intensity Properties** building blocks.

➤ Adjust the region of interest to the area of maximum tubulin intensity, which corresponds to the Microtubule Organization Center (MTOC), in the **Select Cell Region** building block. Use an untreated control image to define the MTOC

region, as treatment with the compound leads to cytoskeletal rearrangement and therefore loss of the MTOC.

➤ Readout parameters can be freely configured in the **Define Results** building block. You can rename the readout value "Mean Internalized Intensity" to "Tubulin Intensity Distribution Index" in this application to correctly reflect the meaning of the value.

2.16.3 Image Analysis in Detail

The readout values of this assay are:

➤ Number of Cells.

➤ Mean cell area, including standard deviation.

➤ Tubulin intensity distribution index, ranging from 0 (homogenous) to 1.0 (intensity concentrated near the nucleus).

2.16.4 Hints and Tips

➤ The signal window of this application is small (index changes of ~0.1), but the noise level (standard deviation) is also very low, delivering good Z' values. The proper adjustment of the MTOC region to a small area around the nucleus is essential for good results. See *Figure 2–24* for an example of the radial intensity distribution inside the cytoplasm. For the MTOC detection, the region with a high intensity difference is very small (~ 10 %).

➤ The "Standard deviation of intensity" readout value, in the RMS "Quantification of Cytoplasmic Marker" (section 2.6), can also be used to quantify the re-distribution of intensity from representing a granular structure to a more homogenous structure.

➤ Also see the RMS "Phenotype Classification – Cytoskeleton" (section 2.23) for an alternative approach to cytoskeleton re-arrangement analysis based on texture analysis of the fiber structure.
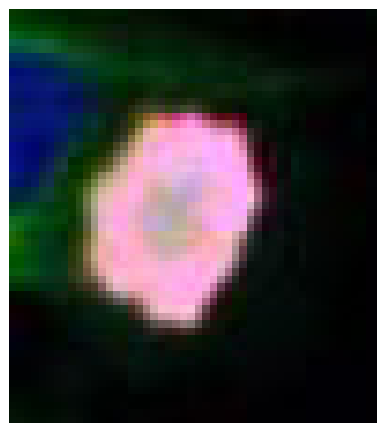
2.17   Mitotic Index

This section describes how to use the RMS "Quantification of Marker in Nucleus" (section 2.5) to measure the mitotic index. For a more detailed description of this RMS please refer to section 2.5.

2.17.1   Introduction and Assay Principle

The mitotic index is a measure of the proliferative status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. Cells in each phase of the cell cycle can be identified using, for example, antibodies against the nuclear antigen Ki-67, a phosphorylated epitope of a core histone protein (pHH3), or Bromodeoxyuridine (BrdU). All these markers lead to specific fluorescence in the nuclei of cells.
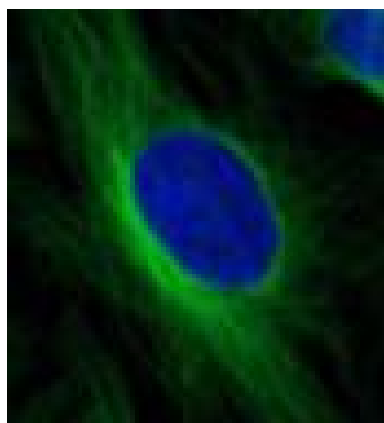
A non mitotic cell stained with Hoechst 33342 (blue) and anti-tubulin-Alexa Fluor® 488 (green).

A mitotic cell stained with Hoechst 33342 (blue), anti-tubulin-Alexa Fluor® 488 (green) and anti-Phospho-Histone-Alexa Fluor® 633 (pink).

Figure 2–38: Example images for calculating the mitotic index.

2.17.2   How to use the "Quantification of Marker in Nucleus" RMS to determine the Mitotic Index

➤ Load the analysis sequence "RMS Quantification of Marker in Nucleus" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the marker dye channel for mitotic cells in the **Calculate Intensity Properties** building block (e.g. pHH3).

➤ Adjust the threshold for "marker positive" cell detection in the **Select Population** building block.

➤ Readout parameters can be freely configured in the **Define Results** building block. You can rename the readout value "Fraction of POS Cells" to "Mitotic Index" in this application. To get a fraction (0.0….1.0) rather than a percentage (0 %...100 %) you can remove the factor of 100 in the output calculation.

2.17.3 Image Analysis in Detail

The assay readout values are:

- ➤ Number of Cells.

- ➤ Mitotic index (fraction of cells in M-phase).

2.17.4 Hints and Tips

- ➤ For a detailed description of threshold adjustment for the selection of marker positive cells, please refer to the RMS "Quantification of Marker in Nucleus" (section 2.5).

- ➤ Quantification of additional markers can easily be determined by adding more **Calculate Intensity Properties** building blocks and selecting the desired properties (see section 1.3.3).

- ➤ Alternatively, mitotic cells can be detected based on the nuclear stain fluorescence only (nuclear size and DNA content). See the RMS "Nuclear Classification – DNA content" (section 2.12).

2.18   Nuclear Spots

This section describes how to use the RMS "Spot Analysis" (section 2.8) to analyze nuclear spots. For a more detailed description of this RMS, please refer to section 2.8.

2.18.1   Introduction and Assay Principle

Analyzing spots in nuclei can be used to monitor the formation of nucleoli (where spots and nuclear dye are in the same channel). It can also be used in fluorescence *in situ* hybridization (FISH) experiments (multi color spots in different channels), see *Figure 2–39*.
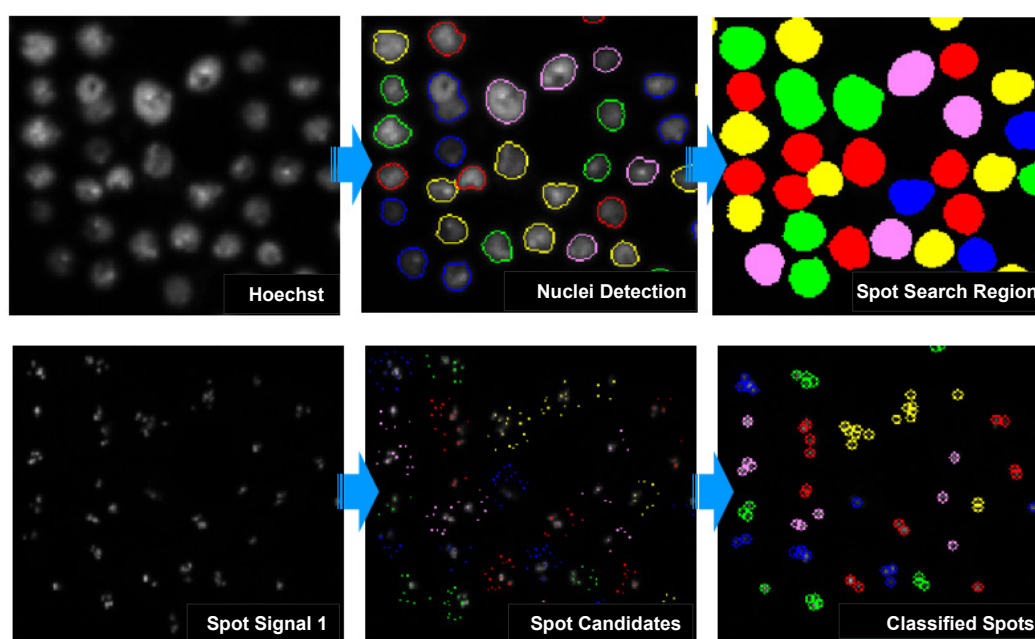

Figure 2–39: Example images of nuclear spots (FISH). Top row: detection of nuclear outlines using the Hoechst 33342 channel. Bottom row: Spot search inside each individual nuclear area. In the first step, candidate spots are detected (intensity maxima), and in the second step the spots are filtered by intensity and size criteria to determine the set of "classified" spots.

2.18.2   How to use the "Spot Analysis" RMS to create a Nuclear Spots RMS

➤   Load the analysis sequence "RMS Spot Analysis" from the Harmony database.

➤   Select the nuclear dye channel in the **Find Nuclei** building block.

➤   Select the spot marker dye channel in the **Find Spots** building block, set the spot search area to "nuclei", and adjust the spot size filter parameters.

➤   Insert additional **Find Spots** building blocks if you have more than one spot marker channel.

➤   Readout parameters can be freely configured in the **Define Results** building block. You can add, remove and rename the readout values to assign meaningful names for your specific application.

2.18.3 Image Analysis in Detail

Typical assay readout values for this application are:

➤ Number of cells.

➤ Mean number of spots per cell.

➤ Single cell results, including the number of spots for each detected cell.

2.18.4 Hints and Tips

➤ An arbitrary number of additional spot marker channels can be included by adding further **Find Spots** building blocks.

➤ More spot statistics can be calculated on demand by adding the **Select Population** building blocks to determine specific populations of cells, e.g. "cells with at least one spot identified with marker 1".

➤ A set of tailored results for each well can be defined using the **Define Results** building block, e.g. "Percentage of cells with at least one spot".

2.19　Nuclei Counting

This section describes how to use the RMS "Cell Counting" (section 2.2) to count nuclei. For a detailed description of this RMS please refer to section 2.2.

2.19.1　Introduction and Assay Principle

Counting nuclei is a variant of counting cells, but using a nuclear dye. It is suitable for use when the number of nuclei per cell is expected to be "1", and if no specific cytoplasmic stain will be used.
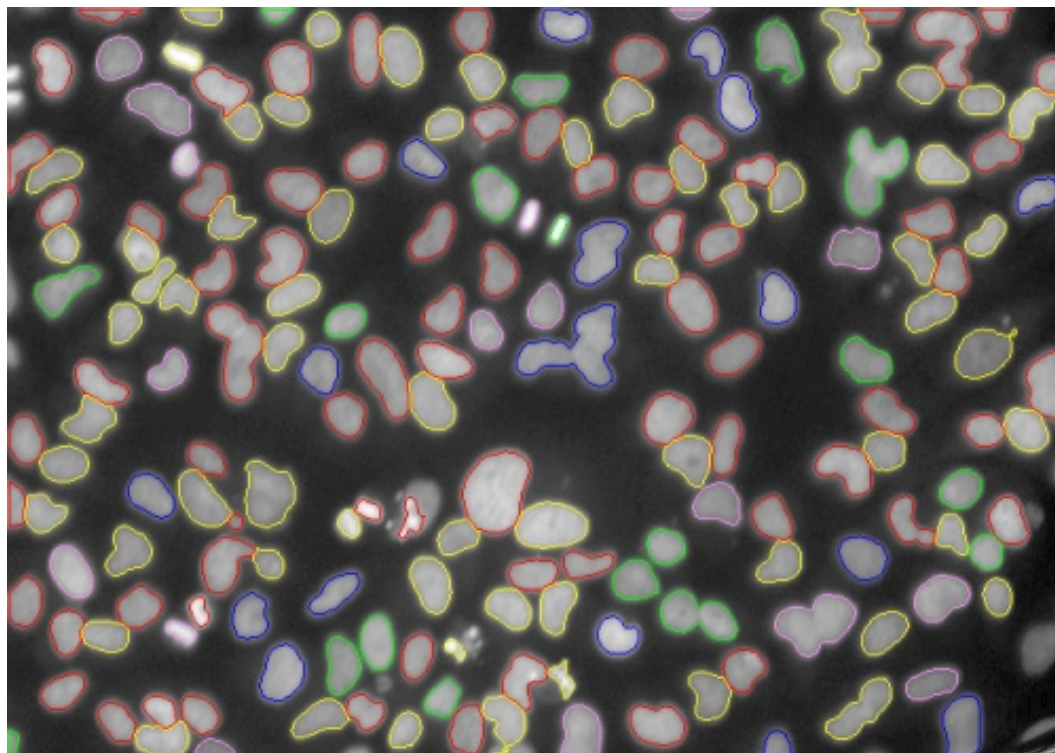

Figure 2–40: Example of identified nuclei in a nuclear stain image showing a very high cell density and heterogeneity. Adjustable parameters allow you to specify criteria in which objects considered to be intact nuclei are counted.

2.19.2　How to use the Cell Counting RMS for Nuclei Counting

➤ Load the analysis sequence "RMS Cell Counting" from the Harmony database.

   OR

➤ Create a new analysis sequence and add a **Find Nuclei** building block. You can refer to the "Operation Manual" or the "Harmony Help function" for information on creating and modifying analysis sequences.

➤ Select the channel for nuclear stain in the **Find Nuclei** building block.

➤ Option: Adjust the nuclei detection parameters (following the instructions in the **Find Nuclei** building block help function) to optimize the detection results.

2.19.3 Hints and Tips

➤ Homogeneous populations of well separated nuclei (similar size, shape, staining intensity) are most reliably counted.

➤ The **Find Nuclei** building block is designed to cope with difficult situations. You can use the "Method" parameter of the building block to select from a set of different methods, with different characteristics, to adapt to different imaging situations. Select alternative methods in the Harmony user interface and choose the one which gives the best results.

➤ Touching nuclei will be separated by any of the methods in the **Find Nuclei** building block. Depending on the imaging conditions it might be impossible in some situations to determine if an object consists of two touching nuclei or is only one nucleus. Try using different methods to find which one works best for your images. Additionally you might use the "Splitting Adjustment" parameter for fine tuning.

➤ When interpreting the results of the automated analysis, keep in mind that there might be ambiguous situations where the number of nuclei is difficult to define, for example:

- If there are fragments of nuclei due to apoptotic cells.

- During cell division.

- If there are other bright objects (e.g. agglomerates of dye) which cannot be distinguished from nuclei.

➤ Be sure to include a sufficient number of nuclei in the analysis to obtain a robust statistic. Check the standard deviation of the results of control wells to determine the reliability of the analysis.

➤ Do not worry about some wrongly detected nuclei in your images as long as the statistical error is sufficiently low for your application.

Image Analysis Guide

2.20 Online Quality Control

This RMS is designed to provide quality control of a sample during measurement, even when no other RMS is available for evaluation of the specific signal(s). It can be used for online quality monitoring during data acquisition and provides key information about cell number, a quick health check of the cells, and the overall signal intensity.

2.20.1 Image Analysis in Detail

The readout values are:

➤ Number of cells.

➤ Nuclear area (Mean and StdDev).

➤ Cell area (Mean and StdDev).

➤ Degree of confluency (Percentage of the image area covered by cells).

➤ Marker intensity (Mean and StdDev).

2.20.2 How to use the "Online Quality Control" RMS

➤ Load the analysis sequence "RMS Online Quality Control" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic dye channel in the **Find Cytoplasm** building block.

➤ Select the marker channel in the **Calculate Intensity Properties** building block.

➤ Save the analysis sequence in the Harmony database and select it to be used for online analysis when setting up the experiment.

2.20.3 Hints and Tips

➤ Additional marker channels can be monitored by adding additional **Calculate Intensity Properties** building blocks.

➤ To determine the degree of confluency a "Whole Image" region is created using the **Select Region** building block. The image area is then calculated using the **Calculate Morphology Properties** building block. Finally, the image area is used in the **Define Results** building block to calculate the percentage of the area that is covered by cells.

2.21   Neurite Outgrowth Analysis

This RMS allows detection and quantification of dendritic stuctures, e.g. neurites, through a variety of different shape and intensity readouts.

2.21.1   Introduction and Assay Principle

Neurite outgrowth assays are used in drug discovery for identifying drugs that target spinal injuries and neuronal disorders such as Alzheimer's and Parkinson's disease.

A neuronal cell line or primary cultured neurons are used for measuring neurite outgrowth. Neuronal growth factor (NGF) can be used for stimulation of neurite outgrowth, for example as a positive control. Neuronal bodies and neurites can be visualized by immunofluorescence using anti-α-tubulin antibody and a suitable fluorescently labeled secondary antibody. Nuclei are counterstained using, for example, Hoechst 33342.
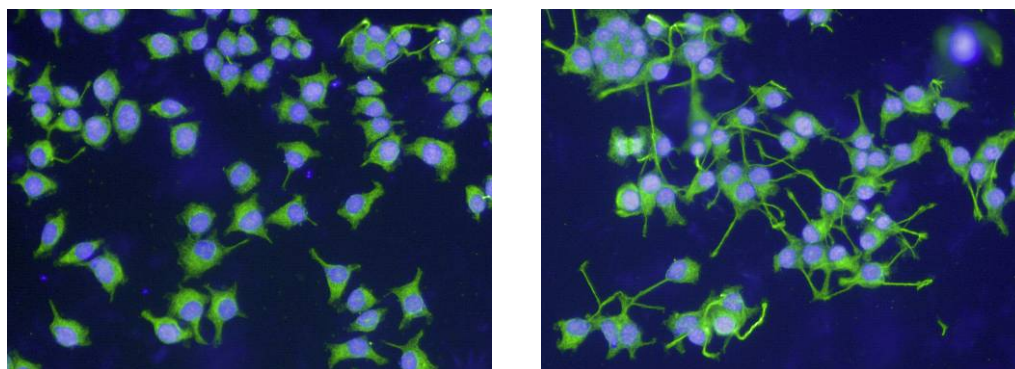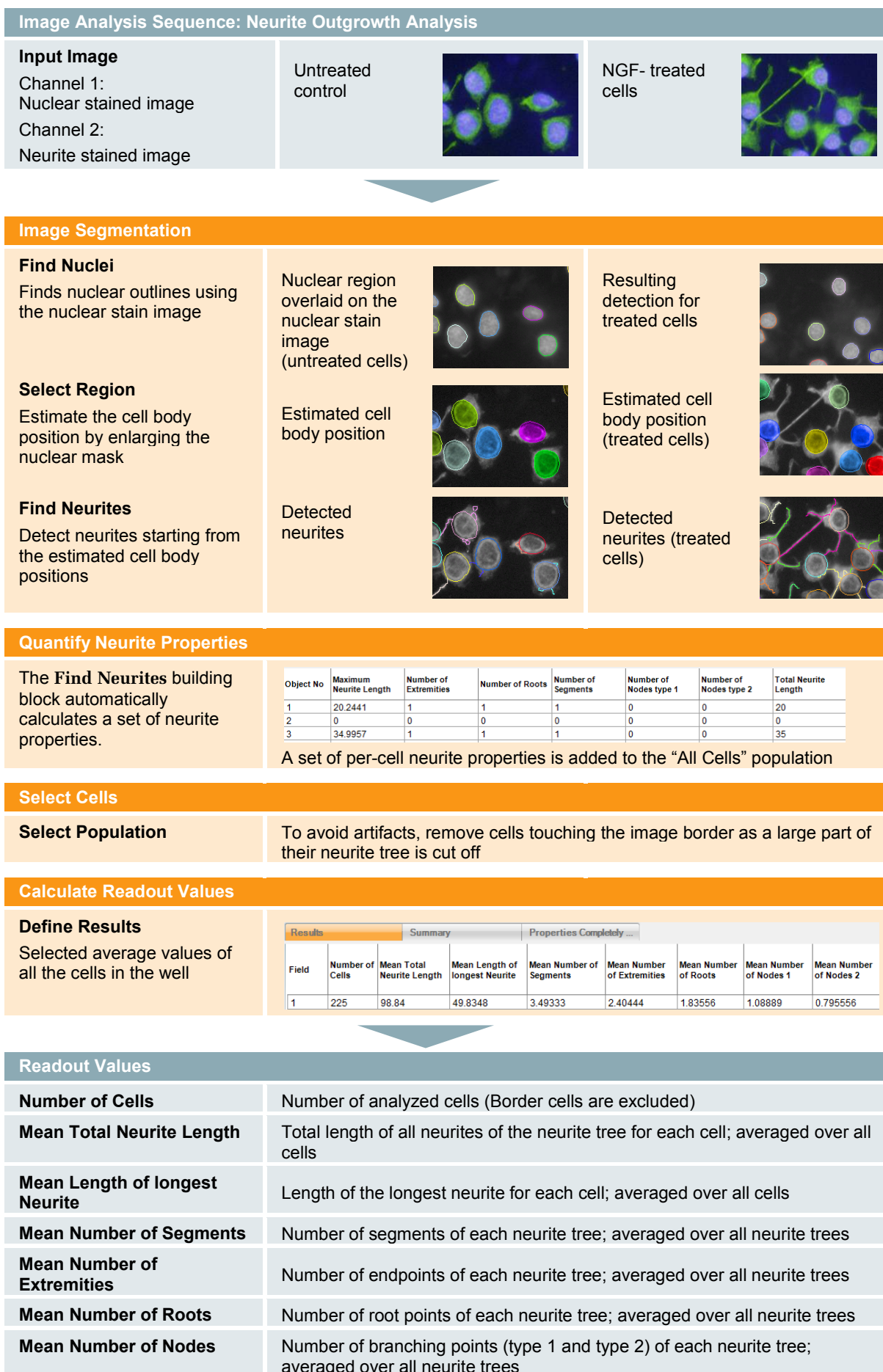

3 ng/ml NGF   60 ng/ml NGF

Figure 2–41: Example images of NGF treated NS-1 cells. Hoechst 33342 stained nuclei are shown in blue and α-tubulin labeled with Alexa Flour® 488 is shown in green. The images show the effect of treatment with different concentrations of NGF on neurite outgrowth. Details of the example dataset ("Plate P011-N Neurite Outgrowth") can be found in the Appendix on page 311.

2.21.2   How to use the Neurite Outgrowth RMS

➤ Load the analysis sequence "RMS Neurite Outgrowth Analysis 2" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the neurite stain channel in the **Find Neurites** building block.

➤ Check the neurite detection results and adjust the **Find Neurites** and **Select Region** building block parameters if needed.

Image Analysis Sequence: Neurite Outgrowth Analysis

Input Image

Channel 1:
Nuclear stained image

Channel 2:
Neurite stained image

Untreated control

NGF- treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Nuclear region overlaid on the nuclear stain image (untreated cells)

Resulting detection for treated cells

Select Region

Estimate the cell body position by enlarging the nuclear mask

Estimated cell body position

Estimated cell body position (treated cells)

Find Neurites

Detect neurites starting from the estimated cell body positions

Detected neurites

Detected neurites (treated cells)

Quantify Neurite Properties

The **Find Neurites** building block automatically calculates a set of neurite properties.

Object No	Maximum Neurite Length	Number of Extremities	Number of Roots	Number of Segments	Number of Nodes type 1	Number of Nodes type 2	Total Neurite Length
1	20.2441	1	1	1	0	0	20
2	0	0	0	0	0	0	0
3	34.9957	1	1	1	0	0	35

A set of per-cell neurite properties is added to the "All Cells" population

Select Cells

Select Population

To avoid artifacts, remove cells touching the image border as a large part of their neurite tree is cut off

Calculate Readout Values

Define Results

Selected average values of all the cells in the well

Results	Summary		Properties Completely ...					
Field	Number of Cells	Mean Total Neurite Length	Mean Length of longest Neurite	Mean Number of Segments	Mean Number of Extremities	Mean Number of Roots	Mean Number of Nodes 1	Mean Number of Nodes 2
1	225	98.84	49.8348	3.49333	2.40444	1.83556	1.08889	0.795556

Readout Values

Number of Cells	Number of analyzed cells (Border cells are excluded)
Mean Total Neurite Length	Total length of all neurites of the neurite tree for each cell; averaged over all cells
Mean Length of longest Neurite	Length of the longest neurite for each cell; averaged over all cells
Mean Number of Segments	Number of segments of each neurite tree; averaged over all neurite trees
Mean Number of Extremities	Number of endpoints of each neurite tree; averaged over all neurite trees
Mean Number of Roots	Number of root points of each neurite tree; averaged over all neurite trees
Mean Number of Nodes	Number of branching points (type 1 and type 2) of each neurite tree; averaged over all neurite trees

2.21.3 Image Analysis in Detail

Image Segmentation

In the first step, nuclei are detected with a **Find Nuclei** building block using the nuclear stain channel.

In the second step, an estimate of the cell body region is calculated, which is needed as a starting point for the neurite detection. A **Select Region** building block is used to enlarge the nuclear region of each cell by an adjustable width, pre-set to 5 pixels in the RMS (see *Figure 2–42* for an example for two different cell lines). To adapt this to different applications, adjust the region to cover the main part of the cell body. Omitting small parts of the cell body or including background will not affect the neurite detection (see *Figure 2–42*, right column).
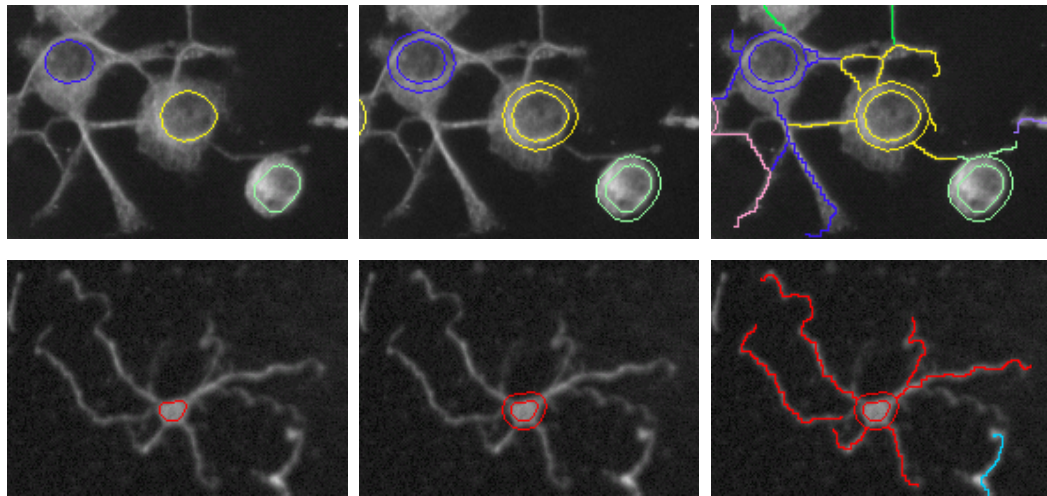
Figure 2–42: Examples of cell body region estimates. Top row: Neuroscreen™-1 cells, bottom row: Primary neurons. Left column: Detected nuclear outlines on the nuclear stain image (not shown here). Middle column: The outlines of the cell body regions, estimated by enlarging the nuclear region, are shown in addition to the nuclear outlines. Right column: Detected neurites assigned to their corresponding cell body region (indicated by color coding).

The third step is the actual neurite detection with the **Find Neurites** building block using the neurite stain channel and the estimated cell body region. The neurite detection algorithm can be adapted to many different applications by using a number of optional tuning parameters (described in detail in the "Harmony Help Function"). For an overview and introduction to the **Find Neurites** building block, please see section 1.8.

Removing Border Cells

A **Select Population** building block is used to remove cells that are not completely visible in the image. This reduces artifacts introduced by cells having large parts of their neurite tree outside the image. This would, for example, lead to underestimation of the mean neurite tree length.

Please note that the removal of border cells is done **after the neurite detection** to allow correct assignment of the neurites to cell bodies. If the cell bodies at the image borders are removed earlier, their neurites could be mistakenly assigned to the wrong cell bodies.

Calculate Readout Values

The single cell results are averaged per well and reported as the final results of the analysis using the **Define Results** building block.

2.21.4 Hints and Tips

➤ Cell body detection can also be done using the **Find Cytoplasm** building block if more precise cell body outlines are needed for the specific application. To remove parts of neurites which are erroneously detected as part of the cytoplasm, use the **Select Region** and **Modify Population** building blocks. See the analysis sequence "RMS Neurite Outgrowth Analysis 2 – with Cell Body Refinement" for an example.

➤ Additional marker stains (recorded in additional channels) can be quantified by adding **Calculate Intensity Properties** building blocks.

➤ Co-localization of Marker Intensity with the detected neurites can be measured using the "Neurite" region in the **Calculate Intensity Properties** building block.

➤ Additional arbitrary geometric properties of the neurite tree can be quantified using the **Calculate Morphology Properties** building block on the neurite region (e.g. width, length, roundness, width to length ratio).

➤ To filter the neurite tree for neurite segments with specific properties, use the "Neurites" population (returned by the **Find Neurites** building block). Use the **Calculate Properties** building block method "**By related Population**" to calculate per-cell statistics of the filtered / selected neurites.

➤ Spots (e.g. synaptic vesicles) can be quantified on neurites by adding a **Select Region** building block to enlarge the neurite region (by a choice of pixels) and then using the **Find Spots** building block to find spots in this region (see *Figure 2–43*).
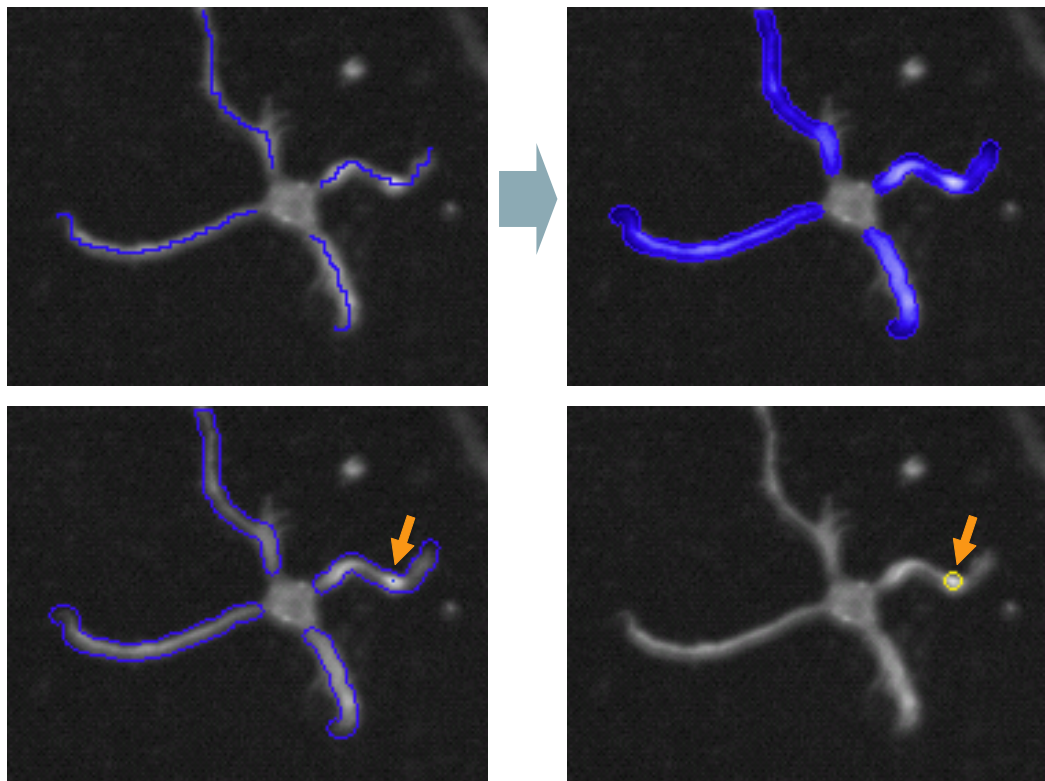
Figure 2–43: Example of spot detection on neurites by adding a **Find Spots** building block to the Neurite Outgrowth Analysis RMS. Top left : Neurite tree detected by the **Find Neurites** building block. Top right: Neurite mask generated using the **Select Region** building block. Bottom left: Spot center (blue dot) found by the **Find Spots** building block in the spot search region (blue overlay). Bottom right: Outline of the detected spot (yellow circle).

2.22　Texture Analysis – Mitochondria Classification

This RMS allows quantification of biological effects by monitoring changes in the texture ("pattern") of a fluorescent dye. The parameters of this RMS are optimized for mitochondria but can also be adapted for any other effect that induces textural changes.

2.22.1　Introduction and Assay Principle

In addition to changes in intensity and morphology, changes in the **texture** of the cellular fluorescence can also be used to quantify biological effects. See **Figure 2–44** for an example of different textures of stained mitochondria. The texture of a region could also be considered as the visual "pattern" of the intensity distribution, e.g. homogeneous, granular, spotty or stripy.

In the Harmony analysis sequences the texture can be easily measured by adding a **Calculate Texture Properties** building block to the sequence. Because the texture of a region does not depend on its absolute intensity or shape, texture parameters can be very robust readout values.

Untreated　　　　　0.1 µM　　　　　3 µM　　　　　10 µM
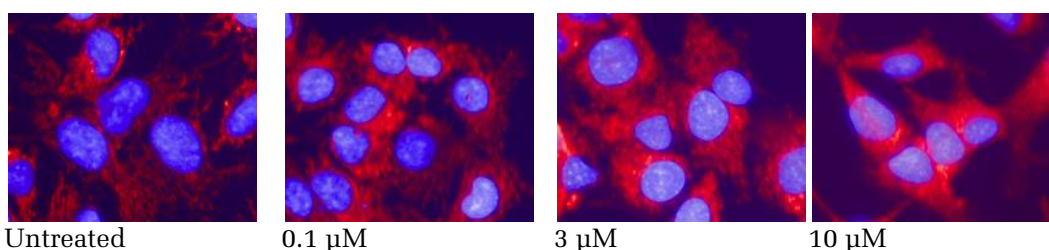
Figure 2–44: Images showing Hoechst 33342 nuclear staining (blue) and MitoTracker™ Deep Red staining (red) of human hepatocellular liver carcinoma (HepG2) cells at different concentrations of a toxic compound (FCCP). As the concentration of the compound is increased (from left to right), there is a notable change in the mitochondrial texture. Details of the example dataset ("Plate P003-CT: Live Tox") can be found in the Appendix on page 311.

2.22.2　How to use the Texture Analysis – Mitochondria Classification RMS

➤　Load the analysis sequence "RMS Texture Analysis – Mitochondria Classification" from the Harmony database.

➤　Select the nuclear dye channel in the **Find Nuclei** building block.

➤　Select the cytoplasmic (if available) or mitochondrial stain channel in the **Find Cytoplasm** building block.

➤　Select the mitochondrial stain channel in the **Calculate Intensity Properties** and **Calculate Texture Properties** building blocks.

➤　Use positive and negative control wells to check the texture readout results. If needed, try different texture properties in the **Calculate Texture Properties** building block and choose the one which gives the best signal to noise ratio.

Image Analysis Sequence: Texture Analysis – Mitochondria Classification
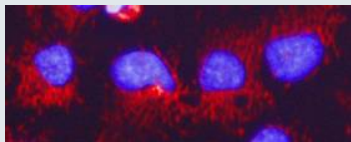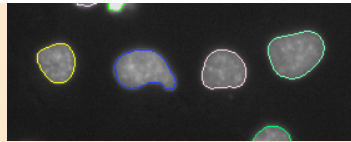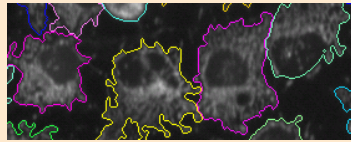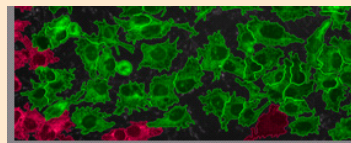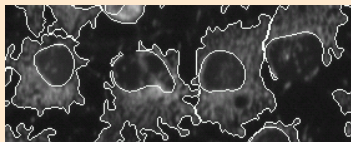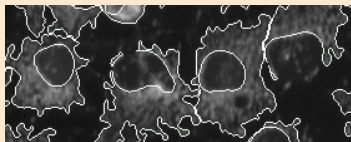
Input Images

Channel 1:Nuclear stain image

Channel 2: Mitochondrial stain

Color overlay of nuclear stain image (blue) and mitochondrial stain image (red)

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Detected nuclear outlines overlaid on the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around nuclei using the mitochondrial stain image

Detected cytoplasmic outlines overlaid on the mitochondrial stain image

Select Population

Discard cells that are not completely visible in the image

Green: selected cells

Red: discarded cells at the image border (grey rim on the left and bottom edge)

Quantify Properties in Regions

Calculate Morphology Properties

Area and roundness

Object No	Object No in All Nuclei	Cell Area [μm²]	Cell Roundness	MitoTracker Deep Red Intensity Mean	MitoTracker Deep Red Intensity CV [%]	MitoTracker Deep Red Texture SER Hole 1 px
1	7	1308.09	0.783947	4318.46	39.7236	0.00177472
2	9	419.754	0.723381	3494.56	55.9168	0.000840187
3	10	597.323	0.72847	2834.41	21.3514	0.00158835

Calculate Intensity Properties

Mitochondrial marker intensity and homogeneity

Analyzed region marked on the mitochondrial stain image

Calculate Texture Properties

Texture feature measuring mitochondrial damage

Intermediate illustration of the texture analysis, showing structures used for texture quantification

Calculate Readout Values

Define Results

Selected averaged values of all the cells in the well

		Results				Summary	
Field	Number of Cells	Mean Cell Area [μm²]	Mean Cell Roundness	Mean Mito Marker Intensity	Mean Mito Marker Intensity CV [%]	Mean Mito Marker Texture Index
1	257	673.947	0.609269	3146.81	26.0786	0.00391058

Readout Values

Number of Cells	Equivalent to the number of detected nuclei (border cells removed)
Mean Cell Area	Area of whole cell in μm²; averaged over all cells
Mean Cell Roundness	Measure of the roundness of the cell; averaged over all cells
Mean Mito Marker Intensity	Intensity of the mitochondrial marker stain; first averaged over all pixels within each cell and then averaged over all cells
Mean Mito Marker CV%	Homogeneity of the Mitochondria marker; first the coefficient of variation (CV) over all pixels within each cell, then averaged over all cells
Mean Mito Marker Texture Index	Selected texture feature to measure mitochondrial structural changes, e.g. due to treatment with a toxic compound; averaged over all cells

Image Analysis Guide

2.22.3 Image Analysis in Detail

Image Segmentation

In the first step, the nuclear outlines of all cells are determined using a **Find Nuclei** building block, using the nuclear stain image.

In the second step, the cytoplasmic region is determined around each detected nucleus using a **Find Cytoplasm** building block. The mitochondrial stain image can be used in this step if the whole cytoplasmic area is visible on all images, irrespective of whether treatment with a compound leads to mitochondrial damage. If parts of the cytoplasm are not stained, a separate cytoplasmic stain channel has to be used for cytoplasmic detection, e.g. a combined nuclear / cytoplasmic stain such as DRAQ5™ (see Application Guide, section 1.6.1 "Signal versus reference stain").

In the third step, the **Select Population** building block is used to remove all nuclei which are not completely visible in the image. These nuclei would introduce artifacts, e.g. the measured area of the nuclei would be smaller than the actual area.

Quantifying Morphology, Intensity and Texture Properties

The **Calculate Morphology Properties** building block is used to determine the most frequently used cell shape properties – cell area and roundness. For more information on morphology and intensity parameters see the RMS "Nuclear Analysis – Nuclear Shrinking" (section 2.4).

The **Calculate Intensity Properties** building block is used to determine the mean intensity of the mitochondrial stain in the cytoplasmic region and the homogeneity of the intensity, measured by the percentage coefficient of variation (CV) of the intensity of the mean value. For example, a CV of 30 % means that the mitochondrial intensity fluctuates by ± 30 % around the mean value in a cell.

The **Calculate Texture Properties** building block is used to quantify the texture of the mitochondria ("pattern of the stain"). Texture analysis can be used to determine changes in the structure of mitochondria, e.g. as a result of toxic impact. See section 1.7 for an introduction and overview of texture analysis.
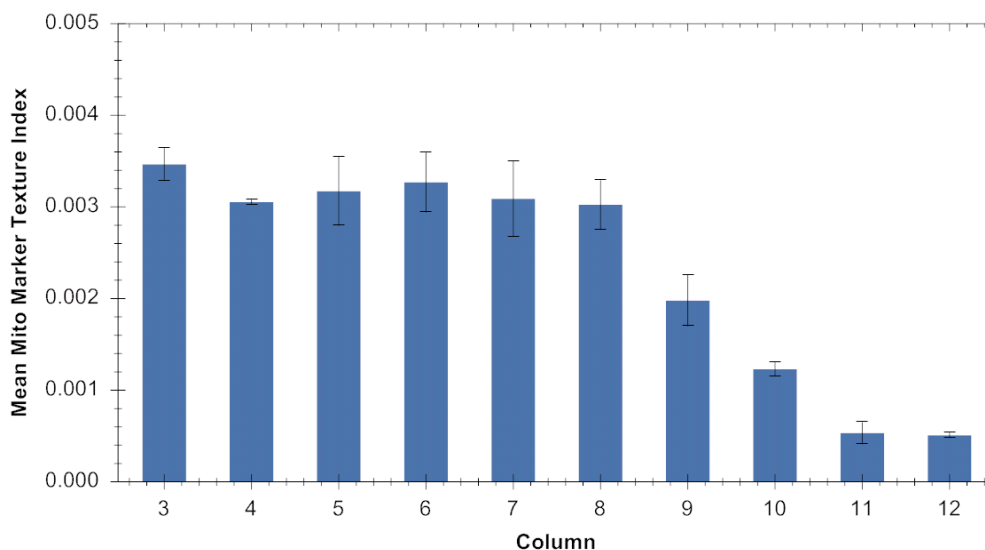

Figure 2–45: Texture readout values calculated by the "Texture Analysis – Mitochondria Classification" RMS for wells containing increasing concentrations of a cytotoxic compound (from left to right). The texture parameter values clearly reflect the impact of the increasing concentration of the compound on the cells. The graph is displayed on the "Evaluation Screen" of the Harmony software to allow selection of the most suitable texture property. A set of 14 properties in three groups are available in the **Calculate Texture Properties** building block.

One texture property which has proved to be well suited to mitochondrial texture quantification has been pre-selected in this RMS. In total, a set of 14 different features are available in the **Calculate Texture Properties** building block. See *Figure 2–45* for an example of a dose response for this parameter.

In many cases, the pre-set texture property (method: "SER Features", feature: "SER holes", scale: 1px) will provide very good results. To adapt to other imaging conditions (e.g. magnifications) or applications (e.g. different cell lines, stains), you might want to change the texture feature or its optional tuning parameters. Please refer to the "Harmony Help Function" for a detailed overview of methods and parameters.

The decision for choosing a parameter can easily be made by looking at the analysis results for a dose response curve on the Harmony "Evaluation Screen" (see *Figure 2–6* for an example). Select multiple texture features in the optional parameters of the building block (see *Figure 1–62*), view the results and choose the most suitable parameter.

The only tuning parameter for the "SER Features" is "scale"; see *Figure 2–46* for an explanation and instructions on how to adjust this parameter.

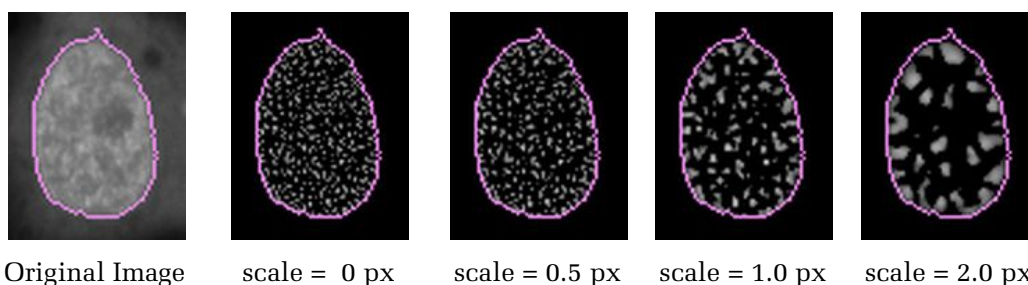| Original Image | scale = 0 px | scale = 0.5 px | scale = 1.0 px | scale = 2.0 px |

Figure 2–46: Illustration of the "scale" parameter – adjusting the typical length scale of the texture pattern in order to quantify it. The image on the left shows the original marker intensity. All other images are filtered images that are provided as an additional illustration by the **Calculate Texture Properties** building block when selecting the "SER Features" method. The scale parameter is increased from left to right in four steps and the images show that the scale of the structures identified by the building block is increased accordingly. To adjust, set the "scale" parameter to a value which matches your visual impression of the size of granular features seen in the image. In this example, the scale values of 0.5 px or 1.0 px seem appropriate. In addition (or instead of), you can evaluate a dose response curve (as shown in *Figure 2–45*) with different scale values and choose the one which gives the best curve.

Calculate Readout Values

In the **Define Results** building block, a set of properties is selected to be averaged over the whole cell population and reported back by the analysis sequence as the final results per well.

2.22.4 Hints and Tips

➤ To calculate more properties: Open the **Calculate Intensity Properties** or **Calculate Morphology Properties** building block options and select more parameters. See the "Harmony Help Function" for a detailed description of all parameters.

➤ To quantify additional markers recorded in other channels, add additional **Calculate Properties** building blocks that work on different channels. For an example, see Application Guide, section 3.2 "Ready-Made Application: Cytotoxicity-1".

➤ Single cell results can be saved in the database by activating the corresponding option in the **Define Results** building block (see section 1.5.4).

2.23   Phenotype Classification – Cytoskeleton

This RMS allows the easy identification of different cellular phenotypes within a cell population using PhenoLOGIC machine learning technology. Training is done by interactive selection of examples for each phenotype. See section 1.15 for details on the use of PhenoLOGIC for classification.

2.23.1   Introduction and Assay Principle

A common class of cellular assays is based on the classification of cells by characteristic properties like morphology, structure or fluorescence intensity of specific markers. The influence of a compound is quantified by monitoring changes in the mixture of phenotypes. An example is shown here with two phenotype classes: untreated cells and cells with a re-arranged cytoskeleton, induced by Demecolcine treatment. Demecolcine causes depolymerization of microtubules and leads to a significant change in the tubulin texture and intensity properties. HeLa cells were stained with Hoechst 33342 and anti-tubulin-Alexa Fluor® 488 and imaged with a 20x objective.
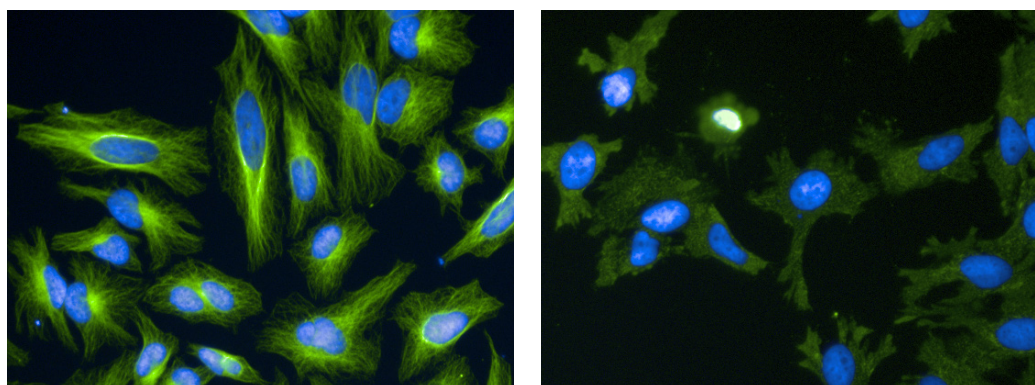
Untreated cells   1 µM Demecolcine

Figure 2–47: Images showing Hoechst 33342 nuclear staining (blue) and tubulin Alexa®488 staining (green) of HeLa cells. Cells are untreated (left image) or treated with 1 µM Demecolcine (right image). Demecolcine depolymerizes microtubules, resulting in a more homogeneous distribution of tubulin compared to the filamentous structure seen in untreated cells. As the concentration of the compound is increased there is a notable change in tubulin texture and intensity properties. Details of the example dataset ("P013-CC: Cytoskeleton") can be found in the Appendix on page 317.

2.23.2 How to use the Phenotype Classification RMS

➤ Load the analysis sequence "RMS Phenotype classification 2" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block.

➤ Select the cytoplasmic stain channel in the **Find Cytoplasm** building block.

➤ Select the cytoskeletal stain channel and the appropriate region in the **Calculate Intensity Properties**, **Calculate Morphology Properties and Calculate Texture Properties** building blocks.

➤ Click on "Train…" in the **Select Population** building block to start the training.

➤ Use "Reset Training" to delete the training of the example dataset and train on a new dataset. Select a class and click on cells in the image representing that phenotype. Train at least 40 examples for both classes (A and B). Cells may be located on different images.

Image Analysis Sequence: Phenotype classification
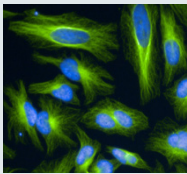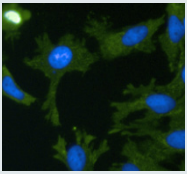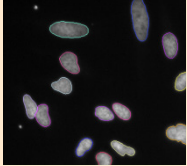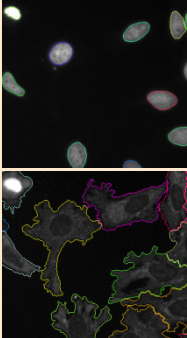
Input Image

Channel 1:
Nuclear stain image

Channel 2:
Tubulin stain image

Untreated control

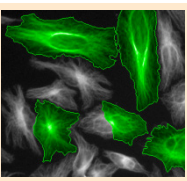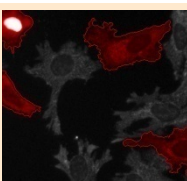

Demecolcine treated cells

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around each of the previously detected nuclei

Nuclear region overlaid on the nuclear stain image (untreated cells)

Detected cytoplasmic outlines

Resulting detection for treated cells

Quantify Properties in Regions

Calculate Morphology Properties

Calculate Intensity Properties

Calculate Texture Properties

Calculate a set of properties for each individual cell including texture features to measure cytoskeletal re-arrangement

Object No	Marker Intensity Mean	Marker Intensity CV [%]	Cell Area [µm²]	Cell Roundness
1	713.804	24.0787	282.631	0.62319
2	1230.19	53.8608	1101.91	0.703505
3	1786.39	121.545	1222.02	0.572648
4	872.29	43.7713	781.797	0.584212
5	1141.15	54.774	1189.47	0.759924
6	1794.28	49.8924	534.68	0.777586
7	1436.48	55.7322	836.054	0.495076

Marker Texture SER Spot 2 px	Marker Texture SER Hole 2 px	Marker Texture SER Edge 2 px	Marker Texture SER Ridge 2 px	Marker T… SER Valley
0.00178653	0.00286584	0.0512984	0.00528866	0.007394
0.00180352	0.00297132	0.0596871	0.00690599	0.005980
0.00115648	0.00202973	0.0887132	0.00522615	0.016661
0.00222077	0.00271713	0.0534024	0.00666911	0.005616
0.00148195	0.00274283	0.0558012	0.00646237	0.009474
0.00310581	0.00426464	0.0708473	0.00861869	0.013282
0.0015509	0.00287968	0.0952157	0.0120645	0.017528

Object No	Marker Intensity Mean	Marker Intensity CV [%]	Cell Area [µm²]	Cell Roundness
1	580.011	35.457	1052.34	0.56519
2	440.488	35.2324	641.962	0.648592
3	681.08	60.9499	2235.89	0.622617
4	692.716	44.3722	1579.63	0.56738
5	605.704	50.0032	2158.2	0.677953
6	568.051	19.6679	1570.5	0.480444
7	625.854	27.3664	1520.44	0.473197

Marker Texture SER Spot 2 px	Marker Texture SER Hole 2 px	Marker Texture SER Edge 2 px	Marker Texture SER Ridge 2 px	Marker SER Vall
0.00356558	0.00297389	0.0604662	0.00796147	0.0075
0.00298867	0.00184693	0.0570467	0.00696718	0.0082
0.00392114	0.00359681	0.0602097	0.00794592	0.0115
0.00451284	0.00358158	0.0886674	0.0116631	0.0126
0.00420582	0.00292604	0.0615412	0.00794067	0.0113
0.00481375	0.000952746	0.0432256	0.00948983	0.0031
0.00416794	0.00232481	0.0573472	0.00857235	0.0073

Select Cells

Select Population

Linear classifier

Classification using pre-calculated object properties

Requires a training phase of selecting cells

Training mode:

Select cells for Phenotype A (green)

Training mode:

Select cells for Phenotype B (red)

Calculate Readout Values

Define Results

Calculate the percentage of cells of phenotype A and B

Number of Cells	Percentage of Phenotype A	Percentage of Phenotype B
41	37.2727	62.7273

Readout Values

Number of Cells	Number of analyzed cells in the well
Percentage of Phenotype A	Percentage of "Phenotype A" classified cells in the well
Percentage of Phenotype B	Percentage of "Phenotype B" classified cells in the well

2.23.3 Image Analysis in Detail

Image Segmentation

➤ First the nuclei are detected, and then the cytoplasmic area around each nucleus is identified.

➤ For the cytoplasmic detection, either a combined nuclear and cytoplasmic stain (e.g. DRAQ5™) or a separate cytoplasmic stain can be used. For advantages and disadvantages of these staining options, see section 1.4.

➤ In this example, the cytoplasm is identified using the tubulin stain, which is also used to classify the cells. See section 2.15.3 in the RMS "Cell Rounding" for advice on avoiding artifacts by using a signal stain also as a reference for segmentation.

Classification of Phenotypes

➤ With the **Calculate Properties** building blocks several properties (e.g. tubulin marker intensity and a set of texture features) are calculated for each cell in the input population which will be used for classification.

➤ Classification of objects requires a training phase in the **Select Population** building block. As a result of the training, a classifier will be created (see **Figure 2–48**).
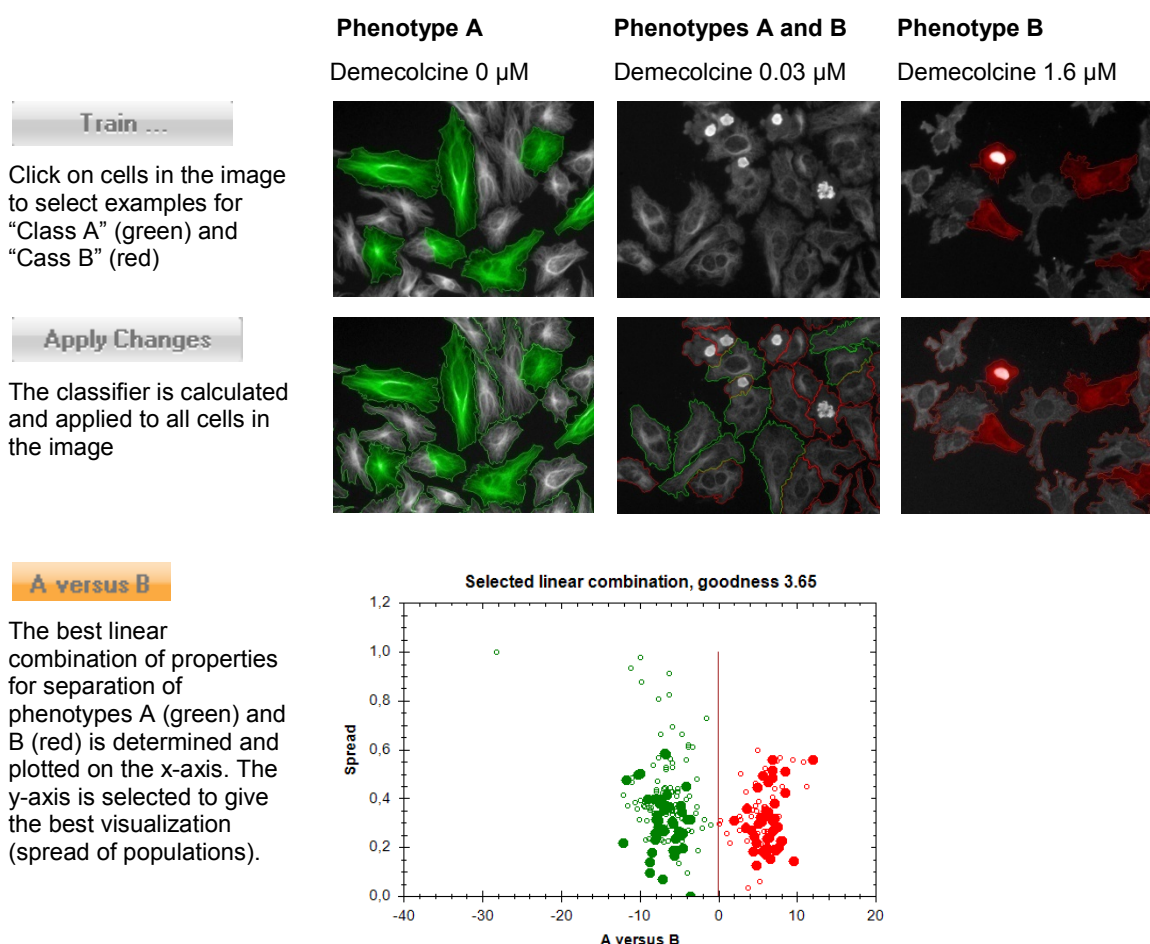
Figure 2–48: Illustration of the process of training the building block. Top row: Example cells, selected during the training, are marked red and green. Middle row: Classification results are added as red and green rims to each cell. Bottom row: scatter plots of property pairs and classifiers. Red and green dots represent the trained cells and black circles represent all other cells.

➤ In the "Train…" mode select at least 40 example cells for each phenotype.

➤ Click on "Apply Changes" to create the classifier and apply it to all objects in the active image. The classification result is shown in the image. Repeat the last two steps until the classification results are correct.

➤ All pre-calculated properties will be evaluated to find the linear combination of properties which separates the two classes best. The result is displayed in a scatter plot and table in the "A versus B" tab for information.

Calculate Readout Values

In the **Define Results** building block the set of results to be reported per well is selected. The "Formula Output" method is used to calculate the percentage of "Phenotype A" and "Phenotype B" cells in the population.
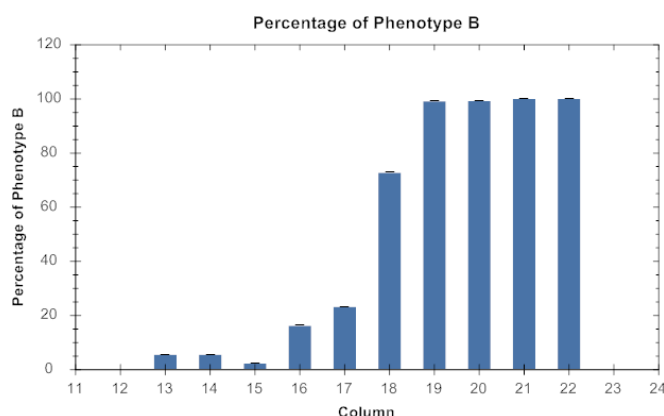
Figure 2–49: Readout values calculated by the "Phenotype classification – Cytoskeleton" RMS for wells with increasing concentrations of Demecolcine (from left to right). The increasing percentage of "Phenotype B" classified cells (cells with re-arranged cytoskeletons) reflects the impact of treatment with Demecolcine.

2.23.4 Hints and Tips

➤ To add more properties for object classification during the training phase: Open the **Calculate Intensity Properties** or **Calculate Morphology Properties** building block options and select more parameters. See the "Harmony Help Function" for a detailed description of all parameters.

➤ To quantify additional markers, recorded in other channels, add additional **Calculate Properties** building blocks that work on different channels. For an example, see Application Guide, section 3.2 "Ready-Made Application: Cytotoxicity-1".

➤ For the training in the **Select Population** building block, make sure to choose objects showing distinct phenotypes. By selecting hybrid forms the probability of generating artifacts increases.

➤ To exclude properties which are known to cause artifacts or which you do not want to use for classification, e.g. intensity or size, deselect the corresponding check boxes in the third level options of the building block.

➤ Single cell results can be saved in the database by activating the corresponding option in the **Define Results** building block, see section 1.5.4.

➤ More phenotype classes can be added (up to six) in the **Select Population** building block by changing the "Number of Classes" parameter.

2.24 Lipid Droplet Analysis – Analysis of various sized spots

This RMS analyzes spot-like features (cellular organelles, endosomes, lipid droplets, etc) of various sizes in any sort of cellular compartment. An example is the drug-induced formation of lipid droplets in the cytoplasm of cells.

2.24.1 Introduction and Assay Principle

Phospholipidosis (PLD) is a phospholipid storage disorder induced by drugs with a cationic amphiphilic structure (CADs). Affected cells show an excessive intracellular accumulation of phospholipids and the appearance of membranous inclusions named lamellar bodies. Although this condition is reversible and there is no evidence that phospholipidosis is harmful to human health, the PLD-inducing potential is considered an important factor during drug development.

In the example dataset shown in **Figure 2–50**, lipid droplets were labeled with a green fluorescent lipophilic tracer (BODIPY® 493/503).

DMSO   Triacsin   JAK3 Inhibitor
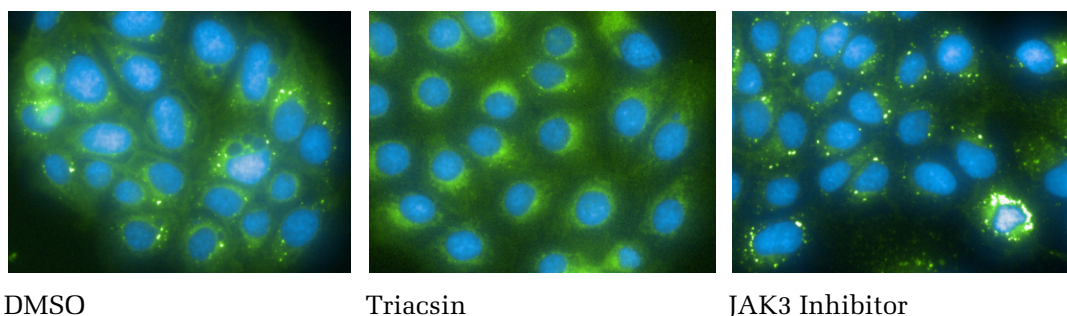
Figure 2–50: Example images showing cells with a different extent of lipid droplet formation. Cells were stained with DAPI (blue) and SYTO®42 (blue). Lipid droplets were detected with the lipophilic tracer BODIPY® 493/503 (green). Left: Cells treated with DMSO alone show a normal level of lipid droplet formation. Middle: Treatment with Triacsin reduces lipid droplet formation. Right: Cells incubated with a JAK3 inhibitor show a significant increase in lipid droplet formation. Details of the example dataset ("Plate P015-Phospholipidosis") can be found in the Appendix on page 319.

2.24.2 How to use the Lipid Droplet Analysis RMS

➤ Load the analysis sequence "RMS Lipid Droplet Analysis" from the Harmony Database.

➤ Select the nuclear dye channel in the **Find Nuclei** building block and the cytoplasmic dye channel in the **Find Cytoplasm** building block.

➤ Select the lipid droplet / spot specific channel in the **Find Spots** building block and modify the parameters to optimize the spot detection according to your application.

➤ Select the lipid droplet / spot specific channel in the **Calculate Intensity Properties** building block.

➤ Optionally: readout parameters to be reported can be freely configured in the **Define Results** building block. It is possible to add, remove and rename readout values according to the needs of your application.

Image Analysis Sequence: Lipid Droplet Analysis

Input Images

Channel 1:
Nuclear stain image

Channel 2:
Lipid droplet stain
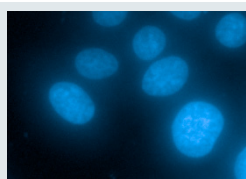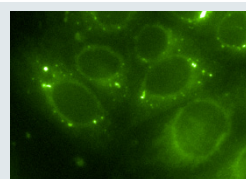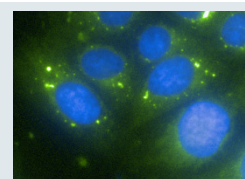
Channel 1   Channel 2   Color overlay

Image Segmentation

Find Nuclei

Finds nuclear outlines using the nuclear stain image

Find Cytoplasm

Finds cytoplasmic outlines around nuclei using the lipid droplet stain image

Find Spots

Find lipid droplets using the lipid droplet stain image
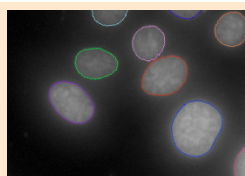
Detected nuclear outlines overlaid on the nuclear stain image
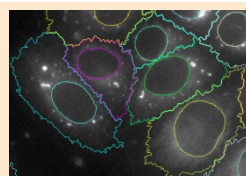
Detected cytoplasmic outlines overlaid on the lipid droplet stain image
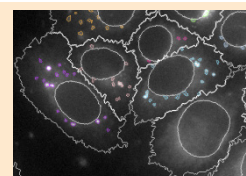
Detected Spots within the cytoplasmic outlines

Quantify Properties

Calculate Morphology Properties

Lipid Droplet Area and Cytoplasm Area

Calculate Intensity Properties

Lipid droplet marker intensity; Mean and Sum

Object No	Lipid Droplet Area [µm²]	Lipid Droplet Intensity Mean	Lipid Droplet Intensity Sum
1	2.95948	569.958	27358
2	2.15796	630.571	22070
3	3.0828	896.1	44805
4	2.65121	671.837	28889
5	2.28127	580.162	21466
6	2.77452	696.689	31351

Property table of the "Lipid Droplets" population

Object No	Cytoplasm Area [µm²]
1	339.108
2	177.322
3	710.4
4	635.426
5	390.097
6	362.352

Property table of the "All Cells" population

Calculate Properties and Calculate Ratios of Properties

Calculate Properties

Lipid droplet properties per cell

Object No	Cytoplasm Area [µm²]	Number of Lipid Droplets- per Cell	Lipid Droplet Area [µm²]- Mean per Cell	Lipid Droplet Intensity Mean- Mean per Cell	Lipid Droplet Area [µm²]- Sum per Cell	Lipid Droplet Intensity Sum- Sum per Cell	Fraction of Droplet Area
1	738.145	2	1.63388	387.399	3.26776	20447	0.004427
2	502.064	1	2.95948	425.938	2.95948	20445	0.00589463
3	855.353	7	2.48385	536.711	17.387	143295	0.0203273
4	314.877	6	1.84968	358.346	11.0981	66696	0.0352457
5	602.687	7	1.9818	462.41	13.8726	111405	0.0230179
6	981.439	15	2.05931	433.575	30.8896	234179	0.0314738

Readout Values

Number of Cells	Equivalent to the number of detected nuclei (border cells removed)
Average number of lipid droplets per cell	Number of lipid droplets per cell; averaged over all cells
Average Lipid droplet area [µm²]	Lipid droplet area in µm²; averaged over all cells
Average Lipid Droplet Intensity	Intensity of the lipid droplet marker stain; first averaged over all pixels within each cell and then averaged over all cells
Total Lipid Droplet Area [µm²] per cell	Sum of lipid droplet area (µm²) per cell; averaged over all cells
Fraction of Lipid Droplet Area	Sum of lipid droplet area divided by the cytoplasm area; averaged over all cells
Total Lipid Droplet Intensity per cell	Sum Intensity of lipid droplets per cell; averaged over all cells

2.24.3 Image Analysis in Detail

Image Segmentation

The nuclei and the surrounding cytoplasms are detected using the **Find Nuclei** and **Find Cytoplasm** building blocks. This creates a set of standard output regions (whole cell, nucleus, cytoplasm and membrane). One of these regions can be used for the subsequent spot detection. In this example, the lipid droplet stain is also used as a reference stain for detecting the cytoplasmic outlines.

Find Spots and Quantify Spot Properties

Spots are detected in the spot signal channel by using the **Find Spots** building block. Several parameters may be adjusted on demand:

➤   There are four different methods (A to D) available. Method A, B and D detect spots of various sizes whereas method C uses a fixed spot radius (For method C, please refer to the RMS Spot Analysis (section 4.8)).

➤   Method A: The "**Relative Spot Intensity**" determines how intense a spot must be in order to be detected. The relative spot intensity is the ratio of the spot peak intensity to the mean intensity of the cell region where the spot is detected.

➤   Methods B and D: The "**Detection Sensitivity**" describes the sensitivity of the detection algorithm. At the lowest value (0) only extremely intense spots are detected. At the highest value (1.0) extremely weak spots are detected.

➤   The "**Splitting Coefficient**" adjusts how aggressive the algorithm tries to split touching droplets. Use it to correct for over or undersegmenation of droplets.

As a result, a new population called "Lipid Droplets" is returned by the building block. For each droplet, the number of the cell in which it was found is recorded for later calculation of "per cell" statistics.

By using the **Calculate Morphology Properties** and **Calculate Intensity Properties** building blocks on the cell and lipid droplet populations, the cytoplasmic area, the lipid droplet area and the lipid droplet intensity (mean and sum values per droplet) are calculated.

Calculate "per cell" Properties

Several **Calculate Properties** building blocks are used to calculate the lipid droplet properties per cell:

➤   The **Calculate Properties** building block's method "**By related population**" allows the allocation of sub-objects (e.g. lipid droplets) to their parent-objects (e.g. cells) to calculate statistical readouts (sum, mean, minimum, maximum, StdDev, etc.). Here it is used to calculate the mean area and intensity of the lipid droplets in each individual cell.

➤   A second **Calculate Properties** building block is used here to calculate the sum value of the lipid droplet intensity per cell and the sum of the lipid droplet areas per cell.

➤ The **Calculate Properties** building block's method "**By Formula"** allows the calculation of combinations of previously determined properties. Here it is used to calculate the fraction of the cell's cytoplasmic area which is covered by lipid droplets:

$$"Fraction\ of\ Droplet\ Area" = \frac{"Lipid\ Droplet\ Area\ [\mu m^2]\ (Sum\ per\ cell)"}{"Cytoplasm\ area\ [\mu m^2]"}$$

This property corrects for different cell sizes.

Define Readout Values

➤ Border cells that are not completely visible in the image falsify the results and are therefore removed by the **Select Population** building block.

➤ In the **Define Results** building block, average values per well are calculated from the single cell properties.

2.24.4 Hints and Tips

➤ If only spots in a certain cell region are of interest, the **Select Cell Region** building block can be used to further refine the search area.

➤ The lipid droplet population can be filtered by their properties, to select specific droplets, using a **Select Population** building block, e.g. to exclude lipid droplets below a size threshold.

➤ Similar to the average fraction of lipid droplet area, a normalized intensity readout can be generated. By dividing the accumulated spot intensity by the mean cytoplasmic intensity, inhomogeneous staining of the cells can be corrected.

➤ The **Find Spots** building block also provides a set of pre-calculated spot properties, which can be enabled by activating the "Calculate Spot Properties" checkbox in the optional parameter section of the building block. These properties also include normalized intensity values corrected for the spot background. The details of the normalization depend on the selected method. For more information, please refer to the "Harmony Help function" and to section 1.18.

➤ You may add more custom readouts by using additional **Calculate Properties** building blocks.

2.25   Colony Formation Analysis

This RMS is designed to analyze the growth and size of any kind of cell colonies labeled with a cytoplasmic live stain and imaged with a 2 x objective. The RMS can be extended to monitor (user-selectable) individual colony properties over time.

2.25.1   Introduction and Assay Principle

Cell colony formation assays are used extensively in research and clinical applications to assess the functional integrity and viability of cells after *in vitro* manipulations. Key areas include hematopoietic stem cell research, malignant cell transformation studies, and prediction of the response of tumors to chemotherapeutic agents.

As an example, we analyzed the formation of colonies by individual, adherent A-431 cells (human epidermoid carcinoma cells) over a time course of 5 days (see ***Figure 2–51***).

The pre-defined readout values for colony classifications include:

➤   The number and mean size of the colonies over time.

➤   A list of individual properties of each colony over time (area, mean intensity, sum intensity, width, length, roundness, contrast).

| Time point 1 / day 1 (T0) | Time point 2 / day 4 (T1) | Time point 3 / day 5 (T2) |
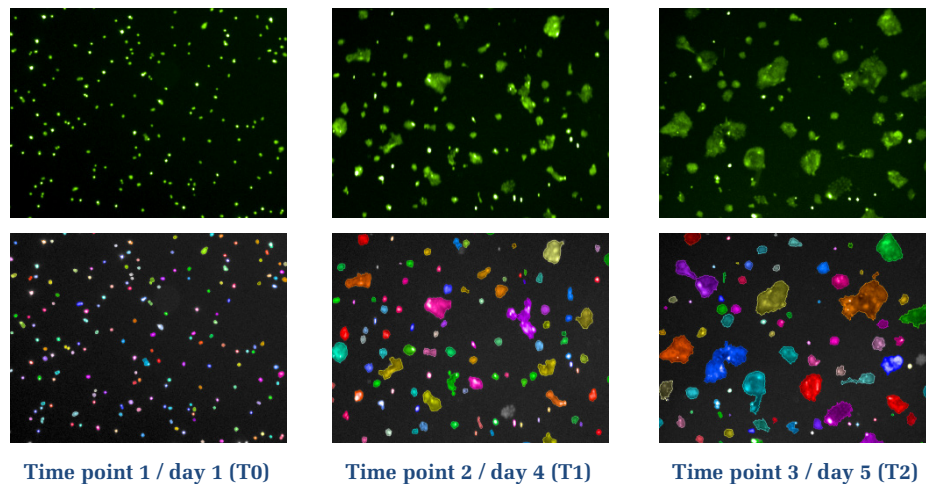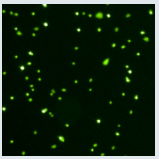
Figure 2–51: Colony formation of A-431 cells, in one well of a 24-well plate, over a time course of 5 days. Cells were stained with CMFDA at day 1. Images were recorded with a 2x objective (example dataset "P018-Colony Formation", for details see the Appendix on page 321). Upper row: CMFDA stained cells. Lower row: Colony detection results of the RMS Colony Formation. Each detected colony is labeled with a different color.

2.25.2 How to use the RMS Colony Formation
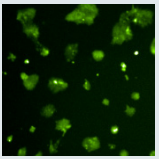
➤ Load the analysis sequence "RMS Colony Formation" from the Harmony database.

➤ Select the cytoplasmic dye channel (CMFDA) in the **Find Cells** building block. Check for different time points that the colonies are correctly detected and adjust parameters if needed. See the "Harmony Help function" of the building block.

➤ If needed, the merging of fragmented colonies can be adjusted using the "distance" parameter in the **Modify Population** building block.

➤ Select the CMFDA or any other channel of interest in the **Calculate Intensity Properties** building block to quantify the colonies' fluorescent marker intensities.

Image Analysis Sequence: Colony Formation

Input Image

Cytoplasmic stain image (CMFDA)

Cells on day 1

Colonies on day 5
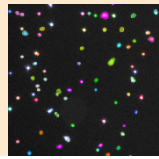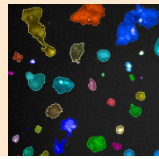
Image Segmentation

Find Cells

Detects single cells, cell clusters or colonies

Refine Colony Detection

Modify Population

Merging of fragmented colonies using the **Cluster by Distance** method

Distance set to 1 px: objects closer than 1 px are merged to one object

Quantify Properties of the Colonies
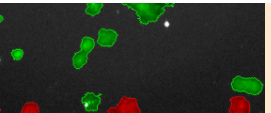
Calculate Morphology and Intensity Properties

Calculate area, roundness, width, length and intensity properties of each colony

Object No	Area [µm²]	Roundness	Width [µm]	Length [µm]
527	665,884	1,13432	28,0926	30,2077
526	1233,12	0,960652	22,2092	47,1128
525	1726,37	0,810421	28,0926	62,817
524	838,521	0,952725	22,2092	33,3138
523	1800,35	1,00263	39,729	51,8478
522	1233,12	0,960652	28,0926	46,8503
521	3206,11	0,941838	44,4184	82,6528
520	1159,13	0,919579	29,7967	40,0382

Object No	Area [µm²]	Roundness	Width [µm]	Length [µm]
194	58597,8	0,601378	203,793	407,343
195	7867,3	0,838112	72,3078	110,266
196	5302,41	0,898263	63,5974	87,8597
197	18940,7	0,444823	93,7006	343,812
198	43948,3	0,807996	191,051	278,546
199	6980,8	0,832056	63,5974	110,266
200	11418,7	0,902606	98,3242	127,679
201	4044,63	0,836827	50,6447	88,4193

Select Population

The common filter **Remove Border Objects** excludes objects crossing the image border.

Complete colonies are shown in green and discarded border colonies in red

Calculate Readout Values

Define Results

Calculate averaged population results per well.

Field	Number of Colonies	Mean Area [µm²]	Sum Area [µm²]	Mean Contrast
1	883	1139,08	1005810	0,756754

Field	Number of Colonies	Mean Area [µm²]	Sum Area [µm²]	Mean Contrast
1	377	10601,7	3996860	0,185258

Readout Values

Number of Colonies	Equivalent to the number of cells at the beginning of the time series and the number of colonies as time proceeds
Mean Area [µm²]	Average size of cells and / or colonies per well
Sum Area [µm²]	Total area covered by cells and / or colonies per well
Mean Contrast	A measure of staining intensity against background

In addition to the well averages, the set of individual colony properties is stored in the database and can be exported for secondary analysis.

2.25.3 Image Analysis in Detail

Image Segmentation

The **Find Cells** building block is used to detect the outlines of cells, cell clusters, and colonies, based on the CMFDA stain image. The subsequent analysis is reliant on this detection step. Image details may strongly vary over time therefore it is important to check the correct detection for different time points and object sizes. Adjust the building block parameters to optimize the detection (see section 1.4).

Further refinement of the segmented cells and colonies is achieved by using a second building block, **Modify Population**. Fragmented colonies are merged, based on a distance threshold. Adjust this value if needed.

Quantification of Properties

Depending on the biological set-up of the colony formation assay, different readouts may be of interest. The RMS calculates a set of commonly used readouts; however more customized readouts can be added by using additional building blocks (see section 1.3).

The **Calculate Morphology Properties** building block is used to calculate a set of morphology features: area, roundness, width, length, and width to length ratio of the colonies. For more information, see the RMS "Nuclear Analysis – Nuclear Shrinking" (section 2.4).

The **Calculate Intensity Properties** building block is used to determine the mean intensity as well as the contrast (third level parameter) of the cytoplasmic stain (CMFDA channel). The contrast can be used as an indicator of the staining quality (the rate of segmentation errors gets higher at lower contrast). In the example experiment, the contrast decreases over time because the cells were stained on the first day and the dye is then distributed to daughter cells over time.

Select Population

The common filter **Remove Border Objects** is used to exclude objects from the analysis which are not completely visible in the image. These objects would compromise, for example, the mean colony area per well readout.

Calculate Readout Values

In the **Define Results** building block a set of readouts is selected to be averaged and returned as the final results per well. For our example dataset the selected readouts are the number of cells or colonies, the mean and sum area in µm² per well, and the contrast of the staining.

In addition to the averaged readouts, Single Cell Results can be saved in the database by activating the corresponding option in the **Define Results** building block (see section 1.5.4). Selecting the 'ALL' option saves all object properties which are calculated by any of the building blocks.

For most colony formation assays the size distribution of the colonies over time is of interest: To access the single cell results, use the export format "Evaluation Results per Well and Object" to create a comma separated text file which can be directly used by any third party software e.g. Excel® (for details, see section 1.5.4 and the Harmony help function). ***Figure 2–52*** shows an example of a histogram of the colony area distribution.
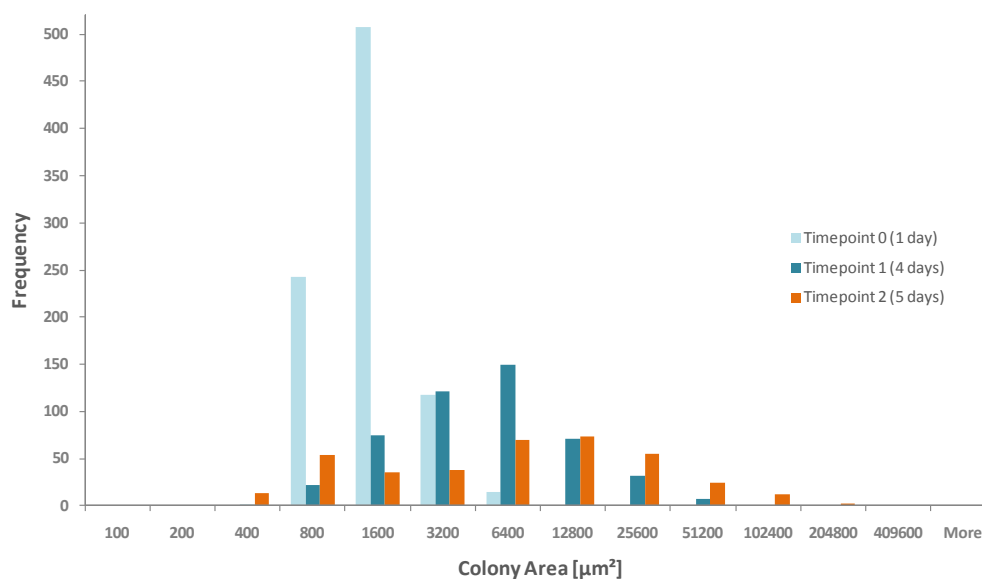
Figure 2–52: Histogram of the colony area distribution at three different time points (day 1, day 4 and day 5). Single Cell results were exported by Harmony (see section 1.5.4) and the histogram was created in Excel®. On day 1, many small cells and some cell clusters were detected. As time proceeds, some cells grow into larger colonies, reflected by an increasing number of larger colonies (orange distribution).

2.25.4 Hints and Tips

➤ Further classify colonies which have a specific feature or a different fluorescence marker, using additional **Calculate Intensity** or **Calculate Texture Properties** building blocks (more details on the intensity independent texture analysis can be found in section 1.7).

➤ Use the **Select Population** building block's "Filter by Property" or the "Linear Classifier" (PhenoLOGIC) methods to filter colonies by their properties, e.g. discard small colonies or classify colonies by the presence of a marker or texture feature.

➤ For a time series data display of dose response experiments on the Harmony Evaluation Screen, consider using "Time" as an x-axis and group the plots by the concentration. You will get a set of time series for each concentration, allowing you to assess the compound dose effect at a glance. Also see the RMS "Migration" (section 2.26).

➤ Analyze selected colonies in depth with higher magnifications using the Pre-scan / Re-scan option (for details see Application Guide, section "Manual PreScan/ReScan option").

➤ Consider using normalized readout values to improve the statistics of your results.

➤ For 3D assay formats (semi-solid matrices), consider using z-stack maximum intensity projection (see section 1.17).

➤ To create a time series measurement from a set of individual measurements at different points over time, use the **Combine Measurements** function (for details, see Harmony Help function and Application Guide, section "Long kinetic measurements").

2.26 Migration

This section covers two different RMSs for analysis of cell migration, depending on the objective used for image acquisition. Both RMSs are compatible with scratch wound assays and other types of migration assays.

2.26.1 Introduction and Assay Principle

Cell migration plays a major role in physiological events such as the immune response and wound healing, but can also contribute to pathological processes, including metastasis. Understanding the molecular components of migration is crucial for discovering new therapeutics that affect the migration of cancer cells. As an example, we used the Oris™ Pro Cell Migration Assay from Platypus Technologies, LLC. The assay uses a 96-well format with a dissolving bio-compatible gel droplet which forms a cell free zone in the center of the wells. Two different datasets were created where migration of HT-1080 fibrosarcoma cells was imaged with a 2x and a 10x objective. The cells were labeled with Cell Tracker™ Green CMFDA and images were acquired in the Alexa 488 channel and in the Brightfield channel. Time series measurements were created from individual measurements using the Combine Measurement function (please see Harmony Help function and Application Guide, section "Long kinetic measurements").
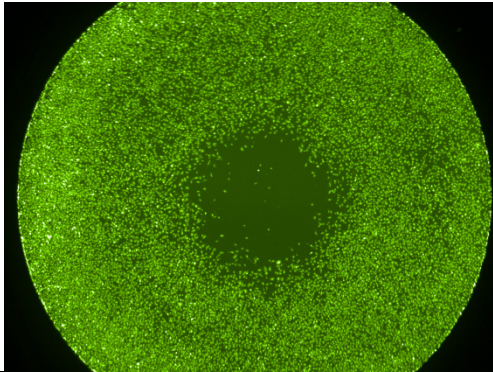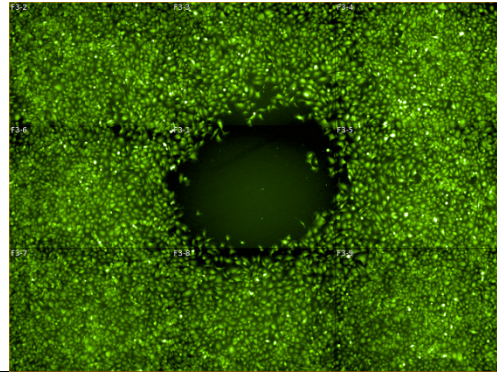
2x objective	10x objective (or higher magnification)
Description: One image represents one entire well and the cell layer contains a hole of varying size and shape.	Description: Individual images represent only a section of the well. The example shows a montage of 9 images.
Channel: Fluorescence	Channel: Brightfield **or** Fluorescence
Use: RMS Migration – Hole in Cell Layer	Use: RMS Migration – Confluency

Table 2-2: Selection of a suitable migration RMS.

2.26.2 RMS Migration – Hole in Cell Layer

The RMS Migration- Hole in Cell Layer is tailored for images of fluorescent cells that were acquired using the 2x objective. The RMS assumes the cell layer to have a hole, which can be of varying size and shape.

The cells in the corresponding dataset ("P019-Migration") were incubated in the presence of various concentrations of Cytochalasin D, inhibiting migration into the cell free zone to different extents.

T0: ~3 h after cell seeding   T1: ~24 h after cell seeding   T2: ~43 h after cell seeding
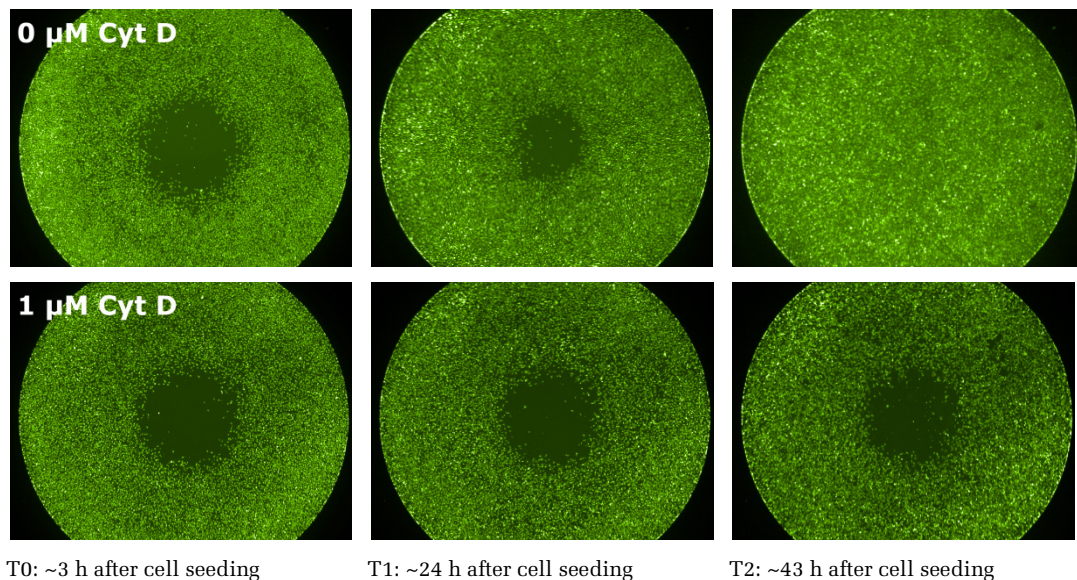
Figure 2–53: Example images of an Oris™ Pro Cell Migration time series acquired with a 2x objective. HT-1080 cells were incubated in the absence or presence of Cytochalasin D (1 µM) and labeled with Cell Tracker™ Green CMFDA. Cytochalasin D shows a strong inhibiting effect on cell migration, preventing closure of the cell free zone during the time course of the experiment. Details of the example dataset ("Plate P019-Migration") can be found in the Appendix on page 311.

2.26.3 How to use the RMS Migration – Hole in Cell Layer

➤ Load the analysis sequence "RMS Migration – Hole in Cell Layer" from the Harmony database.

➤ Select the fluorescent dye channel in the **Find Image Region** building block for recognition of the cell layer.

➤ Adjust the **Select Region** building block parameters to fill small holes in the cell layer (if needed). See section 2.26.4 for details.

Image Analysis Sequence: Migration – Hole in Cell Layer

Input Images

Channel 1:
Cytoplasmic stain
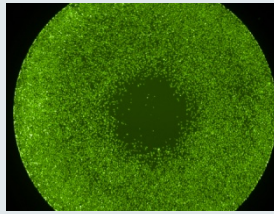
(Cell Tracker™ Green CMFDA)

T0 ~ 3 h after cell seeding
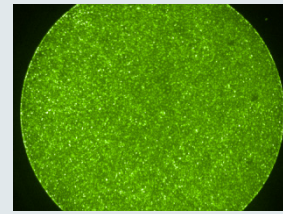
T2 ~ 43 h after cell seeding

Image Segmentation

Find Image Region

Allows you to define the whole image, or parts of the image, as the region.
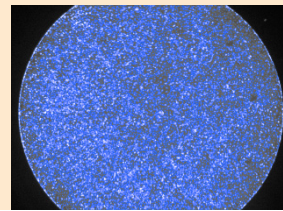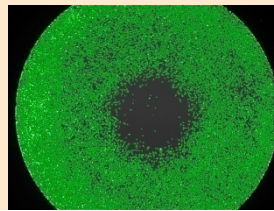
Detected cell layer region overlaid on the cytoplasmic stain image

Define Region of Interest

Select Region (1, 2)

Allows the creation of a region of interest.
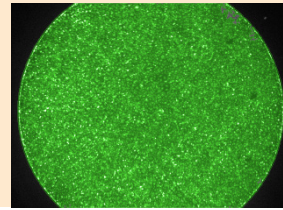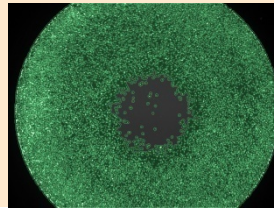
Detected cell layer enlarged by 5 px and small holes filled up

Select Region (3)

Allows the creation of a region of interest.
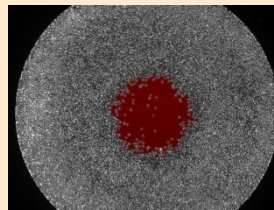
Detected Hole in Cell Layer
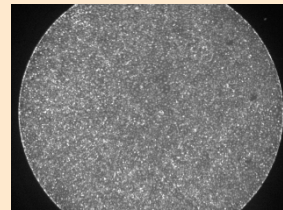
No Hole is detected

Quantify Properties of Regions

Calculate Morphology Properties

Cell Layer Area and Open Area [µm^2]

Object No	Cell Layer Area [µm²]	Open Area [µm²]
1	2,72007E+07	2406550

Calculate Readout Values

Define Results

Calculation of Cell Layer Area and Open Area in mm^2; Calculation of Confluency [%]

Field	Cell Layer Area [mm2]	Open Area [mm2]	Confluency [%]
1	27,2007	2,40655	91,8717

Readout Values

Cell Layer Area	Area covered by the Cell Layer, in mm^2
Open Area	Area not covered by Cells, in mm^2
Confluency	Coverage of the well by cells, given as a %

2.26.4 Image Analysis in Detail

Image segmentation

➤ The **Find Image Region** building block is used to find the region covered with fluorescently labeled cells. The common threshold method allows you to define the image region based on an intensity threshold.

Define Region of Interest

➤ Small dark areas of low staining intensity in the closed cell layer may cause detection errors (a hole in the detected cell layer). Two **Select Region** building blocks are used to correct the detection.

➤ The **Select Region** building block allows modification of the detected image region. The method "Resize Region [µm / px]" is used to enlarge the outer border of the region by 5 px. This closes small gaps between the cells.

Resize Region [µm / px]

Outer Border -5 px   Outer Border 0 px   Outer Border +5 px
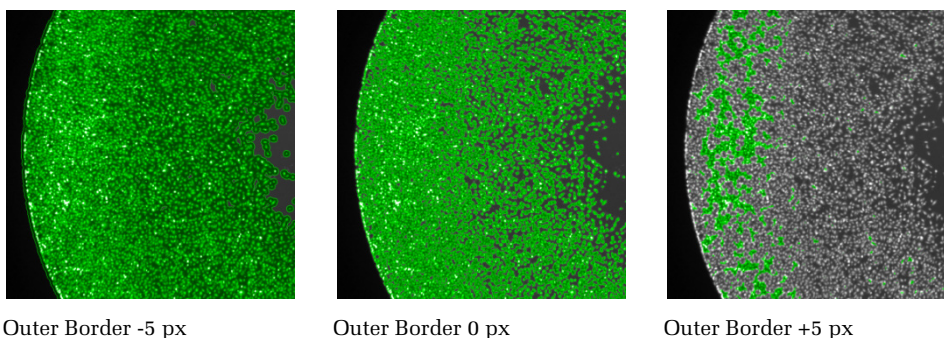
Figure 2–54: Examples of the adjustment of the Outer Border using the Resize Region [µm / px] method. Negative values (left: -5 px) enlarge the image region relative to its original size (middle: 0 px), while positive values (right: +5 px) shrink the image region.

➤ A second **Select Region** building block with the standard method "Fill Holes" can be used to close any remaining small holes in the cell layer image region. Using a threshold prevents filling up larger, correctly detected zones without cells. A suitable threshold is the area of one cell.

➤ The cell free zone can then be detected using a third **Select Region** building block and the standard method "Holes". If a threshold is used, holes of a certain size will be detected. By leaving the threshold on "INF" (infinity), all holes will be detected, independent of their size.

Quantify Properties and Calculate Readout Values

➤ Properties of the previously defined regions of interest can now easily be calculated using the **Calculate Morphology Properties** building block. A separate building block for each region of interest is needed. By default, areas are calculated in μm^2.

➤ To calculate the areas in mm^2, the formula output of the **Define Results** building block can be used. Additionally, the formula output allows the calculation of the well confluency. The confluency is the coverage of well area by cells, given as a percentage.

2.26.5 RMS Migration – Confluency

The RMS Migration – Confluency uses the **Find Texture Regions** building block, which is part of the interactive **PhenoLOGIC** technology. The segmentation of this RMS is based on the texture properties of the objects; therefore it is compatible with brightfield and fluorescence images. It is also compatible with images acquired using different objectives.

The example dataset ("P020 – Migration") shows the migration of HT-1080 cells over 24 h, without compound treatment. Brightfield and Fluorescence images were acquired using a 10x objective.
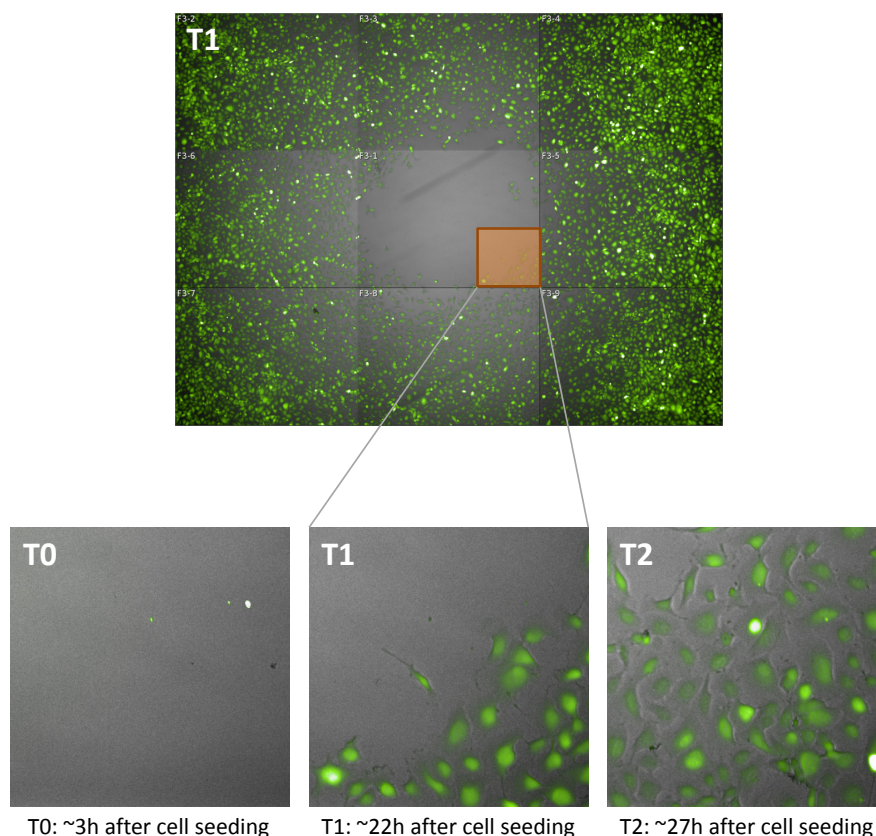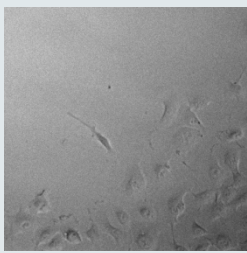
Figure 2–55: Overview of an Oris™ Pro Cell Migration time series, imaged in the brightfield and AlexaFluor®488 channels with a 10x objective. Enlarged images of the highlighted region are shown in the bottom panels. HT-1080 cells were labeled with Cell Tracker™ Green CMFDA and migration was followed up to ~27 h after cell seeding. Details of the example dataset ("P020-Migration") can be found in the Appendix on page 311.

2.26.6 How to use the Migration – Confluency RMS

➤ Load the analysis sequence "RMS Migration – Confluency" from the Harmony database.

➤ Select the Training mode in the **Find Texture Regions** building block and reset the training. Selecting new training points by clicking on the image will allow you to individually adapt your image segmentation. The output populations can be named according to your needs.

➤ Adjust the intensity threshold in the **Select Population** building block. This threshold is intended to exclude regions that do not cover the well, e.g. if the images contain the well edges and the black surrounding of the wells. These regions should be excluded from the image analysis.
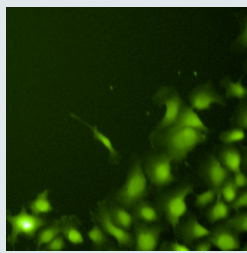
Image Analysis Sequence: Migration – Confluency

Input Images

Channel 1:
Brightfield image

or

Channel 2:
Cytoplasmic Stain
(Cell Tracker™ Green CMFDA)

Brightfield image
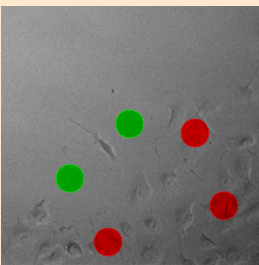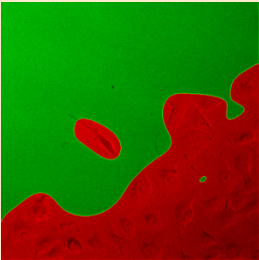
or
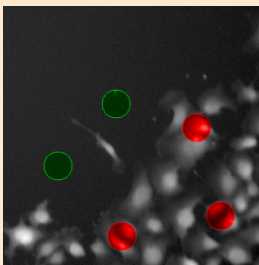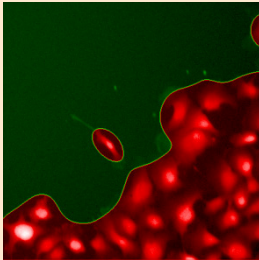
CMFDA

Image Segmentation

Find Texture Regions

Allows you to separate regions of an image with different texture properties

Interactive Training mode
(method: split into classes)

Detected texture regions after training on the brightfield or CMFDA channel

Quantify Properties

Calculate Morphology Properties

Open Area and Cell Area

Calculate Intensity Properties

Open Area Intensity and Cell Area Intensity

Object No	Open Area [µm²]	Open Area Intensity Mean
1	1083620	7993,74
2	214,069	7523

Object No	Cell Layer Area [µm²]	Cell Layer Intensity Mean
1	94523	7596,64
2	1839,81	7784,03
3	4390,89	7594,07
4	274,246	7423,22

Calculate Readout Values

Define Results

Sum of Cell Layer Area and Open Area, in mm²; Calculation of Confluency [%]

Field	Open Area [mm2]	Cell Area [mm2]	Confluency [%]
1	1,08383	0,2853	20,838

Readout Values

Cell Layer Area	Area covered by the Cell Layer, in mm²
Open Area	Area not covered with Cells, in mm²
Confluency	Coverage of the well by cells, given as a %

2.26.7 Image Analysis in Detail

Image Segmentation

➤ Image Segmentation is performed using the interactive **PhenoLOGIC** building block **Find Texture Regions**. In this building block you are able to train the image segmentation by clicking onto the image and defining examples of a single, or a few, texture classes. The **Find Texture Regions** building block then divides each image into regions, according to the user-defined classes. In this example dataset we have divided the open area and the cell layer. If the regions are not connected, e.g. single cells or groups of cells move into the cell free zone, they will appear as individual objects in the properties table. For further information on the training methods please refer to the "Harmony Help function".

Quantification of Properties

➤ The area and intensity of the previously defined regions can now be calculated with the **Calculate Morphology Properties** and **Calculate Intensity Properties** building blocks.

➤ The Intensity is calculated so as to exclude image regions from outside wells from the image analysis. These regions have a very low intensity and can therefore be excluded by introducing a threshold in the **Select Population** building block. Individual **Select Population** building blocks must be used to exclude regions that were wrongly assigned to the Open Area region and the Cell Layer region.

Calculate Readout Values

➤ To calculate the final readouts it is important to use the sum of the region areas, because the regions can be split up into individual objects if they are not connected in the image. By default, areas are calculated in μm^2. The formula output of the **Define Results** building block allows the conversion of μm^2 into mm^2. Additionally, it is possible to calculate the percentage of covered area (Confluency).

2.26.8 Hints and Tips

➤ If the image segmentation of the **Find Texture Regions** building block is not appropriate, it is possible to re-train on the same image, or train on more than one image of the dataset.

➤ Training points that seem to be inappropriate can be removed by repeating the same click.

➤ A migration assay imaged with a 2x objective can be analyzed very quickly, because one well is represented by a single image. If a similar analysis speed is needed for images acquired with 10x magnification (or higher), it is possible to analyze only the center well of the Oris™ Pro Cell Migration Assay plate.

➤ Brightfield image analysis can be used to measure the open area in each individual well before the migration is started, **without staining the cells**. This value can be used as an exact reference value to assess cell growth after the migration phase.

➤ The RMS Migration – Confluency is also applicable for fluorescence images acquired with the 2x objective.

2.27 Micronucleus Analysis

This RMS quantifies the fraction of binucleated cells containing micronuclei in a cell population.

2.27.1 Introduction and Assay Principle

Genotoxic side effects of compounds induce the formation of micronuclei (MN) in cells. *In vitro* micronucleus assays have therefore been established as important tools for assessing the mutagenic potential of drug candidates.

A micronucleus is a small DNA fragment in the cytoplasm of interphase cells – an erratic, small, extra nucleus. Micronuclei contain fragments, or whole chromosomes that were not incorporated into the daughter cell nuclei during mitosis.

The example dataset for this RMS includes images from mammalian cells treated with different concentrations of a genotoxic compound. Cytochalasin B was also added after compound incubation in order to 'trap' cells in their bi- (multi-) nucleated state after one division, by blocking mitosis (cytokinesis-block method). The quantification of binucleated cells containing one or more MN is then used as a direct measure of the genome damage caused by the compound.
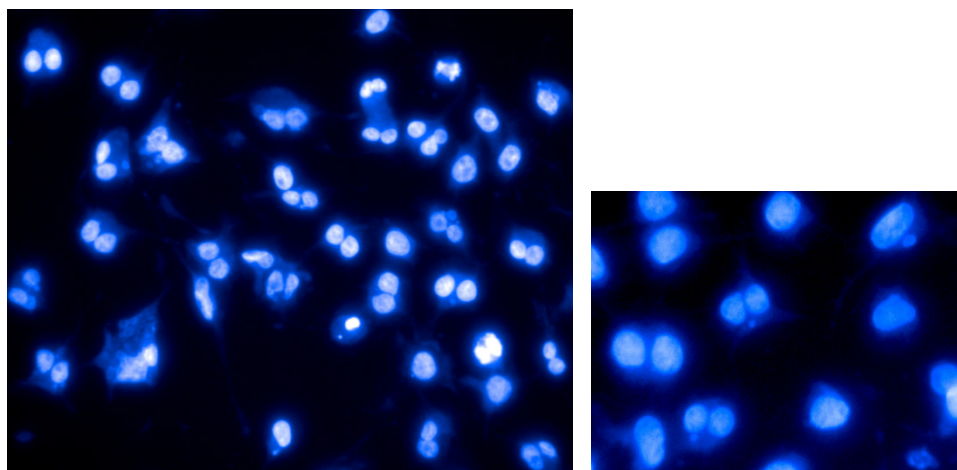
Figure 2–56: DAPI-stained nuclei imaged with a 20x objective after exposure to a genotoxic compound (full field of view on the left figure, detail in the right figure). The image displays a multitude of morphologically distinct nuclear features caused by different cell cycle states, genotoxic effects, and cytotoxic effects such as apoptosis or necrosis. Applying the cytokinesis-block method resulted in the appearance of many binucleated cells. See the example dataset "P016-Micronucleus", details of which can be found in the Appendix on page 319.

2.27.2 How to use the Micronucleus Analysis RMS

➤ Load the analysis sequence "RMS Micronucleus Analysis" from the Harmony database.

➤ Select the nuclear dye channel in the **Find Nuclei, Find Cytoplasm and Find Micronuclei** building blocks.

➤ The criteria to select valid cells and valid micronuclei are dependent on your specific assay. Adjust the **Select Population** and **Select Population (2)** building blocks as needed.

Image Analysis Sequence: Micronucleus Analysis

Input Image

Channel 1: Nuclear stain (DAPI)

Image Segmentation – 3 Populations are Created

Find Nuclei

Finds nuclear outlines

Find Cytoplasm

Finds cytoplasmic outlines around nuclei using the cell´s autofluorescence

Find Micronuclei

Finds all potential micronuclei candidates

For reliably separating touching or nearby nuclei, algorithm A is used ("**Nuclei**")

Cytoplasmic outlines found after applying algorithm E, which allows cells to contain more than one nucleus ("**All Cells**")

Micronuclei candidates found using the whole cell as the search region ("**Micronuclei Candidates**")

Quantify Specific Properties of Populations

Calculate Morphology and Intensity Properties

For the population "Nuclei", the area, roundness and intensity properties are calculated. For the population "Micronuclei Candidates", the roundness is calculated

Calculate Properties by Related Population

Allocation of the sub-objects ("Nuclei" and "Micronuclei") to their parent-objects ("All Cells") in order to calculate the number of micronuclei and the minimum nuclear roundness per individual cell

Identify Subpopulations on Basis of Properties

Select Population

Five successive building blocks allow the filtering of, e.g.:
- Valid micronuclei from the population "Micronuclei Candidates"
- Valid cells from the population "All Cells"
- Binucleated cells from the population "Valid Cells"

Valid cells (green) possess the minimum nucleus roundness; otherwise they are discarded (red)

Valid micronuclei (green) meet the required criteria; discarded ones are shown in red

Binuclear cells (green) are identified by the number of nuclei per cell

Calculate Readout Values

Define Results

Select the readout values to be averaged per well. Calculate statistical values by including Formula Outputs

Readout Values

Number of Cells	All cells found by the **Find Cytoplasm** building block (including "invalid" cells)
Number and Fraction of Valid Cells	The number and fraction of cells classified "valid". Indicates toxic impact of the compound and depends on the filtering criterion
Number and Fraction of Binucleated Cells	By adding Cytochalasin B the cell division is interrupted in a bi-/ multinucleated state. Cytotoxic or cell cycle arresting effects of the compound reduce the number of cells reaching the bi-nucleated state
Number of Micronuclei Candidates	All potential MN candidates found by the **Find Micronuclei** building block
Number of Micronuclei	Valid MN after filtering according to user defined properties

Number and Fraction of Valid Cells with Micronuclei	All valid cells (mono-, bi- and multinucleated) containing at least one MN
Number and Fraction of Binucleated Cells with Micronuclei	Corresponds to the number of cells which divided once and formed at least one MN in the process. Reflects the genotoxic impact of the compound

2.27.3 Image Analysis in Detail

The image analysis of micronuclei formation is generally quite challenging as i) it is a rare event, and ii) cells appear in many different morphologies after genotoxic / cytotoxic treatments (see *Figure 2–56*). The visibility of the genotoxic effects of interest is strongly overshadowed by other cytotoxic effects, e.g. leading to apoptotic and necrotic nuclei. Therefore, the RMS Micronucleus Analysis involves two key steps: the identification of regularly shaped binuclear cells, and the detection of valid micronuclei (MN). Commonly recognized criteria are:

➤ MN should be located in the cytoplasmic cellular area.

➤ MN should have a similar intensity to the nuclei.

➤ The MN diameter should be less than 33 % of the diameter of the main nuclei.

➤ For binuclear cell identification: both nuclei are of a similar size, orientation and intensity, and are detached or else only marginally overlapping.

➤ Irregular nuclei (apoptotic, necrotic) are excluded from the evaluation.

Image Segmentation

The segmentation of the image is based on one nuclear stain, here DAPI, and three segmentation building blocks: **Find Nuclei**, **Find Cytoplasm** and **Find Micronuclei**. All three initially generate separate populations which are then filtered and related to each other at a later stage.

The **Find Nuclei** building block is used to detect the outlines of all individual nuclei. In an assay system with many bi- or multinucleated cells, it is important to separate touching or nearby nuclei at this point. Algorithm A offers a good solution for this task.

The **Find Cells** building block is used to detect the cytoplasmic outlines of all cells, based on the autofluorescence of the cytoplasm. It is mandatory to select Method E for the segmentation since it is the only method which allows multiple nuclei inside cells.

The third building block, **Find Micronuclei**, aims to completely capture all potential micronuclei candidates (valid and invalid ones). A set of eight properties for each micronucleus is calculated by default for later filtering, characterizing the intensity, size, distance from the nucleus, and shape of the micronuclei.

Quantifying Morphology and Intensity Properties

In order to choose suitable properties and limits for selecting valid cells and valid micronuclei, a set of properties is calculated by different **Calculate Morphology** and **Intensity Properties** building blocks. This is in addition to the default properties e.g. the area and roundness of the nuclei and micronuclei, and the mean and coefficient of variance of the nuclear intensity.

Quantifying Properties by Related Populations

In order to calculate cell properties which refer to properties of sub-objects (nuclei, micronuclei), the **Calculate Properties** building block method "**By Related Population**" can be used. Here it is used to calculate the minimum roundness of all nuclei in a cell. This is a good indicator for filtering out invalid cells: in a valid cell, ALL nuclei must be sufficiently round enough. The "By Related Population" method is also used in a second **Calculate Properties** building block to determine the number of valid micronuclei in each cell.

Select Population – Filter by Property – Micronuclei

For the selection of valid micronuclei from the population of potential micronuclei candidates, all previously calculated properties can be used. In our example dataset the criteria "Compactness > 0.8" and "Fraction of Nucleus Area > 0.2" gives good results.

Troubleshooting hints:

➤ If a valid micronucleus remains undetected, check the "Micronuclei candidates" population. If it does not appear in this population, tune the parameters in the **Find Micronucleus** building block. Otherwise, open the **Select Population** building block that filters the micronuclei candidates and adjust, add or remove filter criteria.

➤ If an invalid micronucleus is detected in an invalid cell, it will be filtered out later and will not compromise the result so can be ignored.

➤ If an invalid micronucleus is detected in a valid cell: Try to identify a unique property of the invalid micronucleus (e.g. it is not round, it is very dim, it is far away from the nucleus, etc.) and adapt the filtering criteria in the **Select Population** building block to filter it out.

➤ If that does not help: Use stricter sensitivity settings in the **Find Micronuclei** building block to avoid getting too many invalid micronuclei candidates.

Select Population – Filter by Property – Valid Cells

The **Select Population (2)** building block selects the valid cells and discards apoptotic, necrotic and mitotic cells or any other cells with deformed or irregular nuclei. This task is accomplished by filtering cells according to the minimum roundness of their nuclei.

Classification of Cells

Three **Select Population** building blocks are used to successively create the following classes (sub-populations):

➤ Valid binuclear cells

➤ Valid cells containing at least one micronucleus

➤ Valid binuclear cells containing at least one micronucleus

Please note that the detection of binuclear cells is very conservative, resulting in a very low number of false positive cells, but a significant number of false negative cells in the example dataset. To increase the number of valid binuclear cells, assay conditions resulting in a lower number of partly overlapping nuclei could be used.

Calculate Readout Values

In the **Define Results** building block a set of statistical readout values is selected to be reported back as the final results per well, allowing quality control, judgment of general cytotoxic effects and the genotoxic impact. The numerical outputs are accompanied by graphical illustrations, which may be useful for monitoring the results and setting up the filtering parameters. See *Figure 2–57* for an example result.
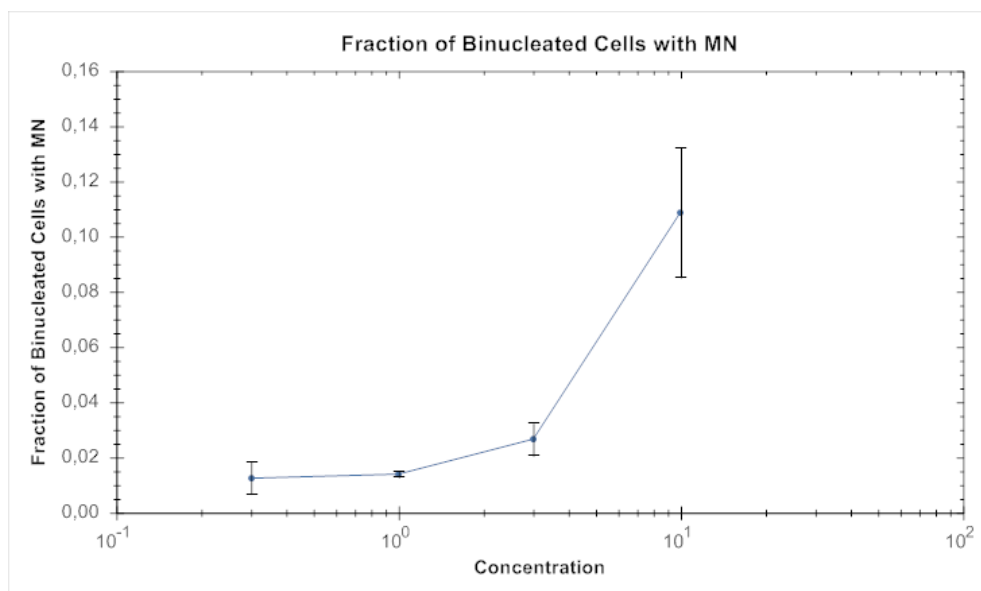
Figure 2–57: Screenshot from the Harmony Evaluation screen showing an example of the analysis results for the example dataset "P016-Micronucleus" (Appendix page 319) analyzed with the RMS "Micronucleus Analysis". The dose dependent increase in the fraction of bi-nucleated cells with micronuclei reflects the genotoxic impact of the compound. The example dataset includes 2 wells per dose and 9 fields with 1600-3200 cells per well.

2.27.4 Hints and Tips

➤ For an in-depth description of many aspects of micronuclei testing, see also:

- **OECD guideline** for the testing of chemicals, draft proposal for a new guideline 487: In Vitro Mammalian Cell Micronucleus Test (MNvit). OECD, 2 November 2009 (http://www.oecd.org/dataoecd/45/51/43996258.pdf)

- **Fenech, M.** (2007) "Cytokinesis-block micronucleus cytome assay". Nature Protocols, 2(5), pp 1084-1104

➤ For a valid statistical analysis, the micronuclei frequencies should be analyzed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per concentration, tests performed in duplicates), according to the OECD guideline. The number of subfields per well can be adjusted accordingly to meet this standard.

➤ Use the scatter plot tabs provided by the **Select Population** building blocks as a graphical aid to adjust the filtering conditions for valid cells and valid micronuclei.

➤ Customized readout values such as the Cytokinesis-Block Proliferation Index, the Nuclear Division Index or the Replicative Index can easily be added by adding more **Select Population** building blocks and adding formula outputs in the **Define Results** building block.

➤ The micronucleus assay is designed to be multiplexed with other indicators, e.g. for apoptosis or cytotoxicity (see for example the RMS "Nuclear Fragmentation", section 2.13 or Application Guide, section 3.2 "Ready-Made Solution: Cytotoxictiy-1".

2.28 Texture Based Segmentation – Stem Cell Colonies

This RMS allows the easy segmentation of an image into regions with different cellular textures, using PhenoLOGIC machine learning. Training is done through the interactive selection of examples for each texture class. See section 1.14 for details on using PhenoLOGIC for image segmentation.

2.28.1 Introduction and Assay Principle

Embryonic stem cells (ESCs) require specific culture conditions for growth and maintenance. To establish and maintain ESCs with a normal karyotype and the ability to fully differentiate into functional cells, researchers generally start by plating cells onto a feeder layer, e.g. mouse embryonic fibroblasts (MEFs). For the analysis of images which include both ESCs and MEFs, a crucial step is to identify both cell types and then separate them from each other to allow further individual analysis of the ESCs. In the example shown here, mouse ESCs (DBA) were seeded on MEFs and fixed after an incubation period of 36 h, and 3 d. Cells were subsequently stained with Hoechst 33342, the stem cell marker Oct4-AlexaFluor®488, and Cytokeratin-AlexaFluor®647. Images were acquired using a 10x objective (***Figure 2–58***).

Colonies after 36 h incubation   Colonies after 3 d incubation
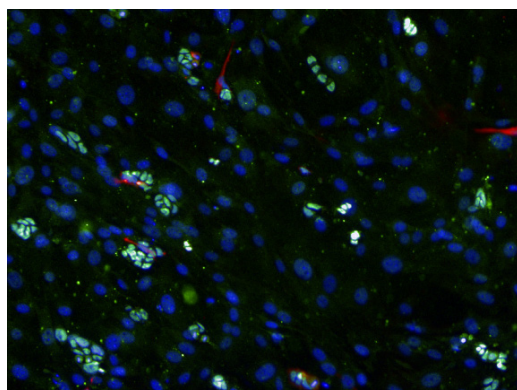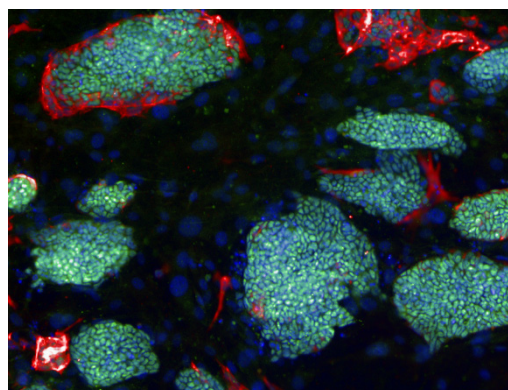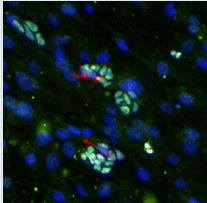
Figure 2–58: The images show mouse embryonic stem cell colonies (DBA) on feeder cells (MEF), fixed after an incubation time of 36 h (left) and 3 d (right). Cells are stained with Hoechst 33342 (blue), Anti Oct4 – AlexaFluor®488 (green) and Anti Cytokeratin-AlexaFluor®647 (red). The images can be found in the example dataset "P017-ESC colonies", see the Appendix page 320.

2.28.2 How to use the Texture based segmentation RMS
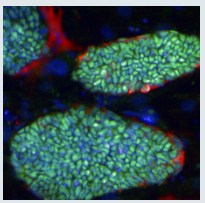
➤ Load the analysis sequence "RMS ESC Colony Analysis" from the Harmony database.

➤ Select the dye channel that you want to use for the colony segmentation in the **Find Texture Regions** building block and click on "Train…" to start the training.

➤ Use "Reset Training" to delete the training of the example dataset and train on a new dataset. Select class A and click on areas in the image representing stem cell colonies, select class B and click on areas in the image representing the feeder layer. Appropriate areas for class A and B may be located on different images.

➤ Adjust the area and distance parameters in the **Modify Population** building block in order to correctly split touching colonies and remove small artifacts.

Image Analysis Sequence: ESC Colony Analysis

Input Image

Channel 1:
Nuclear stained image (blue)

Channel 2:
Oct-4 stained image (green)

Channel3:
Cytokeratin stained image (red)

36 h after cell seeding

3 d after cell seeding

Image Segmentation

Find Texture Regions

Allows you to separate regions of an image with different texture properties.

Use "Train…" mode to select example regions of two texture classes, A and B

Training results:

Detected texture regions on the "36 h" image

Click on different texture regions to set training points for class A (stem cell colonies, green) and class B (feeder layer, red)

Detected texture regions on the "3 d" image

Modification of detected regions

Modify Population

Split detected region into objects, adjust the minimum area and splitting distance for touching regions.

Valid colonies of the "36 h" image

Valid colonies of the "3 d" image

Quantify properties in regions

Calculate Morphology and Intensity Properties

Area and roundness, and marker intensities for Oct4 and Cytokeratin in each colony.

Object No	Colony Area [µm²]	Colony Roundness
1	1855.6	0.92498
2	1407.73	0.906095
3	1485.66	0.951396
4	1075.28	0.956209

Oct-4 Intensity Mean	Oct-4 Intensity Sum	Cytokeratin Intensity Mean	Cytokeratin Intensity Sum
1371.94	2580628	1267.62	2384391
1392.07	1986478	7824.08	1.116496E+07
1377.08	2073876	470.515	708595
1324.03	1443197	587.071	639907

Object No	Colony Area [µm²]	Colony Roundness
1	7477.63	0.925182
2	42439	0.782413
3	37256	0.823627
4	6759.49	0.951005

Oct-4 Intensity Mean	Oct-4 Intensity Sum	Cytokeratin Intensity Mean	Cytokeratin Intensity Sum
1985.91	1.505323E+07	728.873	5524854
2273.48	9.780506E+07	1781.19	7.662677E+07
2729.5	1.030823E+08	1291.93	4.879096E+07
1670.79	1.14465E+07	2626.22	2.491247E+07

Readout Values

Number of Colonies	Number of all analyzed valid colonies in the well
Mean Colony Area	Area of the whole colony in µm²; averaged over all colonies in the well
Mean Oct4 Intensity per colony	Intensity of the Oct4 stem cell marker stain; first averaged over all pixels within each colony and then averaged over all colonies in the well
Mean Cytokeratin Intensity per colony	Intensity of the Cytokeratin marker stain; first averaged over all pixels within each colony and then averaged over all colonies in the well

2.28.3 Image Analysis in Detail

Image Segmentation

➤ Texture based image segmentation is performed by **PhenoLOGIC** machine learning using the **Find Texture Regions** building block. This building block can be trained through interactive selection of different texture classes by clicking into the image. The **Find Texture Regions** building block then divides each image into regions, according to the defined classes. In the example dataset shown here, images are separated into two texture regions: feeder layer and stem cell colonies.

➤ The splitting of the detected regions into objects can be done or modified using the **Modify Population** building block. For this example dataset the detected region "raw stem cell colonies" is split into individual colonies based on a distance threshold. Furthermore, a threshold for the colony area can be set to discard fragments and colonies of insufficient size.

Quantification of Properties

➤ The area of the identified objects (e.g. stem cell colonies) can now be calculated using the **Calculate Morphology Properties** building block. The fluorescence intensity of markers, here stem cell marker Oct-4 and Cytokeratin, can also be calculated for each single colony using **Calculate Intensity Properties** building blocks. Additional measurements can be added here as needed (more channels, further intensity or morphological properties, etc.).

Calculate Readout Values

➤ In the **Define Results** building block the properties of all colonies in a well are averaged to generate the results per well. For the example dataset shown here, the readouts are the number of colonies, the mean colony area and the mean fluorescence intensity per colony of both markers (Oct-4 and Cytokeratin).

➤ All the calculated properties of every individual colony can be saved in the database by setting the single cell result options in the **Define Results** building block (see section 1.5.4). Selecting the option "ALL" saves all object properties which are calculated by any of the building blocks. To access the results, use the export format "Evaluation Results per Well and Object" to create a text file which can be further processed using, for example, Excel®.

2.28.4 Hints and Tips
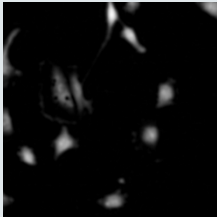
➤ When training in the **Find Texture Regions** building block, be sure to choose regions with a specific texture property for each class. Choosing training points with mixtures of several texture classes increases the probability of generating artifacts.

➤ Segmentation of the image into more than 2 different texture regions is possible. In the **Find Texture Regions** building block, up to 6 different texture classes can be added by changing the "number of classes" parameter in the method "Split into Classes". In addition, with the method "Find a Class" it is possible to detect texture regions of a single texture class only.

➤ To quantify additional markers recorded in other channels, add additional **Calculate Properties** building blocks that work on different channels.

➤ You can add further building blocks to analyze the identified colonies, e.g.:

- Select a ring region in the colonies to quantify a marker intensity in different locations of the colony, e.g. next to the border and/or in the middle. See section 1.3.2.

- Find nuclei, spots, bright regions, etc, in a colony by using the **Find Spots** building block.

- Quantify texture properties of the colony by using the **Calculate Texture Properties** building block to classify colonies according to their structure. This is especially useful in combination with the PhenoLOGIC classification (see section 1.15).

- Use the **Calculate Properties** building block's method "By related Population" to calculate statistical properties of the colonies based on objects found and analyzed inside the colonies, e.g. nuclei. See section 1.10.

➤ Texture based Segmentation also works on brightfield images.

2.29 Cell Tracking – Migration Analysis

This RMS allows users to track cells measured in digital phase contrast over several time points and to calculate parameters such as the speed of cells and the distance covered.

2.29.1 Introduction and Assay Principle

Cell tracking allows monitoring temporal changes in biological systems as it establishes the connection of cells between time points. Assays that benefit from cell tracking are, for example, chemotaxis and chemokinesis assays. While cells migrate towards a chemoattractant in chemotaxis assays and therefore show a directional movement, chemokinesis assays study compounds that stimulate or inhibit random movement. Chemokinesis is, for example, involved in immune cell migration and metastasis.

For the RMS Cell Tracking – Migration Analysis, we performed a chemokinesis assay (example dataset "P021-Cell Tracking"), in which we monitored the effect of Cytochalasin D on migration of HT-1080 fibrosarcoma cells for 2.5 h, using the 10x objective. The cells were unlabeled and imaged in the digital phase contrast (DPC) channel. The DPC image was used to segment the image and then the segmented cells were tracked. As DPC imaging uses red LEDs, phototoxicity is reduced and the culture is kept as undisturbed as possible. The main readout parameters are the kinetic properties of the cells, but other readouts like morphology, intensity or texture can be included if needed.

Untreated   Cytochalasin D (500 nM)

Figure 2–59: Chemokinesis assay performed with digital phase contrast images using single cell tracking. HT-1080 cells were seeded and imaged in the DPC channel, using the 10x objective. Cells were identified using the building block **Find Cells** and migration was monitored for 2.5h using the building block **Track Objects**. Without treatment (left) cells are able to migrate, while in the treated condition they hardly move (right). Details of the example dataset ("P021-Cell Tracking") can be found in the Appendix on page 322.

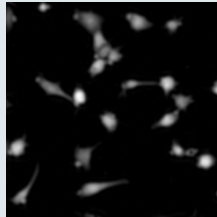## 2.29.2 How to use the Cell Tracking – Migration Analysis RMS

➤ Load the analysis sequence "Cell Tracking – Migration Analysis" from the Harmony database.

➤ Select the DPC channel or another channel with a whole cell stain and a suitable Method in the **Find Cells** building block (Method P for DPC images).

Image Analysis Sequence:	Cell Tracking – Migration Analysis	
Input Image Channel 1: Digital Phase Contrast (DPC)	 Untreated	 Cytochalasin D treated

Image Segmentation

Find Cells Finds cellular outlines (Method P for DPC images)		

Track Objects

Use as time window Selects a subset of time points as time window		Images from these time points will be loaded for tracking
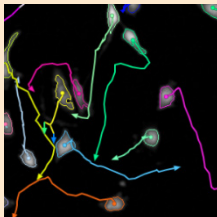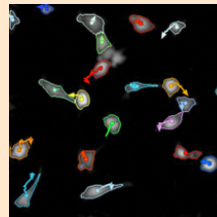
Track Objects Uses the overlap of cells between adjacent time points for tracking	Cell masks overlayed with object tracks	
	 Cell migration	 Cell migration inhibited

Quantify Properties

Calculate Kinetic Properties Calculates Current Speed and Current Step Size		

Object No	Age [s]	Current Displacement X [µm]	Current Displacement Y [µm]	Current Step Size [µm]	Current Speed [µm/s]
1	0	0	0	15,1609	0,0418721
2	0	0	0	2,22092	0,00613383
3	0	0	0	3,14085	0,00867454

Calculate Properties Calculates the Current Square Displacement	Uses a formula to calculate a derived property on a per object basis: Current Square Displacement = (Current Displacement X [µm])^2 + (Current Displacement Y [µm])^2	

Quantify Track Properties

Calculate Track Properties

Calculate accumulated distance, displacement and speed

Calculate Readout Values

Define Results

Select meaningful names for your readouts and save single cell data

Readout Values calculated per time point

Number of Tracked Cells	Number of cells per well
Age [s]	Passed time since the cell was first observed in seconds; averaged over all cells per well
Current Speed [µm/s]	Distance (in µm) a cell traveled per second; averaged over all cells per well
Current Step [µm/s]	Distance (in µm) a cell traveled between two time points; averaged over all cells per well
Mean Square Displacement	Squared norm of a vector from the point of the first to the point of the current observation; averaged over all cells per well
Current Displacement X [µm]	X component of a vector from the point of the first to the point of the current observation; averaged over all cells per well
Current Displacement Y [µm]	Y component of a vector from the point of the first to the point of the current observation; averaged over all cells per well

Readout Values calculated per track

Number of Tracks	Number of detected cell tracks per well
Number of Timepoints per Track	Number of time points that a cell track was observed; averaged over all tracks per well
Accumulated Distance [µm] per Track	Total length of all segments of an object track in µm; averaged over all tracks per well
Displacement [µm] per Track	Distance from the first to last observation of a cell track in µm; averaged over all tracks per well
Average Speed [µm/s] per Track	Accumulated distance in µm divided by the track duration in seconds; calculated per track and then averaged over all tracks per well

2.29.3 Image Analysis in Detail

Image Segmentation

➤ Image segmentation is performed using the building block **Find Cells** – Method P. Method P is dedicated to the segmentation of digital phase contrast images. Parameters have been adjusted to aim for robust object segmentation rather than precise detection of cell outlines.

Track Objects

➤ Object tracking is performed by the building block **Track Objects**. To evaluate the outcome of object tracking on the image analysis screen, a time window has to be selected. Please refer to chapter 1.16 for more information on object tracking.

Quantification of Properties

➤ The building block **Calculate Kinetic Properties** calculates properties based on the cell's position on the previous and next time point (see also *Figure 1–126*). In the RMS, Current Speed and Current Step Size are calculated.

➤ Building block **Calculate Track Properties** calculates more measures for cell migration based on entire cell tracks. In the example RMS Accumulated Distance, Speed and Displacement are calculated. Please refer to chapter 1.16 and *Figure 1–127* for more information on these properties.

Calculate Readout Values

➤ Some of the available output properties have not been explicitly calculated by any **Calculate Properties** building block. Instead, properties like *Age [s]* and *Generation* have been calculated automatically by the building block **Track Objects**. See chapter 1.16 for further information on these properties.

➤ When object tracking is performed usually single cell time-resolved data is desired. Consequently, single cell data for all defined output properties will be stored in the Harmony software's database. Storage of singe cell data is activated by setting the parameter **Single Cell Results** to "Selected".

2.29.4 Hints and Tips

Example Results

➤ Cell segmentation is a crucial step for object tracking. As a general rule, always aim for robust segmentation when adjusting the parameters for image segmentation, since segmentation errors are the most frequent cause for lost cell tracks.

➤ When properties for different regions are being calculated, define these regions after the building block **Track Objects**. All regions that were identified beforehand will be lost after object tracking, except for the one that was used to measure the object overlap, i.e. when the cytoplasmic-to-nuclear ratio is being calculated, add the building block **Find Cytoplasm** after the building

blocks **Find Nuclei** and **Track Objects**. For further information please refer to section 1.16.3.

➤ Plotting the mean square displacement versus the time gives an indication of what kind of cellular movement is being observed. A linear correlation indicates random movement (see *Figure 2–60*, A), whereas a stronger than linear increase in the mean square displacement indicates a directional movement. For a reference see Beltman *et al.*, 2009.

➤ This RMS is provided together with an example evaluation that contains single cell data. This evaluation can be used to learn how to visualize single cell data in the Harmony software. Examples of how this data can be used to visualize cell migration are shown in *Figure 2–60*, B and C.
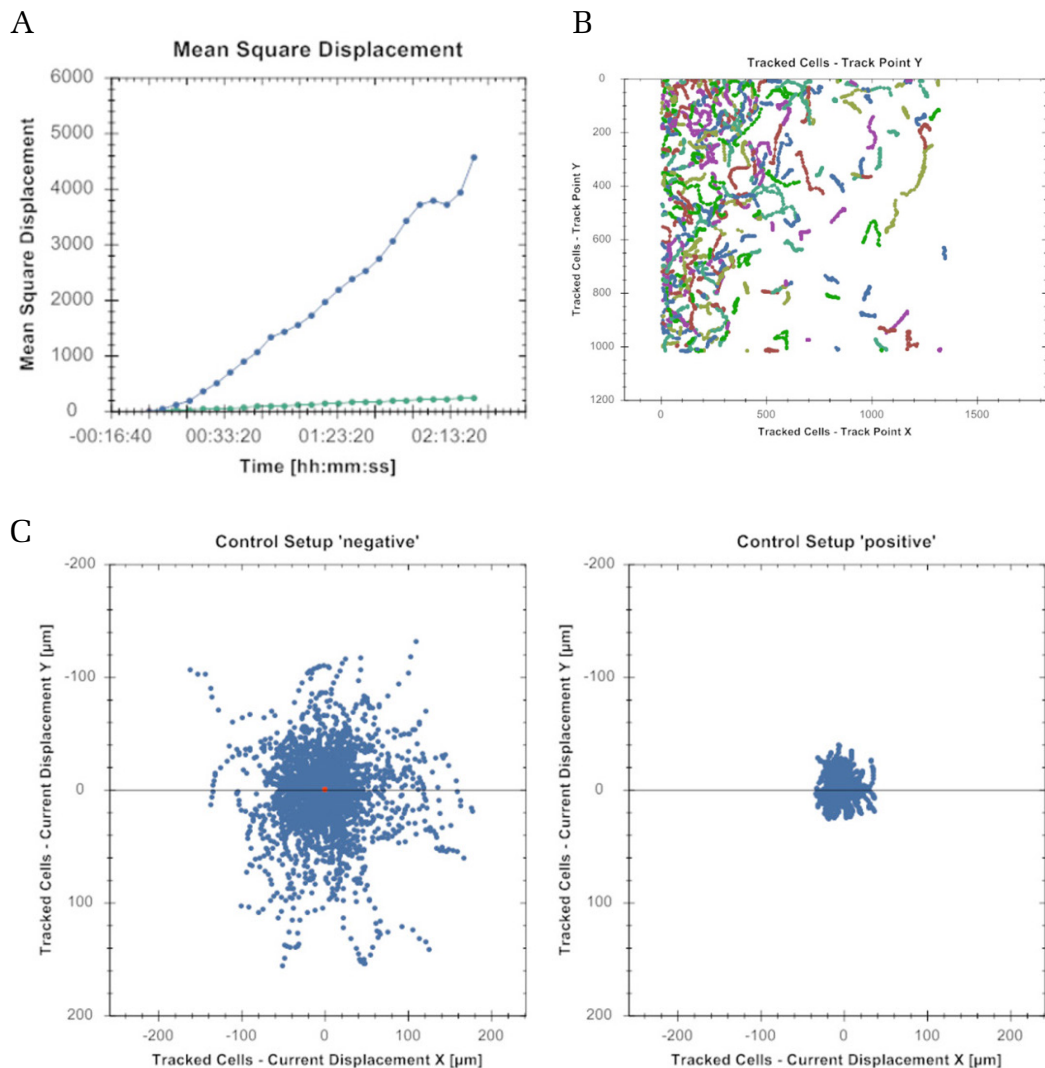
Figure 2–60: Visualization of cell tracking results. **A)** Using well level data, the mean square displacement was plotted versus observation time. The graph was grouped by *Control Setup*. Although both treatments exhibit a linear correlation between mean square displacement and observation time, the steepness of the curve is much higher for the untreated cells (blue curve) than for the Cytochalasin D treated cells. **B)** Single cell data from one field of view was loaded to visualize cell trajectories. Track Point Y was plotted against Track Point X and the grouping was set to *Object*. Note that this visualization will fail if there is data from more than one field of view loaded. **C)** Visualization of cell displacement. Current Displacement Y was plotted against Current Displacement X and grouping was set to *Control Setup* using *Multiple Graphs* for display. Each point corresponds to the displacement of a cell at a given time point. In the untreated condition (left) cells migrate further away from their initial start position than in the treated condition (right). This plot type can also be used to visualize chemotaxis.

2.29.5 References

➤ **Beltman, J. B., Marée, A. F. M., & de Boer, R. J.** (2009). Analysing immune cell migration. *Nature reviews. Immunology, 9*(11), 789–98. doi:10.1038/nri2638

2.30   Cell Tracking – Cell Division

This RMS allows tracking of cells measured in digital phase contrast over several rounds of cell cycle. It is focused on the calculation of genealogical information and the generation of cellular family trees.

2.30.1   Introduction and Assay Principle

Cell tracking allows the monitoring of temporal changes in biological systems as it establishes the connection of cells between time points. Tracking cells across cell division events enables the generation of a genealogical tree or analysis of the different phases of the cell cycle.

As an example for the RMS Cell Tracking – Cell Division, we used HeLa cells that were either left untreated or were treated with 330 ng/ml Aphidicolin (APC) (example dataset "P022-Cell Tracking"). Aphidicolin is an inhibitor of DNA polymerases α and δ thereby preventing DNA replication and causing a cell cycle arrest in early S-phase. Cells were imaged over 24 hours using a 10x objective and an imaging interval of 10 minutes. To minimize phototoxicity cells were not labeled fluorescently. Instead, digital phase contrast (DPC) images were acquired.

untreated

330 ng /ml APC

| 0 hrs | 12 hrs | 24 hrs |
| (Time point 0) | (Time point 72) | (Time point 143) |

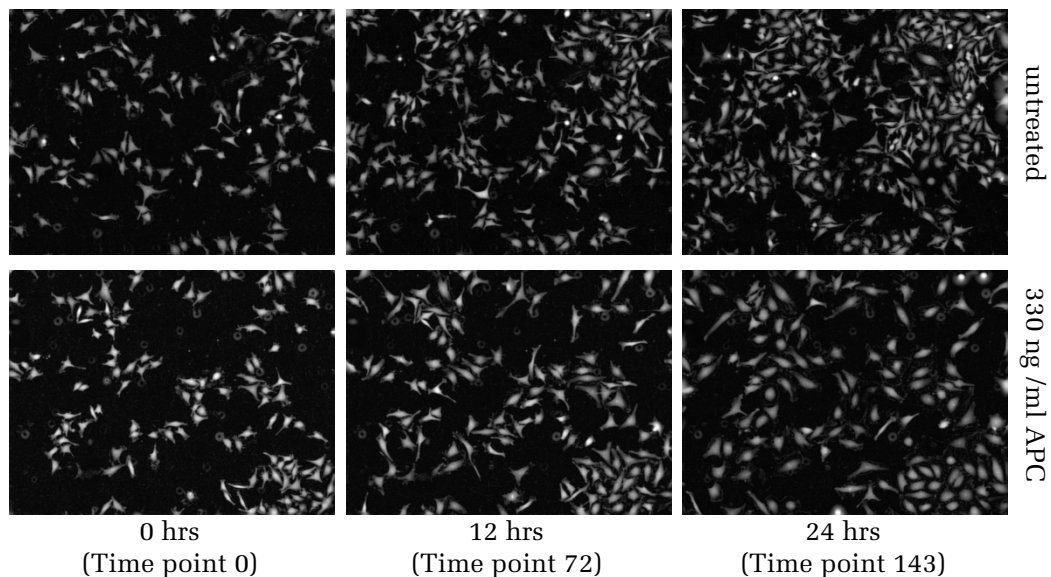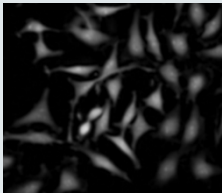***Figure 2–61:*** Following cell division of HeLa cells. Cells were either treated with APC or left untreated and imaged over 24 hours using DPC imaging. Without treatment, cell number strongly increases within 24 hours and cells do not exhibit the increased size that is observed for APC treated cells. Please refer to the Appendix page 322 for details on the example dataset "P022-Cell Tracking".

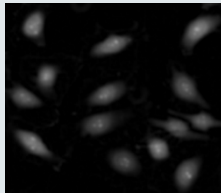## 2.30.2 How to use the RMS Cell Tracking – Cell Division

➤ Load the analysis sequence "Cell Tracking – Cell Division" from the Harmony database.

➤ Select the DPC channel or another channel with a whole cell stain and a suitable Method in the **Find Cells** building block (Method P for DPC images).

Image Analysis Sequence:	Cell Tracking – Migration Analysis	

Input Image

Channel 1:
Digital Phase Contrast
(DPC)

Untreated

Aphidicolin treated
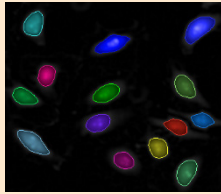
Image Segmentation

Find Cells

Finds cellular outlines
(Method P for DPC images)

Object Tracking

Use as time window
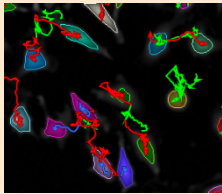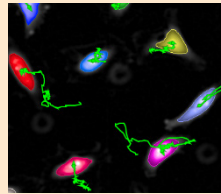
Selects a subset of time points as time window

Images from these time points will be loaded for tracking
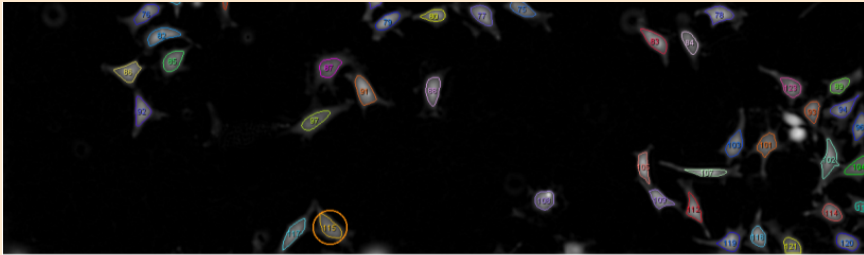
Track Objects

Uses the overlap of cells between adjacent time points for tracking

Cell masks overlayed with object tracks

Quantify Properties

Calculate Intensity Properties

Calculates mean intensity and contrast of DPC signal

Calculate Morphology Properties

Calculates cell area and cell roundness

Calculate Kinetic Properties

Calculates current speed of cell migration

Image Analysis Results

Summary	Properties Tracked Cells							
Object No	Age [s]	Current Displacement X [µm]	Current Displacement Y [µm]	Intensity Cell Digital Phase Contrast Mean	Intensity Cell Digital Phase Contrast Contrast	Cell Area [µm²]	Cell Roundness	Current Speed [µm/s]
114	3001,47	-2,97967	-0,993225	475,214	0,861988	489,301	0,987042	0,00165563
115	3001,47	5,95935	1,98645	398,332	0,861534	658,979	0,871677	0,00165563
116	3001,47	-2,97967	5,95935	370,06	0,837114	310,746	0,91495	0,00234142

Quantify Track Properties

Calculate Track Properties

Calculate accumulated distance, displacement and speed

Track No	First Timepoint	Last Timepoint	Start Time	End Time	Number of Timepoints	Duration [s]	Start Type	End Type	Generation	Parent ID	Root ID	Accumulated Distance [µm]	Displacement [µm]	Speed [µm/s]
1	0	5	00:00:00	00:50:01	5	3001,47	Begin	End	1	1	1	24,4142	12,9501	0,00813408
2	0	5	00:00:00	00:50:01	5	3001,47	Begin	End	1	2	2	5,78894	3,58112	0,0019287
3	0	5	00:00:00	00:50:01	5	3001,47	Begin	End	1	3	3	12,8121	6,35974	0,00426861

Calculate Readout Values

Define Results

Select meaningful names for your readouts and save single cell data

Field	Number of Tracked Cells	Age [s]	Current Displacement X [µm]	Current Displacement Y [µm]	Current Speed [µm/s]	Intensity Cell Mean - Digital Phase Contrast	Intensity Cell Contrast - Digital Phase Contrast	Cell Area [µm
1	122	2971,936	2,987815	1,595672	0,003388078	501,8335	0,8892133	606,9693

Single Cell Results: **Selected**

Readout Values calculated per time point

Number of tracked cells	Number of cells per well
Age [s]	Time passed since the cell was first observed in seconds; averaged over all cells per well
Current Displacement X [µm]	X component of a vector from the point of the first to the point of the current observation; averaged over all cells per well
Current Displacement Y [µm]	Y component of a vector from the point of the first to the point of the current observation; averaged over all cells per well
Current Speed [µm/s]	Distance (in µm) a cell traveled per second; averaged over all cells per well
Intensity Cell Mean – Digital Phase Contrast	Intensity DPC signal; first averaged over all pixels per cell and then averaged over all cells per well
Intensity Cell Contrast – Digital Phase Contrast	Contrast of the DPC signal calculated per cell and then averaged over all cells per well
Cell Area [µm²]	Average cell size over all cells per well
Cell Roundness	Average cell roundness over all cells per well

Readout Values calculated per track

Number of Tracks	Number of detected cell tracks per well
Number of Timepoints per Track	Number of time points at which a cell track was observed; averaged over all tracks per well
Track Duration [s]	Time span from the first to the last observation of a cell track in seconds; averaged over all tracks per well
Tracked Cells – Generation	Generation of the cell that belongs to a given object track; averaged over all tracks per well
Accumulated Distance [µm] per Track	Total length of all segments of an object track in µm; averaged over all tracks per well
Displacement [µm] per Track	Distance from the first to last observation of a cell track in µm; averaged over all tracks per well
Average Speed [µm/s] per Track	Accumulated distance in µm divided by the track duration in seconds; calculated per track and then averaged over all tracks per well

2.30.3 Image Analysis in Detail

Image Segmentation

➤ Image segmentation is performed using the building block **Find Cells – Method P**. Method P is dedicated to the segmentation of digital phase contrast images. Parameters have been adjusted to aim for robust object segmentation rather than for precise detection of cell outlines.

Object Tracking

➤ Object tracking is performed by the building block **Track Objects**. To evaluate the outcome of object tracking on the image analysis screen, a time window has to be selected. Please refer to chapter 1.16 for more information on object tracking.

Quantify Properties

➤ Typically, mitotic cells round up and therefore have a smaller cell area and higher cell roundness. Cell rounding also affects cell height, resulting in a strong increase of the digital phase signal.

➤ Both effects are quantified by the building blocks **Calculate Morphology Properties** and **Calculate Intensity Properties**.

➤ The building block **Calculate Kinetic Properties** calculates the current speed based on the distance travelled from the previous and to the next time point (see also *Figure 1–126*). Some compounds that inhibit cell division affect the cellular cytoskeleton and thereby strongly inhibit cell migration. Therefore, current speed can be an indicator for the mode of action of a potential cell cycle inhibitor.

➤ Building block **Calculate Track Properties** calculates more measures for cell migration based on entire cell tracks.

Calculate Readout Values

➤ Some of the available output properties have not been explicitly calculated by any **Calculate Properties** building block. Instead, properties like *Age [s]* and *Generation* have been calculated automatically by the building block **Track Objects**. See chapter 1.16 for further information on these properties.

➤ When object tracking is performed usually single cell time-resolved data is desired. Consequently, single cell data for all defined output properties will be stored in the Harmony software's database. Storage of singe cell data is activated by setting the parameter **Single Cell Results** to "Selected".

2.30.4 Hints and Tips

➤ Cell segmentation is a crucial step for object tracking. Always aim for a robust segmentation when adjusting the parameters for image segmentation, since segmentation errors are the most frequent cause for lost cell tracks.

➤ When properties for different regions are to be calculated, define these regions after applying the building block **Track Objects**. All regions that were identified beforehand will be lost after object tracking except for the one that was used to measure the object overlap, i.e. when the cytoplasmic-to-nuclear

ratio is being calculated, add the building block **Find Cytoplasm** after the building blocks **Find Nuclei** and **Track Objects**. For further information please refer to section 1.16.3.

➤ While having the appropriate time window selected and the building block **Track Objects** activated, different overlays can be used to visualize different aspects of object tracking. Use **Track Coloring by Generation** to immediately identify cells in the second or third generation. Use **Track Coloring by Root ID** to have all cells with the same progenitor connected with trajectories of identical colors. See *Figure 2–62* (A) for an example.

➤ This RMS is provided together with an example evaluation that contains single cell data. This evaluation can be used to learn how to visualize single cell data in the Harmony software. Examples that reflect characteristics of dividing cells are shown in *Figure 2–62* (B).
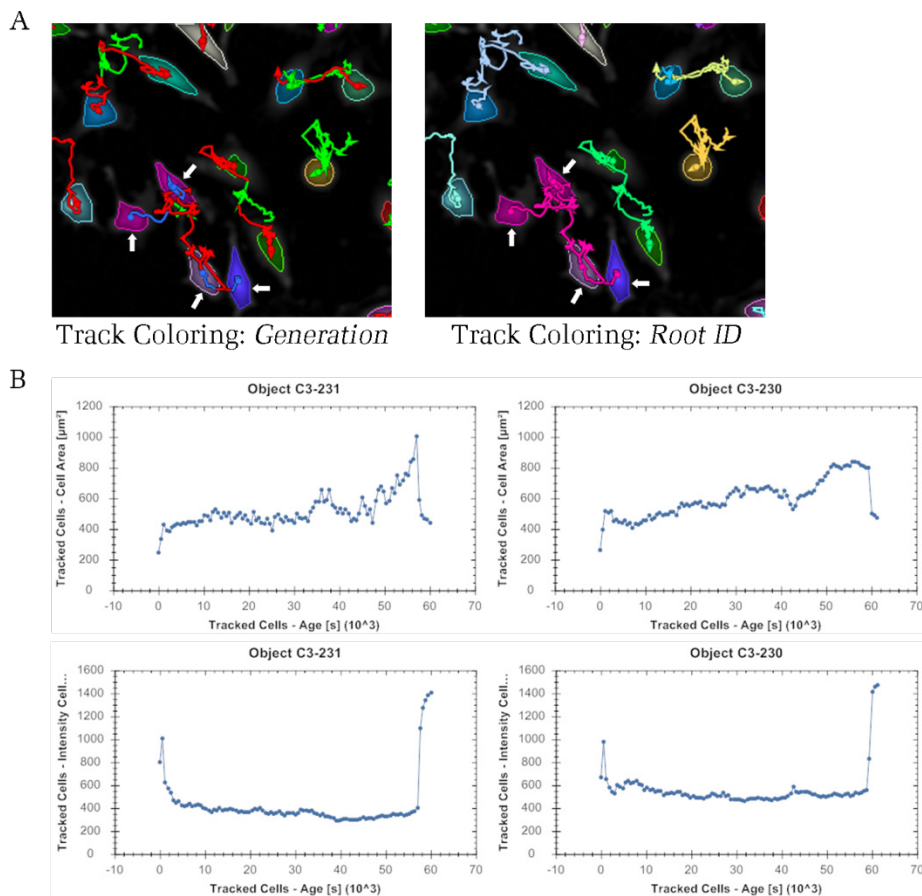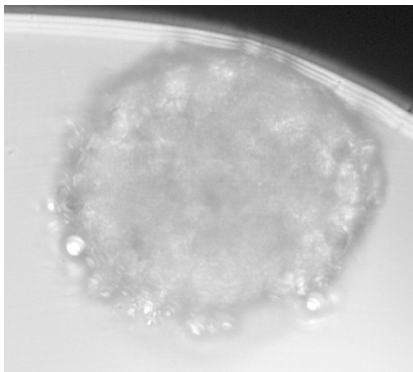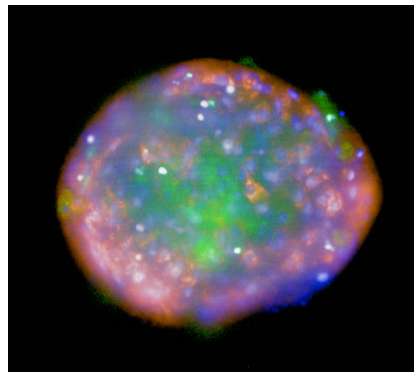
Figure 2–62: Visualization of cell tracking results. **A)** Visual feedback provided by track coloring on the image analysis screen. In the image there are four cells that originate from a common progenitor (white arrows). Note that the coloring of the tracks by generation results in a twofold change in the track color due to two rounds of cell division (from green to red to blue). Coloring the tracks by Root ID will connect the four daughter cells with tracks of identical color (here: purple). **B)** Numerical data from the evaluation provided was used to visualize characteristics of dividing cells. To generate the plots, the track results table was sorted by start type and end type. Only cells with start and end type *Split* were displayed. Grouping was set to *Object*. Cell area or mean intensity DPC were plotted against cell age. Note how cells increase in cell area over the cell cycle progression. Cell rounding during mitosis is reflected by a pronounced decrease in cell size and a strong increase in the DPC signal.

2.31 Microtissue Analysis

This section covers two RMSs for the analysis of 3D microtissues that differ in the required staining. The techniques used here for object segmentation can be applied to other 3D samples like worms, tissue sections or embryoid bodies.

2.31.1 Introduction and assay principle

3D cell culture models are believed to have the highest biological relevance among all cell-based *in vitro* models. They have been successfully employed to study pathologies like drug-induced liver injury or solid tumors. In the assay presented here, we use 3D InSight™ Liver Microtissues from InSphero®. The microtissues were treated for 24 hrs with different concentrations of Tacrin, which is known to be hepatotoxic. The live microtissues were stained with CellEvent™ Caspase-3/7 Green Detection Reagent for apoptotic cells and TMRM for active mitochondria. Hoechst 33342 was used as a counterstain and in addition we acquired a brightfield image. Images from the 3 different planes (5, 20, 35 µm) were acquired using a 20x objective. Here, we provide two RMSs to analyze microtissues either using brightfield (chapter 0) or fluorescence imaging modes (chapter 2.31.6).

RMS Microtissue Analysis – Brightfield	RMS Microtissue Analysis – Fluorescence
Potential Application: Analysis in brightfield can be of interest when only the size or the shape of the microtissue is to be measured. Additionally, imaging in brightfield does not result in phototoxicity, making it the perfect choice for time series measurements.	Potential Application: By using different fluorescent stains measurements that reflect biological functions can be obtained. Such measurements might include mitochondrial activity, cell death, apoptosis, hypoxia or vascularization. Multiplexing of different stains is possible.

2.31.2 RMS Microtissue Analysis – Brightfield

The RMS Microtissue Analysis – Brightfield is tailored to brightfield images of microtissues acquired using a 20x objective. Particular attention was given to the segmentation of microtissues that are close to the well border. The RMS assumes that there is only one microtissue per well.

The liver microtissues in the associated dataset ("P023-Microtissue Analysis") do not show differences in size or shape as they have not been treated accordingly. The effect of cytostatic or cytotoxic compounds on tumor microtissues is a good example of when the microtissue size could be used as readout.
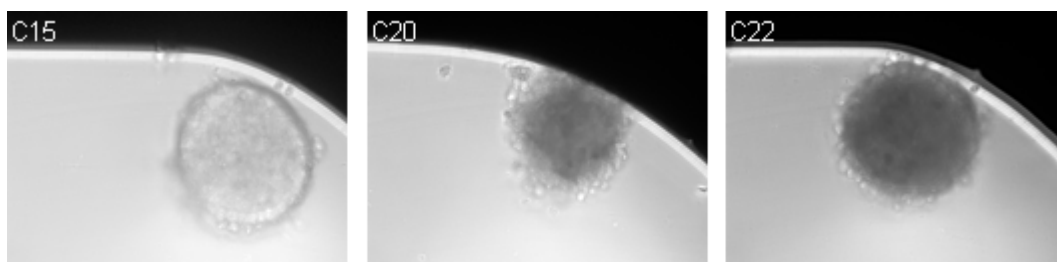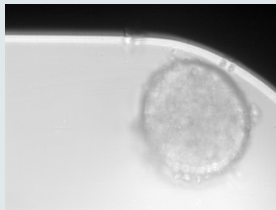
Figure 2–63: Example images of InSight™ Liver Microtissues acquired using brightfield imaging and a 20x hNA objective on the Operetta system. The tissues show no pronounced difference in size as they have not been treated accordingly. The differences in the optical density of the tissues in brightfield are associated with the treatment with a hepatotoxic compound.

2.31.3 How to use the RMS Microtissue Analysis – Brightfield

➤ Load the analysis sequence "RMS Microtissue Analysis – Brightfield" from the Harmony database.

➤ Select the brightfield channel in the **Find Texture Region** building block and train the machine-learning algorithm PhenoLOGIC to segment the microtissue.

Image Analysis Sequence: Microtissue Analysis – Fluorescence

Input Images

Channel 1:
Brightfield

(Maximum intensity projection of 3 stack planes)

Microtissue not touching well border
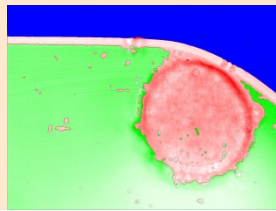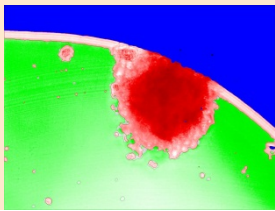
Microtissue touching well border

Image Segmentation

Find Texture Region

Allows you to separate regions of an image with different texture properties.
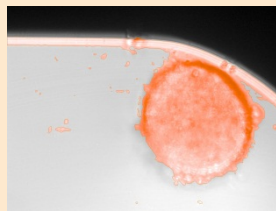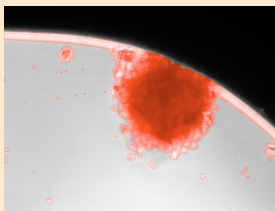
Segment the microtissue using machine learning. Three defined Regions: Well border (blue), well background and foreground (red).

Morphological Refinement
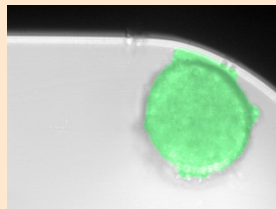
Select Region

Allows the creation of a region of interest.
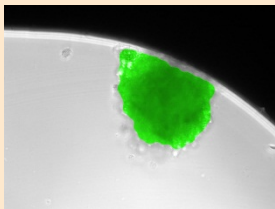
Fill small holes in the microtissue region
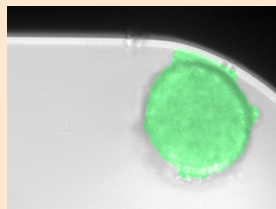
Select Region (2,3)

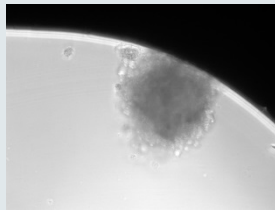Allows the creation of a region of interest.
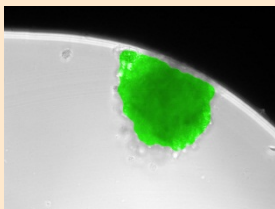
Refine the microtissue border by an erosion and a dilation step

Modify Population

Split detected region into objects, adjust the minimum area and splitting distance for touching regions.

Exclude smaller tissue fragments or debris and generate the final microtissue population

Quantify Properties of Regions

Calculate Morphology Properties

Calculates different morphology measures like area, roundness and length

Object No	Microtissue Area [µm²]	Microtissue Roundness
1	63433.1	0.951185

Calculate Readout Values	
Define Results Select the readout values to be averaged per well. Calculate statistical values by including Formula Outputs	<table><tr><td>Field</td><td>Microtissue Area [µm²]</td><td>Microtissue Roundness</td></tr><tr><td>1</td><td>63433.11</td><td>0.9511846</td></tr></table>

Readout Values	
Microtissue Area [µm²]	Area of the microtissue
Microtissue Roundness	Roundness of the microtissue

2.31.4 Image Analysis in Detail

Image Segmentation

➤ As an initial step the image is segmented using the interactive **PhenoLOGIC** building block **Find Texture Regions**. In this building block you are able to train the image segmentation by clicking onto the image and defining examples of a single, or several, texture classes. The **Find Texture Regions** building block then divides each image into regions, according to the user-defined classes. In this example dataset, we have divided the image into the following regions: the well border, the well background and the well foreground, i.e. the microtissue. By default, the classification into the different regions is exclusively based on texture, which makes the classification very robust towards intensity variation. However, in the analysis sequence presented here, we have activated the option **Include Intensity Information** as it facilitates the segmentation of the well border.

➤ We performed several steps to refine the image segmentation. First, we filled remaining holes within the microtissue region using the building block **Select Region**. Next, we added two more **Select Region** building blocks. One erodes the tissue region by a few px and the second dilates the region by several px. This procedure is called Morphological Opening and removes smaller extensions of the initial region, resulting in a rounder shape for the mircotissue region.

➤ As the final step in the segmentation, we added the building block **Modify Population**. This building block allows smaller fragments to be removed, such as debris or dirt from your initial segmentation. By splitting non-connected regions of the initial segmentation mask into different objects and subsequently excluding objects below a certain size, only the microtissue remains.

Quantification of Properties

➤ The area of the refined microtissue can be calculated using the **Calculate Morphology Properties** building block.

2.31.5 Hints and Tips

➤ Depending on your assay, the well border might not be present in your images. In this case, you can omit the third training region well border and split only into two classes (background and foreground). In this situation, the method **Find a Class** might provide good results when the training points are placed on the background.

➤ If the image segmentation of the Find Texture Regions building block is not appropriate, it is possible to re-train on the same image, or train on more than one image of the dataset.

➤ Training points that seem to be inappropriate can be removed by clicking again on the set training point.

➤ If the microtissues in your assay fragment into several pieces (e.g. due to treatment with cytotoxic compounds), several or all fragments may be excluded from the analysis due to their small size. In this case, it could be a solution to adjust the Area parameter in the **Modify Population** building block. If this value is set to zero, all small fragments will be considered for further analysis and you will obtain the number of tissue fragments in your image as an additional readout value.

2.31.6   RMS Microtissue Analysis – Fluorescence

The RMS Microtissue Analysis – Fluorescence segments microtissues based on a fluorescent staining. It is compatible with different magnifications and different fluorescent stainings.

The liver microtissues in the associated dataset ("P023-Microtissue Analysis") have been stained with Hoechst33342 (nuclear stain), TMRM (mitochondrial membrane potential) and CellEvent™ Caspase-3/7 Green Detection Reagent (apoptosis marker). The liver microtissues were treated with increasing concentrations of the hepatotoxic drug Tacrine.
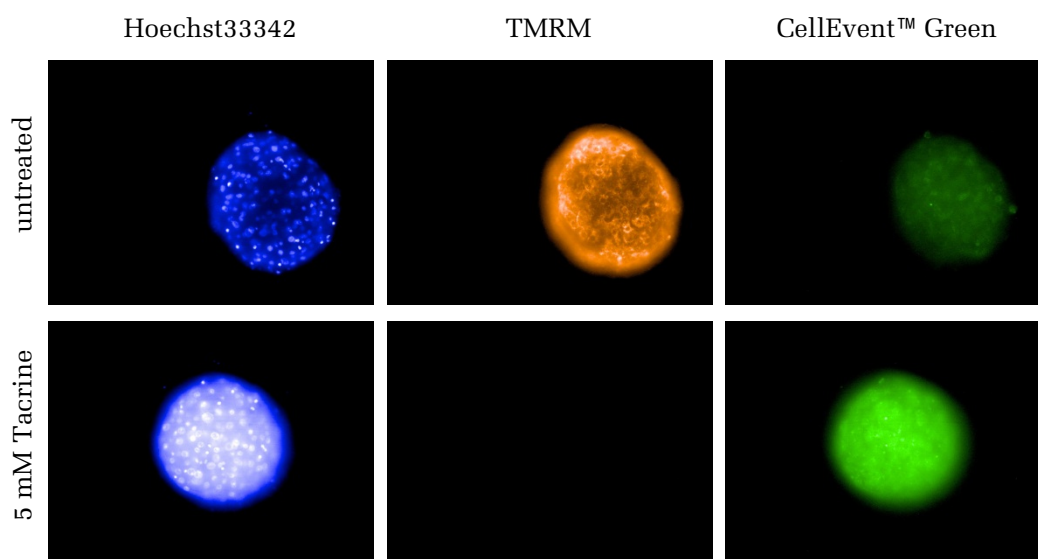
Figure 2–64: Example images of InSight™ Liver Microtissues that were left untreated or treated with 5 mM Tacrine. Three confocal stack planes (5 µm, 20 µm, 35 µm) were acquired using a 20x hNA objective on the Operetta system, and a maximum intensity projections of each image stack was calculated. For each channel, identical contrast settings were applied. Tacrine is a hepatotoxin causing apoptosis in the liver microtissue. This is reflected by an increase in the Hoechst33342 intensity (nuclear shrinkage), a decrease of the TMRM signal (loss of mitochondrial membrane potential) and a pronounced staining with the CellEvent™ Caspase-3/7 Green Detection Reagent.

2.31.7   How to use the RMS Microtissue Analysis – Fluorescence

➤   Load the analysis sequence "RMS Microtissue Analysis – Fluorescence" from the Harmony database.

➤   Select a suitable channel in the **Find Image Region** building block to segment the microtissue.

Image Analysis Sequence: Microtissue Analysis – Fluorescence
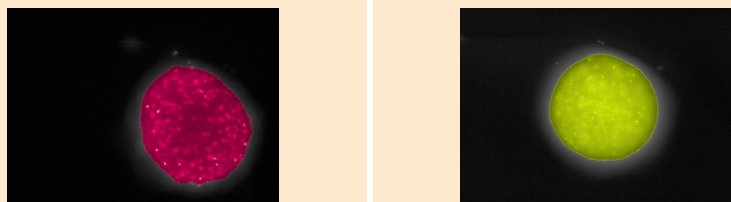
Input Images

Channel 1: Hoechst33342
Channel 2: TMRM
Channel 3: CellEvent™ Green

(Maximum intensity projection of 3 stack planes)

untreated

5 mM Tacrine

Image Segmentation

Find Image Region

Allows you to define the whole image, or parts of the image, as the region.
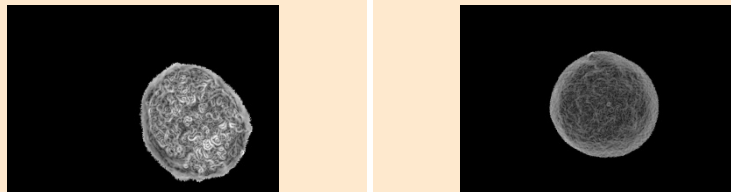
Segment the microtissue based on the Hoechst33342 staining.

Quantify Properties of Regions

Calculate Intensity Properties (1,2,3)

Calculate the mean intensity of Hoechst33342, TMRM and CellEvent™ Green

Object No	Intensity Microtissue Cell Event Green Mean	Intensity Microtissue TMRM Mean	Intensity Microtissue HOECHST 33342 Mean
1	798.066	3261.41	1782.57

Calculate Texture Properties

Texture feature measuring mitochondrial damage

Calculate Readout Values

Define Results

Select the readout values to be averaged per well. Calculate statistical values by including Formula Outputs

Field	Mean Intensity CellEvent Green	Mean Intensity TMRM	Mean Intensity HOECHST33342	TMRM Texture SER Edge
1	798.066	3261.411	1782.574	0.01611018

Readout Values

Mean Intensity CellEvent™ Green	Reflecting the degree of apoptosis; Averaged over all pixels within the microtissue
Mean Intensity TMRM	Reflecting the mitochondrial membrane potential; Averaged over all pixels within the microtissue
Mean Intensity Hoechst33342	Reflecting the condensation of DNA upon apoptosis; Averaged over all pixels within the microtissue
TMRM Texture SER Edge	Quantifies the pattern of the TMRM staining

Image Analysis Guide

2.31.8 Image Analysis in Detail

Image Segmentation

➤ The **Find Image Region** building block is used to find the region covered with fluorescently labeled cells. The common threshold method allows you to define the image region based on an intensity threshold. During this step, smaller objects are removed as defined by the area threshold.

Quantification of Properties

➤ The **Calculate Intensity Properties** building blocks are used to determine the mean intensities of the mitochondrial stain TMRM, the apoptosis marker CellEvent™ Green and the nuclear stain Hoechst33342.

➤ The **Calculate Texture Properties** building block is used to quantify the texture of the mitochondria ("pattern of the stain"). Texture analysis can be used to determine changes in the structure of mitochondria, e.g. as a result of toxic impact. See section 1.7 for an introduction and overview of texture analysis.

Calculated Readout Values

➤ Among the 4 readout values there are 3 absolute intensities. Although they show the expected dose-dependent changes, these kinds of measures are prone to variations due to different batches of the staining reagent, different incubation times and ageing of the light source. However, texture is a measure that is independent of absolute intensities and so is a more robust technique.
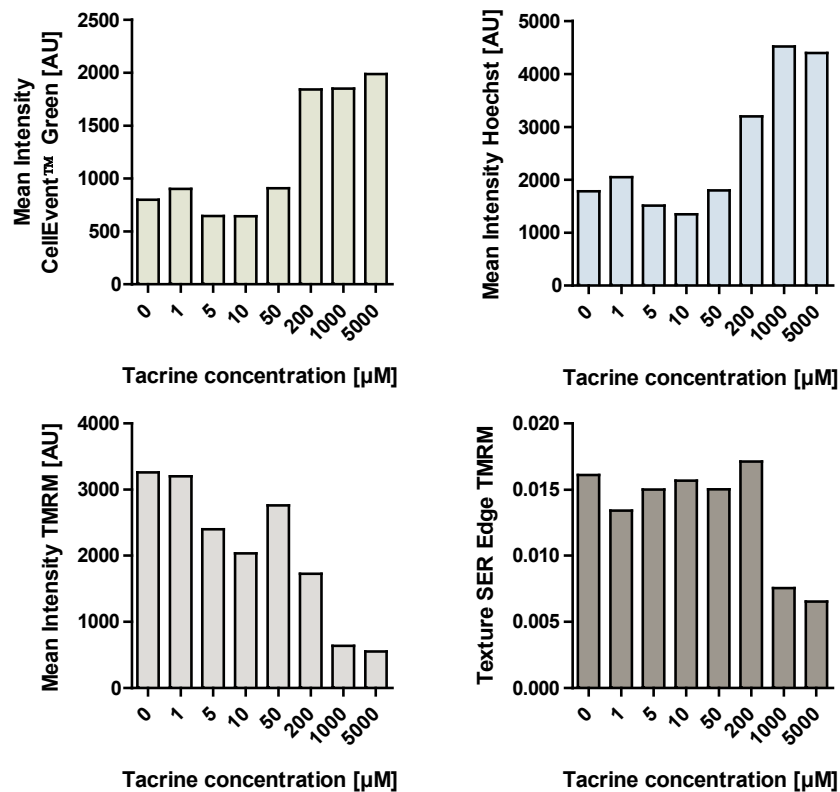

Figure 2–65 Evaluation of the measurement "P023-Microtissue Analysis" using the "RMS Microtissue Analysis – Fluorescence". All of the defined readout values show dose-dependent changes that are in

accordance with the initial observations (***Figure 2–64***). Note that all but the Texture SER Edge are based on absolute intensities, which may result in a higher plate-to-plate or day-to-day variation in screening runs.

2.31.9 Hints and Tips

➤ If the microtissues in your assay fragment into several pieces (e.g. due to treatment with cytotoxic compounds), several or all fragments may be excluded from the analysis due to their small size. In this case, it could be a solution to adjust the Area parameter in the **Find Image Region** building block. If this value is set to zero, all small fragments will be considered for further analysis and you will obtain the number of tissue fragments in your image as an additional readout value.

➤ In some assays, the intensities of the different stainings vary strongly depending on the treatment. Due to the relative threshold used in the building block **Find Image Region,** segmentation is usually still possible. If the differences in the intensities are too great to allow for correct segmentation of treated and untreated images, calculation of an artificial image may help. To this end, you can use the building block **Calculate Image** and sum up two images that exhibit contrary stainings for different treatments. The resulting artificial image could then be used in the building block **Find Image Region**.

Appendix

Setting up the Image Analysis: Workflow

Start on the IMAGE ANALYSIS screen, working with single images at a time.

Browse your images to get an overview of the images to be analyzed.

Select a representative "High" and "Low" image.

Choose a RMS and load it from the database or assemble a new analysis sequence.

Select the "High" image to configure the RMS parameters step by step according to the instructions provided in the respective RMS section in this chapter.

Check the results of this configuration against the "Low" images.

Proceed to the EVALUATION screen, working with multiple images / wells at a time.

Validate the analysis against dose response wells, or a set of High / Low wells.

Optional: to improve results (whilst in the EVALUATION screen)

Identify the most obvious outlying data point and the corresponding image (well, field).

Go back to the IMAGE ANALYSIS screen and view the intermediate results to find a reason for the anomalous results.

Re-adjust your parameters, or add building blocks to fix the problem.

Optional: export and further analyze single cell results to optimize, for example, a threshold for a subpopulation.
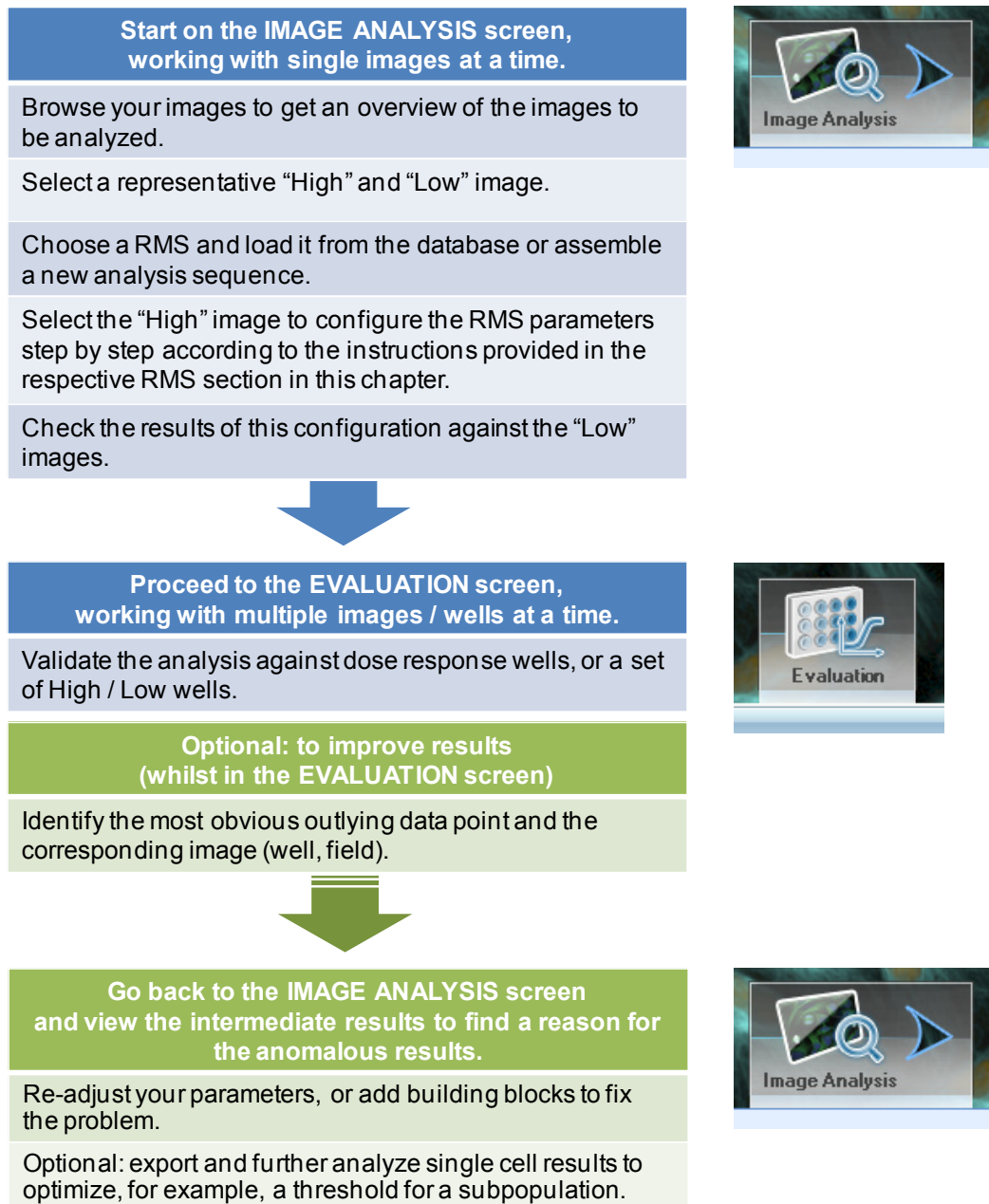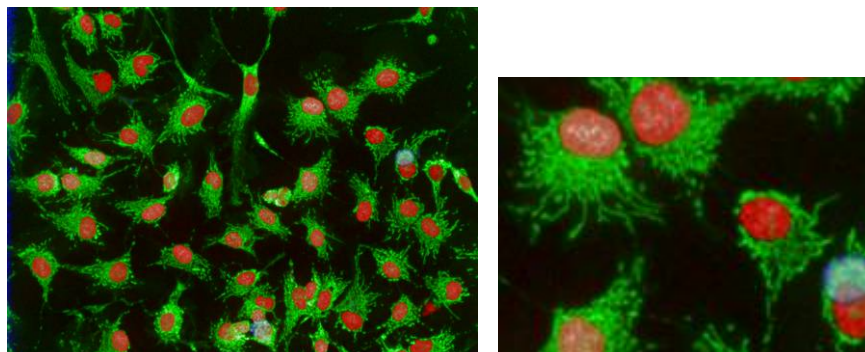
Table 3-1: Flowchart of setting up, optimizing and validating an image analysis sequence. The green parts are optional steps in case optimization of the analysis is desired.
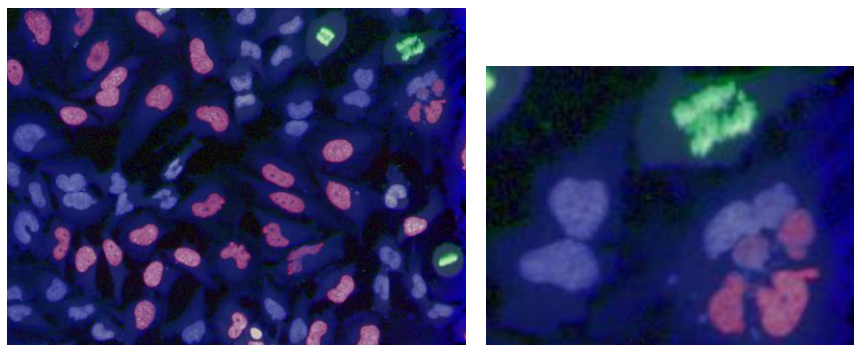
List of Example Datasets

The example datasets are pre-loaded in the Harmony database to allow you to immediately start exploring the ready-made solutions (RMSs).

Example Dataset 1	Plate P003-CT: Live Tox
Cell type	HepG2
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	Hoechst 33342 MitoTracker® Deep Red BOBO™-3
Control Compound(s)	FCCP
Image fields	1 per well
Number of wells	10 (dose-response curve)
Example RMS	Cell Counting (page 177) Live / Dead Cell Counting (page 179) Nuclear Analysis – Nuclear Shrinking (page 183) Texture Analysis – Mitochondria Classification (253) Ready-Made Application: Cytotoxicity-1 (see Application Guide, section 3.2)

Example Dataset 2	Plate P004-CC: EdU-pHH3
Cell type	HeLa
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	EdU, Alexa Fluor® 488 labeled a-pHH3 primary AB, Alexa Fluor® 647 labeled secondary AB Hoechst 33342
Control Compound(s)	Nocodazole
Image fields	3 per well
Number of wells	10 (dose-response curve)
Example RMS	Quantification of Marker in Nucleus (page 187) Nuclear Classification – DNA content (page 219) Cell Cycle Classification (page 229) Ready-Made Application: Cell Cycle-1 (see Application Guide, section 3.3)
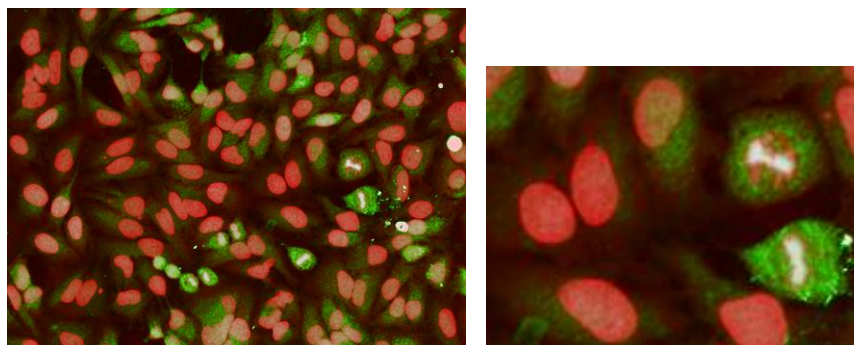
Example Dataset 3	Plate P005-A: Caspase3 Apoptosis
Cell type	HeLa
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	Caspase-3-antibody – Alexa Fluor® 488 conjugated DRAQ5™
Control Compound(s)	Staurosporine
Image fields	1 per well
Number of wells	9 (dose-response curve)
Example RMS	Quantification of Cytoplasmic Marker (page 191) Nuclear Fragmentation (page 225) Ready-Made Application: Apoptosis-1 (see Application Guide, section 3.1)
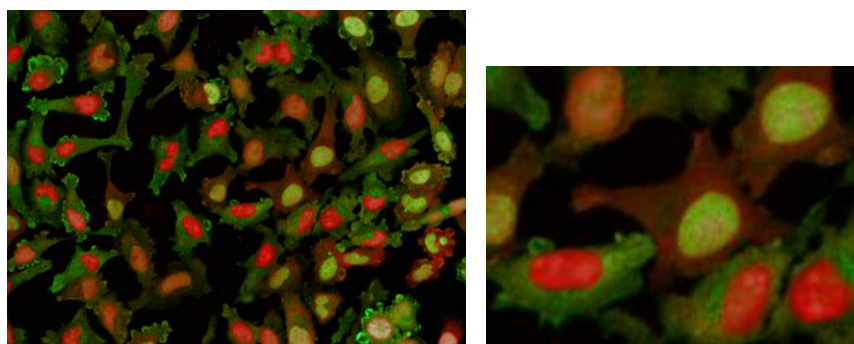
Example Dataset 4	Plate P009-T: NF$_K$B
Cell type	HeLa
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	NF$_K$B primary AB, Alexa Fluor® 488 labeled secondary AB Hoechst 33342
Control Compound(s)	Interleukin 1-a
Image fields	1 per well
Number of wells	12 (dose-response curve)
Example RMS	Cytosol to Nucleus Translocation (page 205)
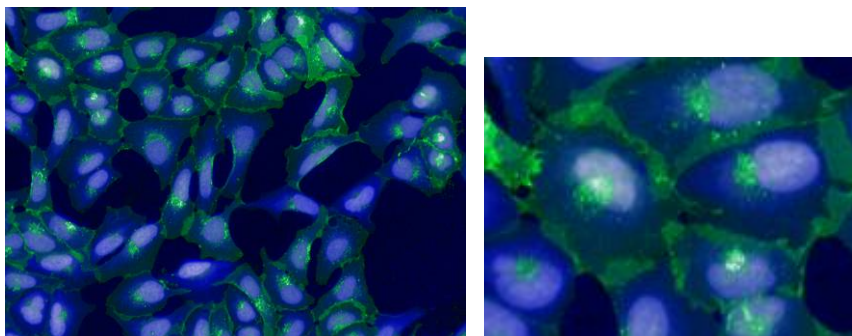
Example Dataset 5	Plate P010-R: ETAR
Cell type	U2OS ETAR
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	Endothelin A-Receptor AFP (488) labeled DRAQ5™
Control Compound(s)	Endothelin-1
Image fields	1 per well
Number of wells	20 (dose-response curve)
Example RMS	Receptor Internalization (page 213) Quantification of Membrane Marker (page 195)
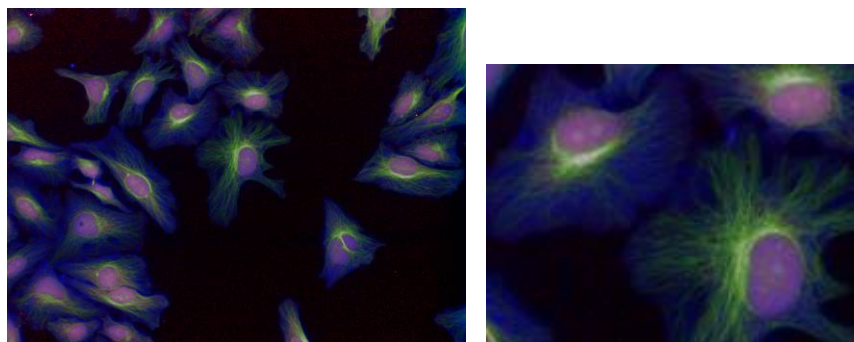
Example Dataset 6	P013-CC: Cytoskeleton
Cell type	HeLa
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	a-α-tubulin primary AB, Alexa Fluor® 488 labeled secondary AB a-pHH3 primary AB, Alexa Fluor® 647 labeled secondary AB (Cytochalasin measurement only) Hoechst 33342
Control Compound(s)	Demecolcine (Measurement 1) Cytochalasin (Measurement 2)
Image fields	1 per well
Number of wells	10 per compound (dose-response curve)
Example RMS	Cell Shape – Cell Rounding (page 235) – Cytochalasin Fluorescence Redistribution – Cytoskeleton (page 239) – Demecolcin Phenotype Classification – Cytoskeleton (257) – Demecolcin
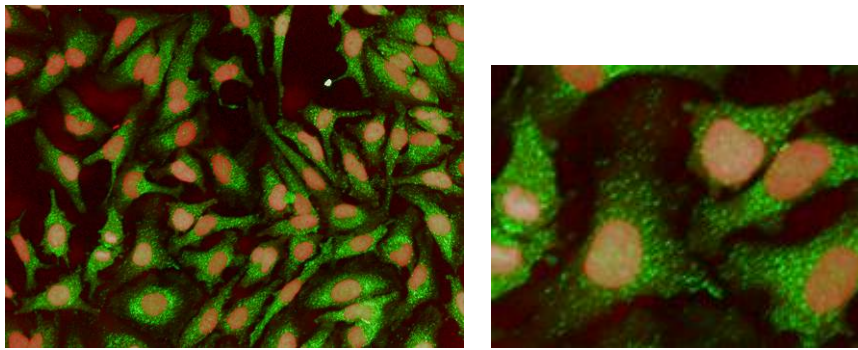
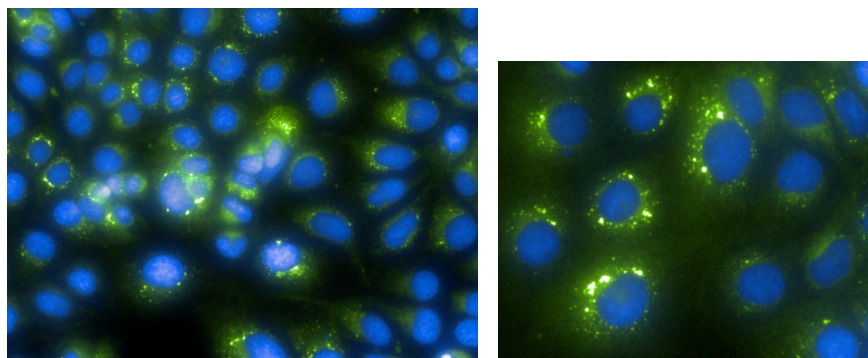| Example Dataset 7	Plate P001-CT: Peroxisomes
Cell type	HeLa (5×10^3 per well)
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD, 60x long WD
Staining(s)/Channel(s)	a-PMP70 primary AB, Alexa Fluor® 488 secondary labeled AB; DRAQ5™
Control Compound(s)	none
Image fields	1 per well (20x), 2 per well (60x)
Number of wells	6 (20x), 3 (60x)
Example RMS	Spot Analysis (page 199)
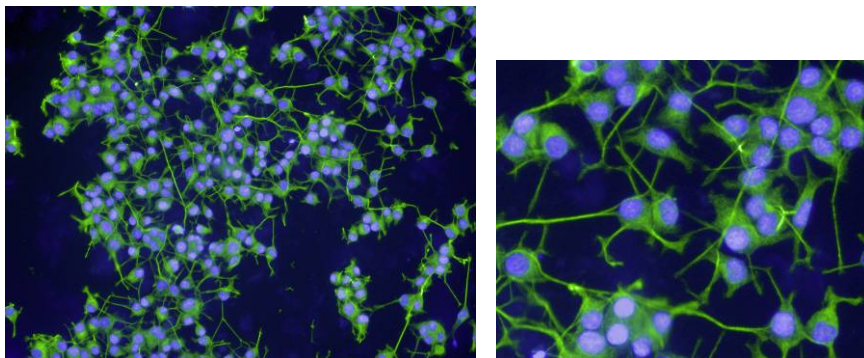
| Example Dataset 8	Plate P011-N: Neurite Outgrowth
Cell type	Neuroscreen™-1 (Thermo Fisher)
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	Hoechst33342 Anti-α-tubulin – AlexaFluor®488
Control Compound(s)	NGF (Nerve Growth Factor)
Image fields	1 per well
Number of wells	11 (dose-response curve)
Example RMS	Neurite Outgrowth Analysis (page 249)

Example Dataset 9	**Plate P015-Phospholipidosis**
Cell type	A-431
Imaging instrument	Operetta High Content Imaging System
Objective	40x long WD
Staining(s)/Channel(s)	DAPI and SYTO®42 (in one channel) BODIPY® 493/503
Control Compound(s)	JAK3 inhibitor, Triacsin, Nocodazole
Image fields	2 per well
Number of wells	6 (3 doses for Nocodazole)
Example RMS	Lipid Droplet Analysis – Analysis of various sized spots (page 263)
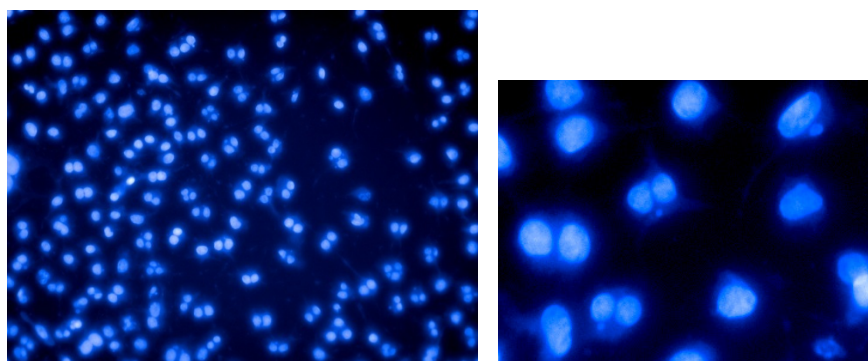
Example Dataset 10	**Plate P016-Micronucleus**
Cell type	Mammalian cells
Imaging instrument	Operetta High Content Imaging System
Objective	20x long WD
Staining(s)/Channel(s)	DAPI
Control Compound(s)	Gentoxic compound
Image fields	9 per well
Number of wells	8 (4 doses, 2 replicates)
Example RMS	Micronucleus Analysis (page 281)
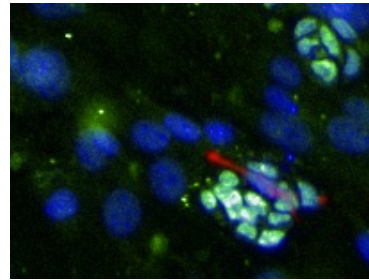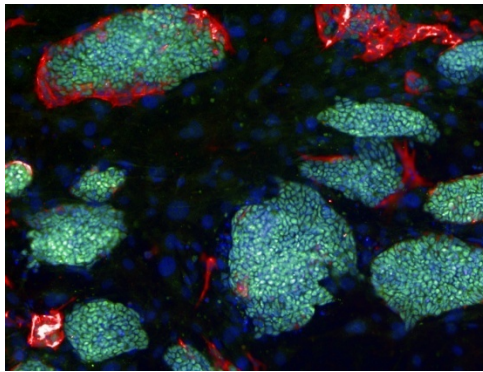
Example Dataset 11	**Plate P017-ESC Colonies**
Cell type	Embryonic stem cells: DBA1 (mouse) Feeder cells: embryonic fibroblasts (mouse)
Imaging instrument	Operetta High Content Imaging System
Objective	10x high NA
Staining(s)/Channel(s)	Brightfield Hoechst33342 Anti Oct4 – AlexaFluor®488 Anti Cytokeratin – AlexaFluor®647
Control Compound(s)	None
Image fields	1 per well
Number of wells	2
Example RMS	Texture Based Segmentation – Stem Cell Colonies (page 287)
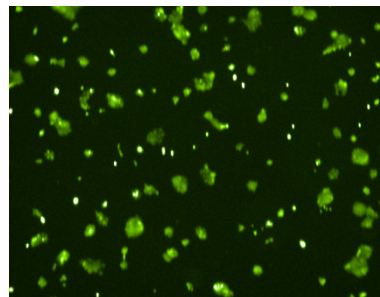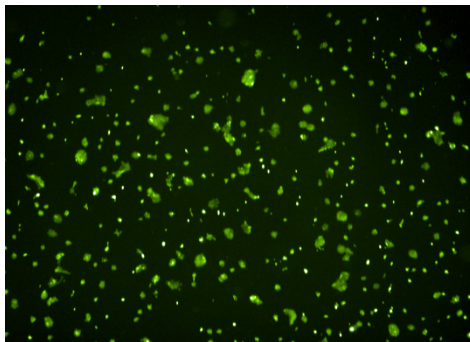
Example Dataset 12	**Plate P018-Colony Formation**
Cell type	A-431 / Human epidermoid carcinoma cells
Imaging instrument	Operetta High Content Imaging System
Objective	2x long WD
Staining(s)/Channel(s)	CMFDA (CellTracker™ Green)
Control Compound(s)	None
Image fields	1 per well
Number of wells	1
Number of time points	3 (0, 69 hr and 93 hr)
Example RMS	Colony Formation Analysis (page 267)
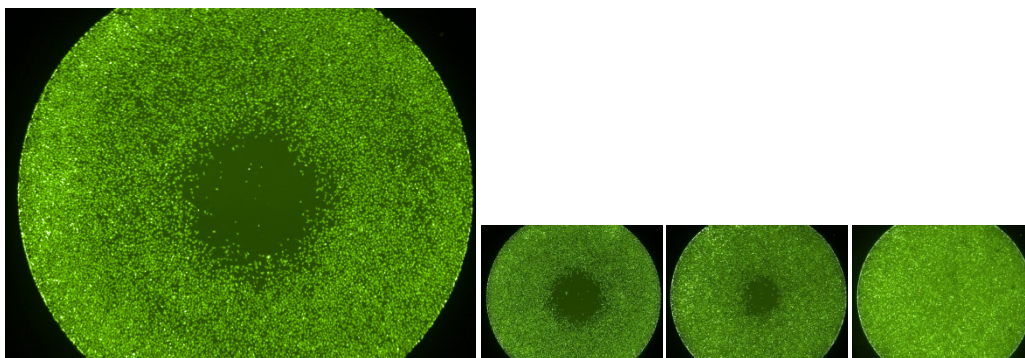
Example Dataset 13	**Plate P019-Migration**
Cell type	HT-1080 fibrosarcoma cells
Imaging instrument	Operetta High Content Imaging System
Objective	2x long WD
Staining(s)/Channel(s)	Brightfield Cell Tracker™ Green CMFDA
Control Compound(s)	Cytochalasin D
Image fields	1 per well
Number of wells	4 (dose-response curve)
Time points	3
Example RMS	Migration– Hole in Cell Layer (page 274)
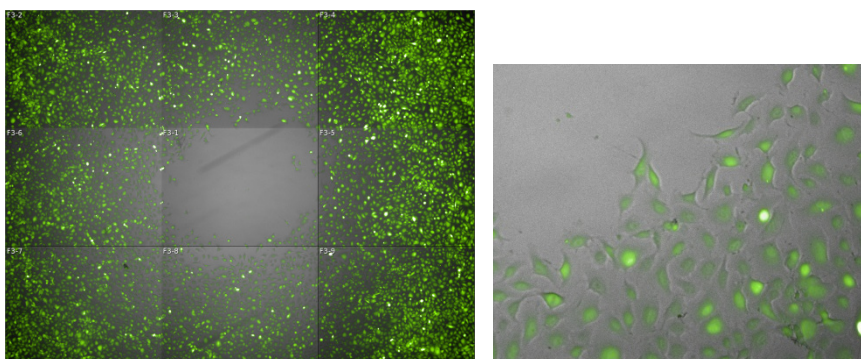
Example Dataset 14	**Plate P020-Migration**
Cell type	HT-1080 fibrosarcoma cells
Imaging instrument	Operetta High Content Imaging System
Objective	10x long WD
Staining(s)/Channel(s)	Brightfield Cell Tracker™ Green CMFDA
Control Compound(s)	–
Image fields	9 per well
Number of wells	1
Time points	3
Example RMS	Migration- Confluency (page 277)
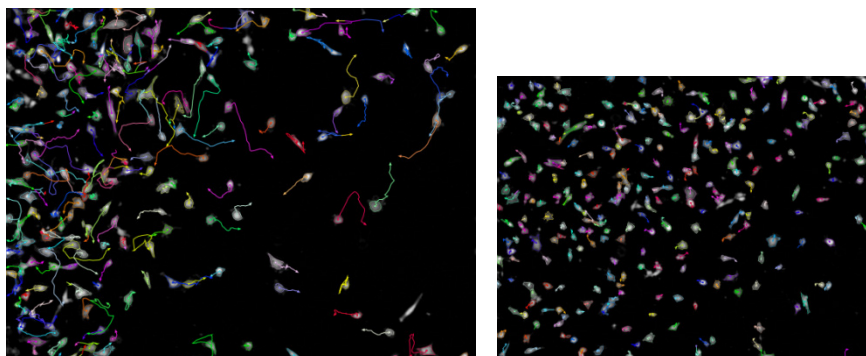
Example Dataset 15	Plate P021-Cell Tracking
Cell type	HT-1080 fibrosarcoma cells
Imaging instrument	Operetta High Content Imaging System
Objective	10x high NA
Staining(s)/Channel(s)	Digital Phase Contrast
Control Compound(s)	Cytochalasin D
Image fields	1 per well
Number of wells	2
Time points	25
Example RMS	Cell Tracking – Migration Analysis (page 291)
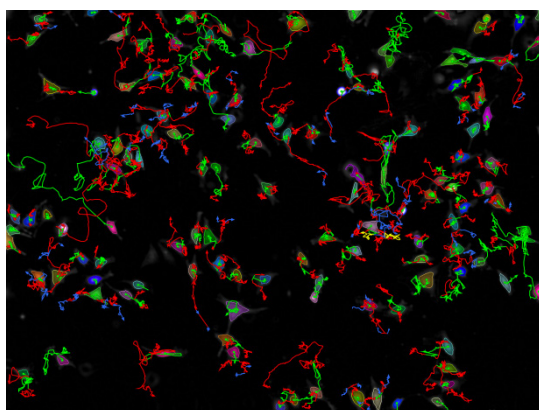
Example Dataset 16	Plate P022-Cell Tracking
Cell type	HeLa
Imaging instrument	Operetta High Content Imaging System
Objective	10x high NA
Staining(s)/Channel(s)	Digital Phase Contrast
Control Compound(s)	Aphidicolin
Image fields	1 per well
Number of wells	2
Time points	144
Example RMS	Cell Tracking – Cell Division (page 297)
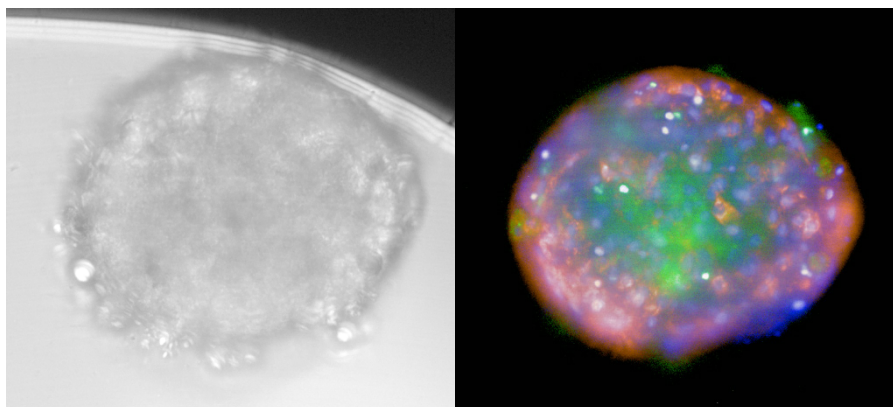
Example Dataset 17	Plate P023-Liver Microtissues
Sample	InSphero 3D InSight™ Liver Microtissues
Imaging instrument	Operetta High Content Imaging System
Objective	20x high NA
Staining(s)/Channel(s)	Brightfield, CellEvent™ Caspase-3/7 Green Detection Reagent, Hoechst33342, TMRM
Control Compound(s)	Tacrine
Image fields	1 per well
Number of wells	8
Number of planes	3
Example RMS	RMS Microtissue Analysis – Brightfield (page 303)
	RMS Microtissue Analysis – Fluorescence (page 307)

Converting Acapella Scripts to Building Block Sequences*

Acapella Scripts can be used for image analysis on the Opera and Columbus system. Columbus and recent Opera versions also support building block sequences in addition to Acapella Scripts. Starting with Opera Phenix only building block sequences will be supported. Acapella Scripts have to be replaced by the corresponding building block sequence. This section gives some hints how to do this.

Most analysis tasks in an Acapella Script can be straight forward done in a building block sequence as well. *Table 3-2* gives an overview of the Harmony building blocks and their equivalent Acapella modules.

Harmony Building Block	Corresponding Acapella 2.0 Modules
Find Nuclei	Nuclei_Detection_select()
Find Cytoplasm	Cytoplasm_Detection_A to F
Find Spots	Spot_Detection_C is equivalent to method "C". The **Find Spots** methods "A" and "B" have no corresponding Acapella 2.0 modules.
Find Cells	Nuclei_Detection_select()
Find Micronuclei	"Micronucleus Analysis" script from the Acapella Script Collection.
Find Neurites	Neurite_Detection_A is equivalent to the method "CSIRO Neurite Analysis". The method "CSIRO Neurite Analysis 2" is not available in Acapella 2.0.
Find Image Region	ThresholdXX() Mask() Mask2Stencil() Stencil2Objects()
Find Texture Regions	No corresponding methods are available in Acapella 2.0.
Find Surrounding Region	Cytoplasm_Detection_A to D
Select Cell Region	CytoplasmRegion(), NucleusRegion(), RingRegion(), MembraneRegion() and WholeObjectRegion() **Please note** that relative regions (% values) are not available in Acapella 2.0. Use absolute regions (measured in pixels) instead.
Select Region	CalcBorder() CalcFillStencil() CalcErosion() to reduce or enlarge the size of a region CalcSkeleton() CalcMassCenter()
Select Region **– Method "Restrict by Mask"**	And(image=stencil, mask=mask) And(image=stencil, mask=!mask)
Calculate Intensity Properties	CalcStat(), CalcIntensity()

Calculate Morphology Properties	Calcarea() CalcWidthLength() for Length, Width and Ratio Width to Length. CalcAttr(Roundness_corrected, 2*sqrt(pi)*sqrt(1.0*area-border_area/2.0)/border_area-0.1)
Calculate Morphology Properties – Method STAR	Morphology::CalcTextuMorphoFeatures()
Calculate Texture Properties	CalcGaborFeatures(), CalcHaralickFeatures(), CalcSERFeatures()
Calculate Properties	CalcAttr()
Select Population	ObjectFilter()
Select Population - Method "Linear Classifier"	No corresponding methods are available in Acapella 2.0.
Modify Population	CalcErosion() Mask2Stencil() Stencil2Objects()
Filter Image	Texture:: CalcSERFeatures(), output SERIllustrations Mean(), Filter::Mean(), Median(), Texture::GaussianFilter() Sliding ParabolaTransform()
Calculate Image	Common expressions like A > 10 iif(A>100, A, 0) iif(A>quantile(A, 0.75).quantile , A, 0) iif(A/A.mean>B/B.mean, A/A.mean, B/B.mean) A/A.mean + B/B.mean
Track Objects	No corresponding methods are available in Acapella 2.0.
Calculate Kinetic Properties	No corresponding methods are available in Acapella 2.0.
Calculate Track Properties	No corresponding methods are available in Acapella 2.0.
Define Results	Output()

Table 3-2: Harmony building blocks and their corresponding Acapella 2.0 modules.

Since Acapella is a programming language with full access to the computer system some advanced functions cannot be converted to building block sequences. Here is a list of examples

➤ Access to the computer hard disk

➤ Manipulating image pixels

➤ Fourier transform of images

➤ Table manipulations

➤ Model fitting

Glossary

Definition of terms as used **in this** booklet. This is not intended to be a tutorial, or a dictionary.

Analysis Algorithm: The description of a step-by-step procedure used to calculate the →assay readout from an →image set. In the →Harmony software the algorithm is described using an →analysis sequence.

Analysis Parameters: The adjustable input parameters of an →analysis algorithm used to adapt the analysis to specific experimental conditions without modifying the algorithm. Do not confuse this with the →readout values!

Analysis Sequence: An image →analysis algorithm consisting of a sequence of building blocks. Each subsequent building block takes the output from the preceding building blocks as an input and refines or generates new information. The last building block returns the →readout values of the analysis.

Assay: A biological test system. Only cellular assays are considered here.

Assay Development: This is the process of defining the Standard Operating Procedure (SOP) for a new assay and includes the biology, measurement, image analysis and data analysis. The result is a SOP with data that supports the assay performance.

Assay Layout: Information about the contents of each well in a plate, e.g. which compounds are added at which concentrations, location of control wells.

Assay Readout: The set of different readout values that are generated by a specific assay. "High Content" means that you require multiple readout values.

Background: The area of the image where no objects of interest are located, e.g. the area not covered by cells.

Batch Analysis: Fully automated analysis of a group of wells or batch of plates. The →assay readout is calculated for each well. Private results are discarded.

Building Block: A ready to use processing module that performs a specific image analysis task, e.g. nuclei detection or measurement of object properties. A building block takes images and/or →populations as an input. It returns →populations and →Illustrations. The behavior of the building block can be adjusted by →analysis parameters. Building blocks can be combined into an →analysis sequence and / or a →Ready-Made Solution.

Canned Solution: A "black box" analysis algorithm for a specific task which cannot be modified by the user. Only the →analysis parameters can be adjusted.

Channel: Independent optical measurement performed at the same position but using different optical settings, e.g. color (wavelength), imaging mode (brightfield / fluorescence), exposure time. Also called "**color**" when referring to different wavelengths.

Classification: Putting each object into one of a set of groups, e.g. "strong responder", "weak responder", "non responder".

Coefficient of Variation (CV): Normalized standard deviation. This is a measure of the fluctuations of a value. The CV is particularly useful when the noise level increases with the signal.

$$CV\% = \frac{stddev}{mean} * 100\%$$

Compounds: Chemical substances that are tested by the assay.

Confluency: The percentage of a given area e.g. the well bottom of a microplate or the bottom of a culture flask, that is covered by a cell monolayer.

Confocal Imaging: Generating an image of a sample by sequential illumination of small focused spots of the sample, collecting light only from these spots and rejecting light from other parts of the sample. The image is generated by sequential scanning of the →field of view. The acquisition speed can be increased, and photobleaching effects minimized, by using multiple points in parallel, e.g. using a →Nipkow Disk. Compared to non-confocal →widefield imaging, confocal imaging allows higher z-resolution and background suppression, resulting in better contrast.

Crosstalk: Lack of independence of different channels ("bleed through").

Data Analysis: Further processing of the →well results or →single cell results in order to arrive at a statistically significant conclusion about the tested compounds. Extract the key information out of the large amount of generated data.

Data Point: One value in a graph or table representing a result derived from one well or one cell.

DPC: Digital Phase Contrast, a channel in Harmony for the generation of digital phase images.

Dose-Response (DR) Curve: The graph showing an →assay readout value as a function of the concentration of the test compound. The correct shape of the DR curve for a known test compound is an important evaluator of the assay and image analysis performance.

EC50: The concentration of a compound that is needed to provoke a response of the →assay readout value halfway between the baseline and maximum response (half maximal effective concentration).

Feature: A property that is visible in an image, e.g. brightness, structure, smoothness, special shape.

Field of View: The area (width and length) that is imaged in the sample during every exposure. This depends on the optical set up of the microscope and the objective magnification.

Flagging: Assigning a new binary →property to an object, e.g. "Does it belongs to the population X?" (Yes / No).

Gamma Correction: Nonlinear intensity enhancement of dark image areas in order to make the structures inside these areas visible.

GUI: Graphical user interface of a software.

Harmony: PerkinElmer's fully integrated image acquisition, image analysis and data management software. For →image analysis, either ready-made solutions (RMSs) can be used or custom algorithms can be created by interactively arranging the ready to use →building blocks in a visual editor.

Illustrations: Graphical representations of →multi channel images, →overlays and →populations on the user interface for visual inspection. A building block or analysis sequence can create pre-defined illustrations for guided data inspection or parameter tuning.

Image Analysis: Calculating the →assay readout for one well by applying an →analysis algorithm to an →image set.

Image Field (Field): The position in a well at which images are taken. The size of the image field depends on the objective magnification.

Image Set: All the images taken in **one well** in order to generate the →assay readout for that well. The image set can include multiple →channels, multiple locations in the well (→image fields), multiple time points (→time series) and multiple z-planes (→stack).

Image: Def. 1) Matrix of intensity values (pixels) generated by a digital camera. Def. 2) General term meaning any kind of picture.

Imaging Assay: An assay that uses microscopy images in order to quantify an effect.

Interactive Analysis: Manual selection of images and the start of an analysis sequence. All →private results can be interactively studied. The impact of changes in the →analysis parameters are immediately visible.

LED: Light emitting diode, e.g. used in the Operetta's transmission light source (690 nm).

Measurement: All the images and →meta information that are collected from one plate during a specific image acquisition. Multiple measurements may exist for the same plate.

Meta Information: Meta data for images: additional information describing the image contents, e.g. date, time, instrument used to acquire the image, instrument settings (e.g. magnification of a microscope), assay type, barcode of a microplate, well, imaged positions in the well, and user annotations.

Method: An →analysis parameter of a →building block that allows you to select different mathematical approaches for solving the task assigned to the building block, e.g. choosing an optimal nuclei detection algorithm in the "Find Nuclei" building block.

Microplate: A flat plate with a collection of 96 or 384 wells (Also just called **plate**).

Multi Channel Image: A set of images captured at the same position but each using a different →channel.

Multiplexing Assay: An assay that combines multiple tests at the same time in the same well, e.g. monitoring two different transcription factors at the same time using two separate channels.

Nipkow Disk: A spinning disk containing a specific pattern of pinholes, that is placed in the optical path of a microscope. A moving pattern of confocal spots is generated to rapidly scan the whole →field of view for →confocal imaging.

Object Detection: Finding objects of interest in an image, e.g. whole cells, nuclei, spots, beads. Each detected object is represented by →regions, which define the position of the object in the image and its →properties (numbers associated with the object), e.g. the object number, size, mean intensity. All detected objects are summarized in a →population.

Object: Any kind of spatially defined similar items that are visible in an image, e.g. cells, nuclei, spots, the cytosol, beads, clusters of cells, bright areas, etc.

Object List: A table listing the individual →properties of all objects in a →population (one line per object, one column per property).

Overlay: Marks image parts in visualizations, e.g. the currently selected →object.

Parameter Tuning: Adjustment of the →analysis parameters in order to optimize the image analysis results.

Pixel (px): "Picture element". The smallest piece of information in an image generated by a digital camera.

Plane: The x-y image at a specific height in a →stack (z-position).

Population: A set of objects, e.g. found by an object detection step. A population is represented by an →object list and an associated →overlay which specifies the object locations.

Positive / Negative Control: Control wells that are designed to either show or not show the expected effect respectively. If compounds are used to induce an effect then, by convention, the well without the compound added is called the negative control (even though the effect might be visible e.g. if the assay is designed to test an antagonistic compound). To avoid ambiguity, the terms "high" and "low" control are avoided here.

Private Results: Intermediate images and →overlays generated by a building block which are not returned as a →readout value. The private results can be displayed for control and parameter adjustment purposes in an interactive analysis.

Property: Numerical values associated with an object, e.g. size, fluorescence intensity, number of spots inside the object.

Quality Control Information: Readout values that are used to monitor the uniformity of samples e.g. cell number or average staining intensity.

Range: Specification of a set of wells, fields, stack planes and time points to be used for an interactive analysis in the →Harmony software.

Readout Value: A number quantifying a biological feature of interest, e.g. the cell number, the intensity of a marker or the fraction of cells showing an effect.

Ready-Made Solution (RMS): An analysis algorithm which **can be modified** by the user, e.g. to add additional →readout values or to combine multiple analysis algorithms for a →multiplexing assay.

Region: Defines the parts of an image that belong to an →object. Each object may have multiple different regions, e.g. nucleus region, cytoplasm region, membrane region.

Region of Interest (ROI): A defined region in an image to which subsequent image analysis steps are restricted, e.g. object detection steps. Image parts outside any ROI are ignored. There may be multiple ROIs in one image.

Screening: The use of an assay to test a (large) set of compounds.

Segmentation: Breaking an image down into discrete objects of interest, e.g. whole cells, nuclei, spots and background. Segmentation is frequently done by →object detection(s).

Signal to Noise Ratio: A measure of how big the desired signal is compared to the noise level

$$SNR = \frac{\left| mean(\text{Negative Control}) - mean(\text{Positive Control}) \right|}{(stddev(\text{Negative Control}) + stddev(\text{Positive Control}))}$$

Signal Window: A measure of how big the difference between the minimum and maximum →readout value is. In case of multiple readout values, each readout value has a different signal window.

$$W = \left| mean(\text{Negative Control}) - mean(\text{Positive Control}) \right|$$

Single Cell Results: Individual readout values for each cell or object in a well (in contrast to →well results).

Stack: A set of images taken at different heights throughout the sample but at the same x-y-position (→field) in a well. See also →plane.

Sublayout: Defines the positions of image fields inside a well.

Subpopulation: Part of a population that matches a specific criterion, e.g. all cells responding to an agonistic compound.

Texture Analysis: Analysis of the intensity pattern of a fluorescent marker or brightfield transmission image, e.g. its granularity or smoothness, to quantify a biological effect and generate a →readout value.

Time Series: A set of images typically taken at 5-30 different time points in order to monitor changes over time (short time lapse movie). Typical time steps range from 1 minute to 1 hour.

Validation: Proofing the performance of an assay, including the image analysis.

Well Results: The →assay readout values for a single well.

Well: The sample chamber of a microplate.

Widefield Imaging: The standard setup for microscopic image generation, illuminating and observing the whole →field of view at the same time. See also →confocal imaging.

Z' Value (Z prime): A number, which measures the accuracy and sensitivity of an assay (including the image analysis algorithm). High Z' values indicate low noise and a large →signal window. Z' values above ~0.4 are considered sufficient for screening cellular assays, Z' values above ~0.6 are considered a good value. The theoretical maximum Z' value is 1.0 (= no noise). [Zhang *et al., 1999*]

$$Z' = 1 - \frac{3 * (stddev(\text{Negative Control}) + stddev(\text{Positive Control}))}{|mean(\text{Negative Control}) - mean(\text{Positive Control})|} \quad = \quad 1 - \frac{3}{SNR}$$

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References

Operetta CLS User Manual

➤ Operetta CLS Application Guide

➤ Harmony Help Function (built into the Harmony Software)

➤ Operetta CLS User Manual (hardware and software description)

➤ Operetta CLS Safety Manual

➤ Operetta CLS Site Readiness Instructions

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Literature

Zhang, J.H., Chung, T.D., Oldenburg, K.R. (1999) "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays". J. Biomol Screen. 4(2), pp. 67–73.