

# OPERETTA<sup>®</sup>



## Quick Guide

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# **1 Introduction**

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This Quick Guide serves to exemplify the required steps for developing an experiment quickly and easily. It only represents very basic measurements, but it is a good point to start from. The following chapters explain also additional functions and procedures such as creating an analysis sequence, evaluation of a measurement and creating an assay layout.

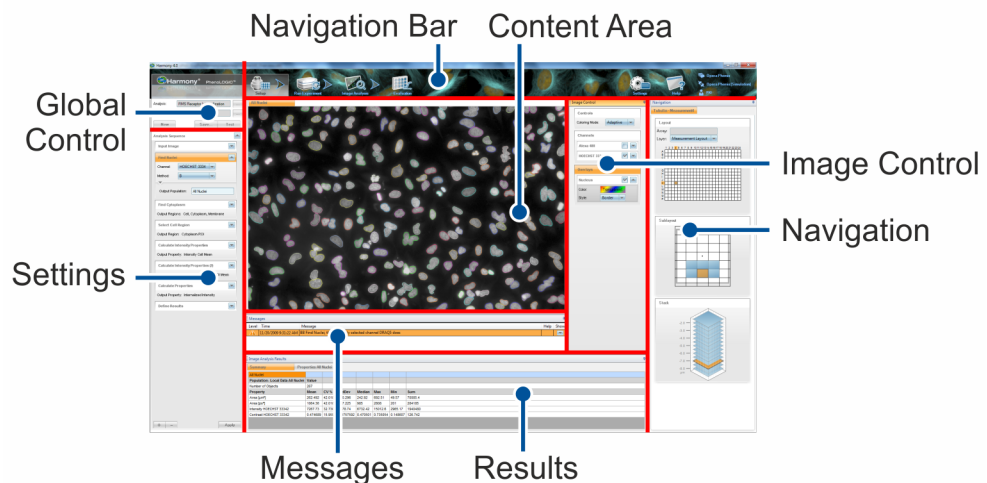
For a detailed description of cellular imaging and image analysis please see the **Operetta Application Guide** and the **Image Analysis Guide**. You can download these PDF guides using the function **Request Application Guide** in Harmony.

This Quick Guide is divided into two parts:

- The first part contains a simple experiment to learn the basic methods for setting up a measurement and for creating an analysis sequence from scratch.
- The more complex experiment in the second part of the Quick Guide should extend the knowledge learned in the first experiment.

## 1.1 **Harmony Overview**

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## **2 Start Up**

---

**Notice**

Do not open the lid without prior permission from Harmony or without using **Open Lid**, especially not during start-up or while components inside the Operetta are being moved. See also **Operetta User Manual**, section "Open Lid".

## 2.1 ***Starting Operetta and Harmony***

---

1. Switch on the Operetta.

The instrument is initialized (status light in balcony is blinking blue). As soon as the status light stops blinking (steady blue), the Operetta is ready and can be used. See also section "Instrument Status".

2. Switch on the Xenon light source.
3. Switch on the Harmony PC and log into Windows®.
4. Double-click the Harmony icon on the desktop.  
Harmony is started and a login dialog appears.
5. Log into Harmony (see section "Login").



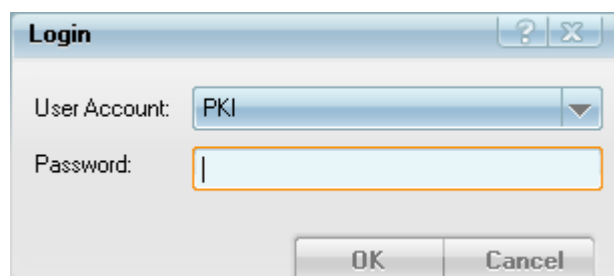
## 2.2 Login

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1. Double click the Harmony icon on the desktop to start the application.



2. After starting the Harmony software, the connections to the database, to the Harmony Engine and to the hardware are checked.
3. After initializing the Operetta, the **Login** dialog is displayed where you can select a user account and enter the corresponding password.



The initial user account is **PKI** with password **PKI**. This account has administrative rights and should only be used for the first login. You can (and should) create new user accounts for each Operetta user. For details see **Operetta User Manual**, section "User Accounts (Harmony)".



## **3    *Simple Experiment***

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## 3.1 Experiment Description

### Goal

In this simple experiment the whole cells should be detected and number of cells, cell area and cell shape should be analyzed.

### Plate Layout

On the **HCS Application Plate** you can find two regions with HeLa cells, which are treated in different ways to enable several readouts.

PerkinElmer For the Better		HCS Application Plate OrderNo: HH12970012																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A																									
B																									
C																									
D				0	0.003	0.008	0.016	0.03	0.08	0.16	0.3	0.8	1.6												
E																									
F																									
G																									
H																									
I																									
J																									
K																									
L																									
M																									
N																									
O																									
P																									

HeLa cells, 5000 cells/well, Demecolcine treated; conc. in [µM], anti- $\alpha$ -tubulin – AlexaFluor® 488 labeled and anti-phospho-histone H3 – AlexaFluor® 647 labeled, nuclei stained with Hoechst 33342 and actin with TRITC – Phalloidin

HeLa cells, [cells/well], untreated, stained with DRAQ5™ and CellMask™ Green

HepG2 cells, [cells/well], untreated, stained with Hoechst 33342

FocalCheck™ Beads, 15 µm green ring stain, dark red throughout

Store at +4°C

For the first experiment described in the Quick Guide we are interested in the region I4 - I13.

### Staining

The cells in this region are just stained with DRAQ5. This dye stains predominantly the nuclei, but also to a lesser extent the cytoplasm, so a robust detection of the whole cell can be achieved.

## 3.2 Setup

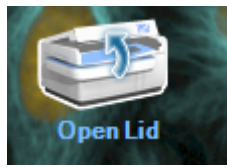
On the **Setup** screen the experiment is defined. The **Global Control Settings** contain all global parameters which are fix during the measurement. In **Channel Selection** you configure the optical parameters relating to the respective dye (exposure time, focus height, filter settings) and the order of taking the images. In **Layout Selection** the plate layout including well layout and stack for the measurement is defined.

### 3.2.1 Insert Plate

#### Notice

To avoid errors or the loss of data, do not open the Operetta's lid without prior permission from Harmony or without using **Open Lid**. Do not open the lid before the Operetta's progress LEDs display an arrow to indicate that the lid may now be opened.

1. Click the **Open Lid** button in the Navigation bar.  
The status light of the balcony starts blinking blue.



2. Wait until the table inside the Operetta has been moved to a defined position and the status light shines blue continuously.
3. The progress LEDs on the front of the lid display an arrow to indicate that the lid may now be opened. A message box is displayed: "You may open the lid now."



4. Confirm the message with **OK**.
5. Open the lid and insert the HCS Application Plate.  
The arrow reminds you of closing the lid.



6. Close the lid when you are finished.  
The progress LEDs are switched off. The next time you select a command which involves moving the table (e.g. snapshot), a table homing is performed first.

### 3.2.2 Global Control Settings

Experiment:  ...

Plate Type: 384 PerkinElmer CellCarrier

Objective: 20x long WD

Opt. Mode: **Non-Confocal** Confocal

Excitation: 50 %

Transmission: 0 %

Live Preview:

Max Duration: 0h 1min

Temperature: off

CO2: off

New Save... Test

Procedure for creating a new experiment:

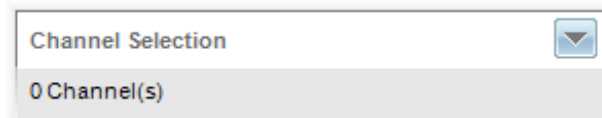
1. Click **Setup** in the Navigation bar if this screen is not already opened.




2. Field **Experiment** stays empty. The experiment name is inserted automatically after saving.
3. Select **Plate Type**: 384 PerkinElmer CellCarrier for the HCS Application Plate.
4. Select **Objective**: 20x long WD.
5. Select **Opt. Mode**: Non-Confocal (only relevant for Operetta with confocal upgrade).
6. Set **Excitation** to 50%. **Transmission** is not used.

### 3.2.3 Channel Selection

1. Activate the **Channel Selection** pane by clicking the disclosure button .

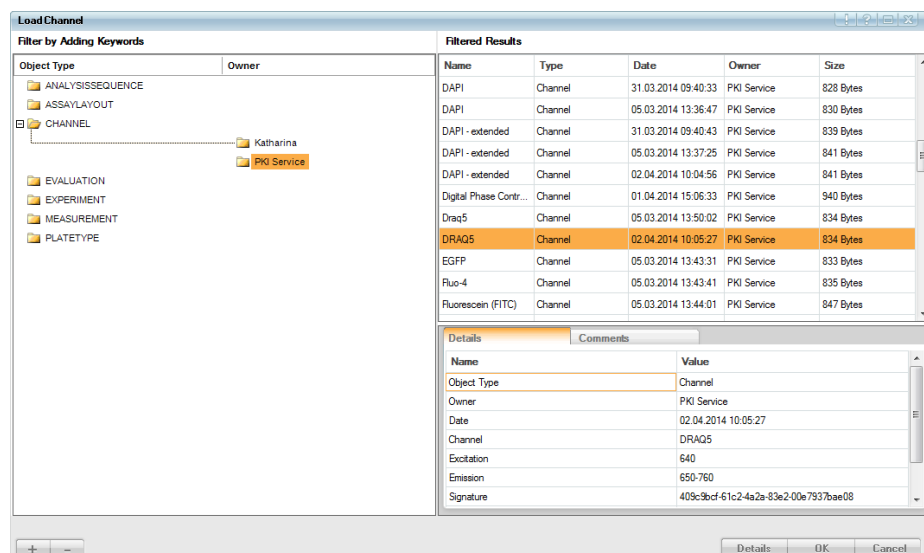



2. Click  to open the database browser for adding the needed channel.

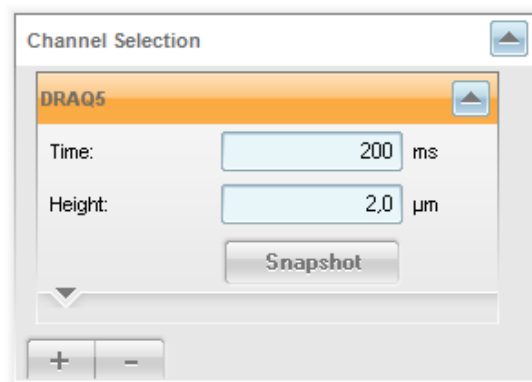


In this experiment you need only the **DRAQ5** channel.

3. Select the predefined channel **DRAQ5**.

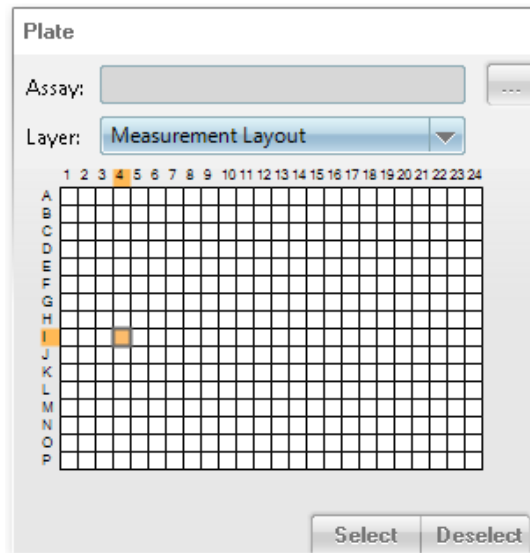


4. Click . The dialog closes and the selected channel is loaded using default values for exposure time (200 ms) and focus height (2.0  $\mu\text{m}$ ).

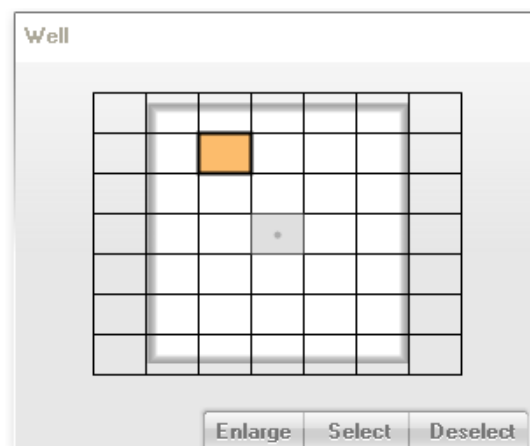


Now you may have to check and modify the channel settings:

1. Activate the **Navigation** pane and open the **Define Layout** window.
2. Select one well in the region of interest (I4 - I13) in the **Plate** window.



3. Select one field in the well layout. It is advisable to choose a well outside the center to avoid bleaching of the cells you want to measure afterwards. If you don't select a well layout field, the central field is taken by default.

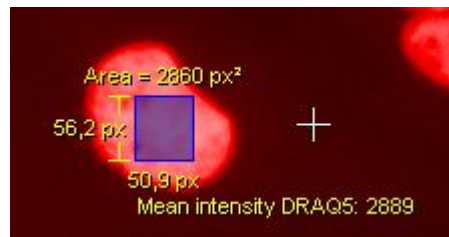


4. Go back to **Channel Selection** and click **Snapshot** to take an image with the corresponding exposure settings of this channel. The image will be shown in the content area.
5. Check visually if the cells are focused. Edit the value for height and take a snapshot image until you are satisfied with the sharpness of the image. In this example the best height was at 5  $\mu\text{m}$ .
6. Check the brightness: Right click into the image to open the context menu and select **Measure Rectangle**.

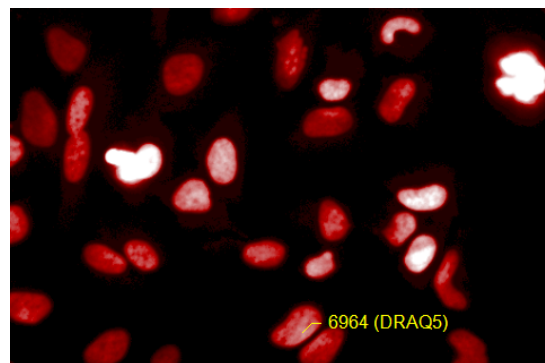
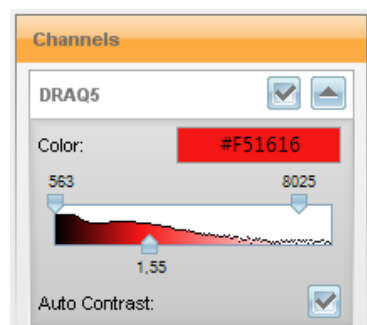
If you now select a small area within a typical object, you will get the mean pixel intensity of the selected area for each channel. The brightest spots should not have more than 16.383 counts. Nuclei should be as bright as possible, most



suitable brightness would be 4000-8000 counts. You can adjust the intensity by changing the exposure time or the excitation power. For details see the **Operetta Application Guide**, section “How to select the focus height, excitation power and exposure time”.



- If you want to adapt the color and the contrast of the image, you can open the image control window to modify these settings.

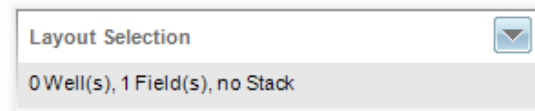


- Please check your settings in another well. Click  to get a new image.
- If you are satisfied with your pictures close the block **Channel Selection** and go on with **Layout Selection** for creating the measurement layout.

### 3.2.4 **Layout Selection**

The **Layout Selection** pane is used for the definition of wells (layout), image fields (well layout) and/or z-planes (stack) which shall be measured. In this experiment we want to measure the wells I4 – I13 to analyze the nuclei which are stained with DRAQ5. Furthermore we want to create a well layout with 2 fields as well as a stack with 3 planes. The creating of the stack is just an exercise as we do not want to use the stack for the measurement.

- Activate the **Layout Selection** pane by clicking the disclosure button .

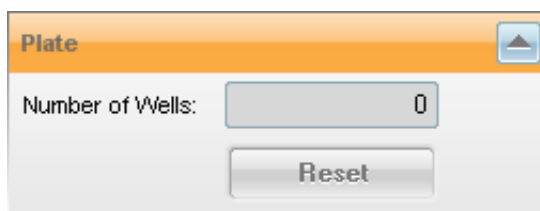


### 3.2.4.1 **Layout definition**

1. Activate the layout block by clicking the disclosure button.

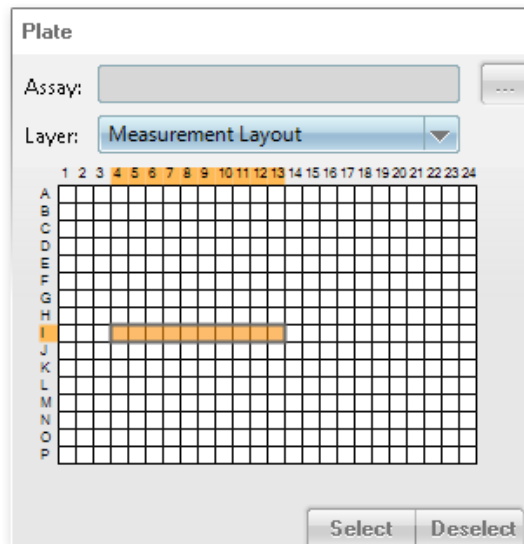


2. The layout block opens and the headline becomes orange to mark the active block.




The **Plate** window (**Navigation - Define Layout**) becomes active and the **Select / Deselect** buttons appear.

3. Define the layout (**Navigation – Define Layout – Plate**) by marking the wells I4 – I13 for this experiment.




4. Click **Select**. The number of selected wells will be displayed in the layout block and the wells will turn gray.

**Plate** 

Number of Wells:

**Plate**

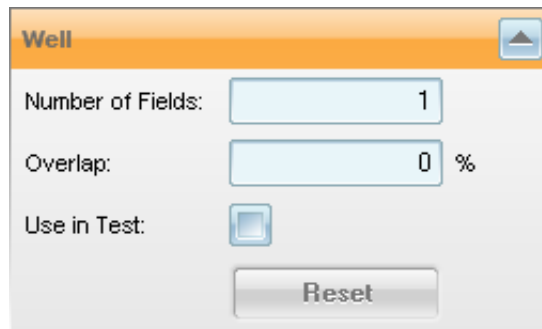
Assay:  

Layer:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

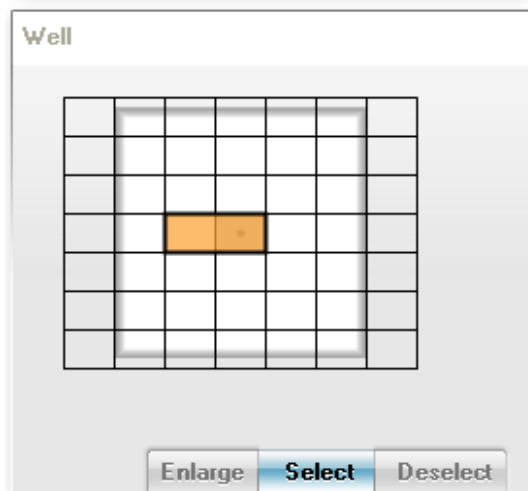
### 3.2.4.2 Well layout definition

1. Activate the **Well** block by clicking the disclosure button. The headline becomes orange to mark the active window.

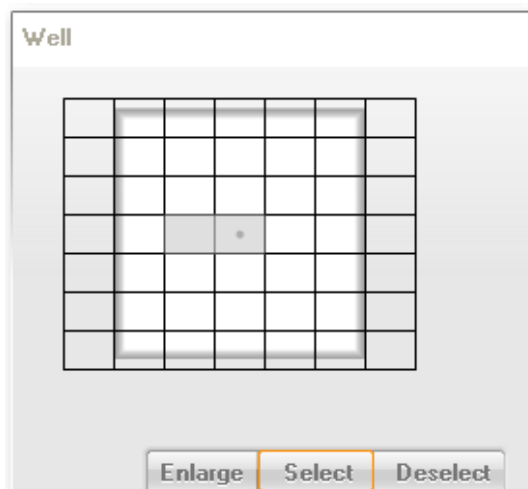


The **Well** window (**Navigation - Define Layout**) becomes active as well.

2. Mark two fields in the middle of the well (**Navigation – Define Layout – Well**).



3. Click **Select**. The number of selected fields are displayed in the **Well** block and the selected fields become gray.



The **Use in Test** checkbox is automatically activated as soon you change the number of fields. The well layout will now be used in the test measurement.

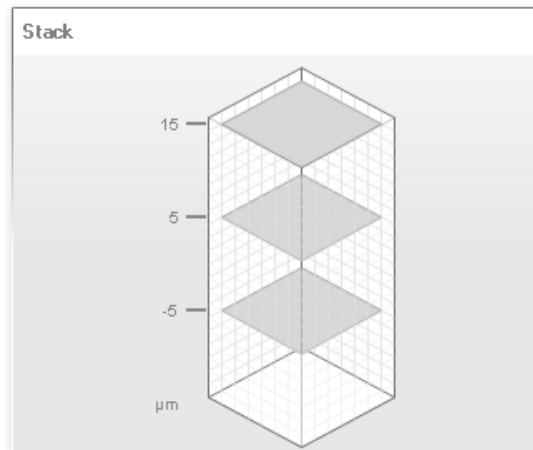
### 3.2.4.3 Stack definition

1. Activate the stack block by clicking the disclosure button.

2. Insert your stack definition:  
 First Plane at: -5  $\mu\text{m}$   
 Number of Planes: 3  
 Distance: 10  $\mu\text{m}$

**Use in Test** should be activated to include the stack in the test measurement.

3. This stack definition is displayed in the **Navigation – Define Layout – Stack** pane.



With these parameters you will get one sharp image at the height of 5  $\mu\text{m}$  and two defocused images underneath and above.

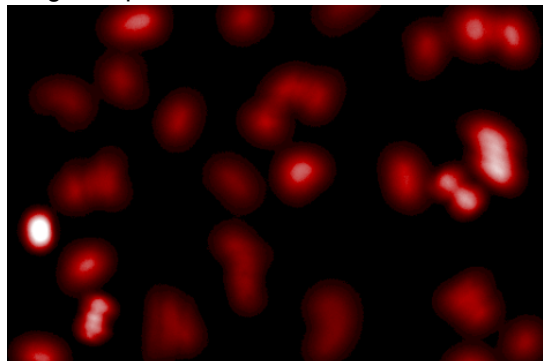
4. Close the **Layout Selection** block.

### 3.2.5 **Test Measurement**

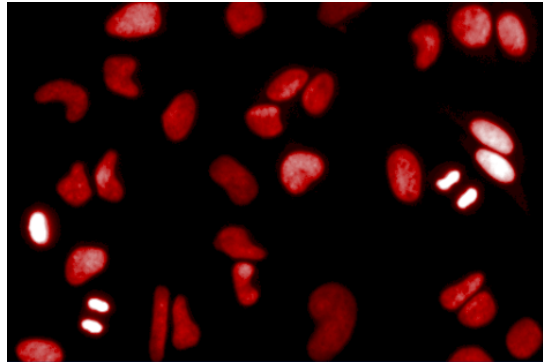
With this function you can check the complete measurement routine in one well. In our example this will create a set of 6 images per well. Under **Test Images** you can select these images for display in the **Content Area**.

1. Select one well in the **Plate** window (**Navigation - Define Layout**) if not selected previously.
2. Click **Test** in the Global Control section to start a test measurement.
3. Open **Navigation - Test Images**.
4. In the **Plate** pane click the blue well. The first image of the well (center field, bottom plane) will be displayed in the **Content Area**.
5. Select a different field in the **Well** pane or a different plane in the **Stack** pane.
6. The following images show the differences in the sharpness depending on the height. As expected, the image is diffused at -5  $\mu\text{m}$ , with a height of 5  $\mu\text{m}$  we have a sharp image and at 10  $\mu\text{m}$  the image is diffused again.

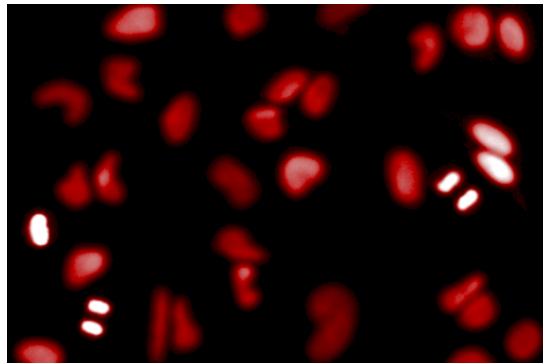
Height: -5  $\mu\text{m}$



Height: 5  $\mu\text{m}$



Height: 15  $\mu\text{m}$



7. To check further images in other wells please select another well in **Navigate - Define Layout – Plate** and click **Test**.
8. As we do not want to use blurred planes in our experiment please open **Layout Selection – Stack** again and click  to deactivate the stack function.
9. Now you can complete the experiment setup by saving it.

### 3.2.6 Save Experiment

1. Click  in the **Global Control** section. The **Save Experiment** dialog appears.
2. Insert an experiment name: *Quickguide Exp1*. Optionally you can add a comment.
3. Click . Your experiment is saved to the database.

### 3.3 **Run Experiment**

On the **Run Experiment** screen you start your automatic measurement. Results can be viewed on the **Evaluation** screen if an online analysis was defined in the experiment.

1. Click **Run Experiment**.



2. The newly created experiment is already loaded and the global parameters are displayed:

**Plate Type:** 384 PerkinElmer CellCarrier  
**Objective:** 20x long WD.

3. Insert **Plate Name:** e.g. Training Plate.
4. Press **Start**.

 A screenshot of a software configuration window titled "Run Experiment". The window has a white background and a grey border. It contains several settings:
 

- Control Mode:** Two buttons, "Local" (highlighted in blue) and "Remote".
- Experiment:** A text box containing "Quickguide Exp1" and a dropdown arrow on the right.
- Analysis:** A section header.
- Plate Type:** A text box containing "384 PerkinElmer CellCarrier".
- Objective:** A text box containing "20x long WD".
- Assay Layout:** A text box with a dropdown arrow on the right.
- Plate Name:** A text box containing "Training Plate".
- Shutdown:** A checkbox that is currently unchecked.
- Max Duration:** A text box containing "0h 13min".
- Temperature:** A text box containing "off".
- CO2:** A text box containing "off".
- Start:** A button located at the bottom right of the window.

5. The measurement starts running. The measurement progress is indicated by blue color in the layout wells, fields or planes.

During and after the measurement you can click on the blue wells in the **Plate** window (**Navigation - Measurement**) and view the images.



### 3.3.1 **View Images**

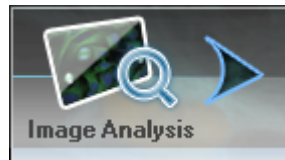
1. Open the **Navigation – Measurement** window.
2. To display an image click a measured well (blue) in the **Plate** pane. The first image in this well is displayed.
3. Select a different field in the **Well** pane if desired.

## 3.4 *Image Analysis*

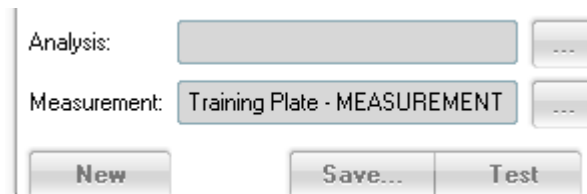
---

The **Image Analysis** pane enables the definition of an image analysis sequence by assembling predefined algorithm parts (so called building blocks). This analysis can be applied to the images after the measurement if no online analysis was defined during setup.

1. Click **Image Analysis** in the Navigation bar.



2. The current measurement is already loaded.



### 3.4.1 *Analysis Sequence*

As a first step you have to select a representative image on which you want to start developing your analysis method.

1. In the **Navigation – Measurement** window browse through different wells using the visual feedback in the **Content Area**.
2. Select one image for developing your analysis method.

We will now create a simple analysis sequence consisting of five building blocks which will give you the read-out results **Number of Cells**, **Cell Shape** and **Cell Area**.

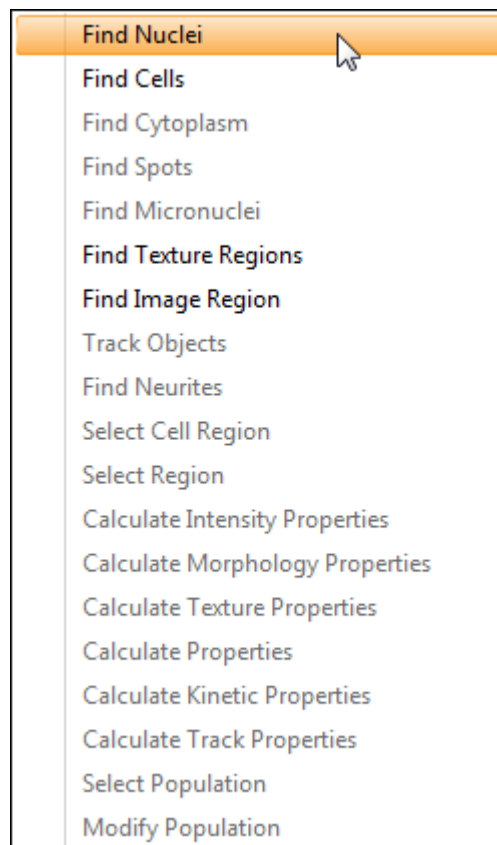
### 3.4.1.1 Building Block: Find Nuclei

1. Open the **Analysis Sequence** pane by clicking the disclosure button.

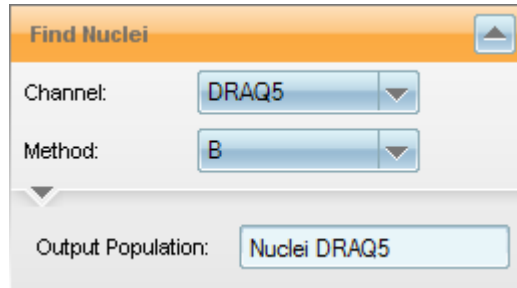


As default there are two building blocks:

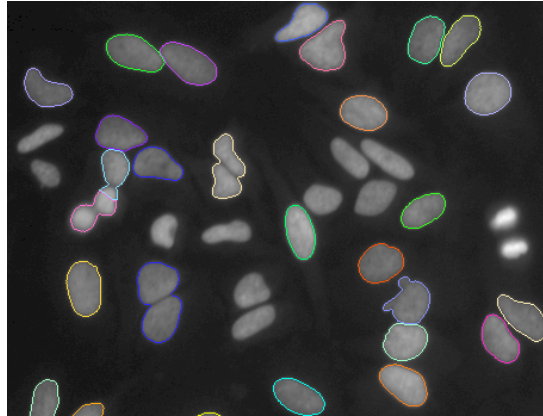
- **Input Image:** Beginning of the analysis sequence. Open this building block to view the original image without overlays.
  - **Define Results:** In this last building block you will define the desired readout parameters. These results will be saved to the database.
2. Click the **Plus** button to add a new building block.
  3. Select the building block **Find Nuclei** from the popup menu.



4. The building block **Find Nuclei** is inserted into the analysis sequence.



5. The nuclei detection is running with these predefined parameters.

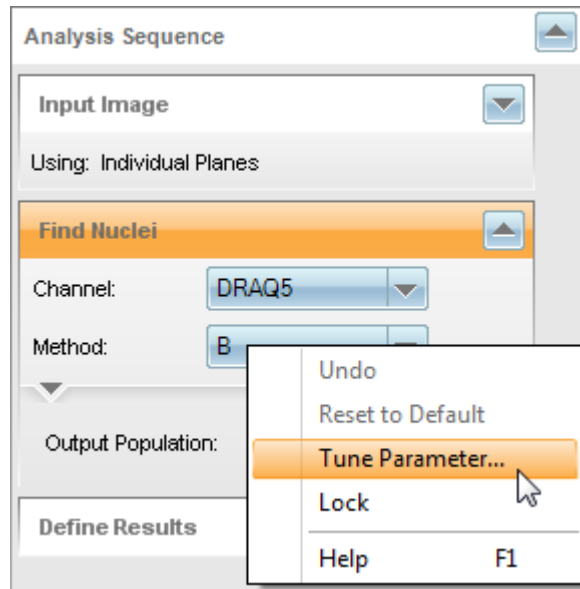


As you can see not all nuclei were detected so you have to tune some parameter for this building block.

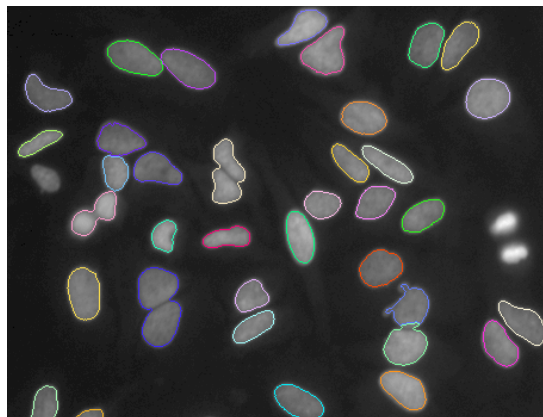
### 3.4.1.2 **Tune parameter: Method**

To get an optimal result you have to tune some parameters. First you need to tune the method:

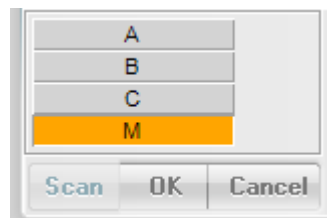
1. Right click on the parameter **Method** to open the context menu.
2. Select **Tune Parameter**: All four methods will be calculated (this may take a while).



- Now the results of all four methods can be displayed to find the most suitable result. Movement of the cursor over the method makes even small changes compared to the selected method (marked orange) visible to the human eye. The selected method serves as a reference to the other methods. In this example method **M** fits the best and can be selected.



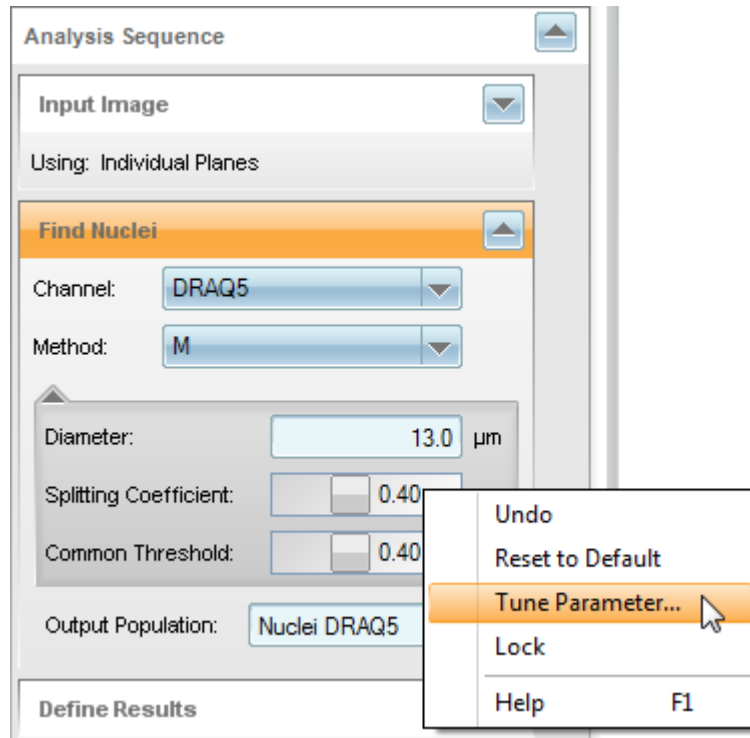
- Click **OK** to apply method **M** to the building block.



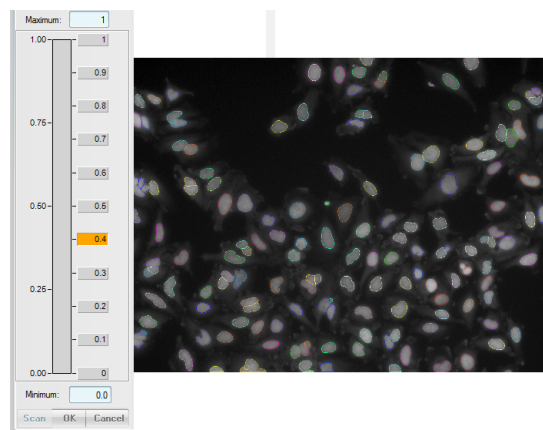
### 3.4.1.3 *Tune parameter: Splitting Coefficient*

As you can see the separation of the nuclei is not sufficient. For this reason, expand the nested pane and go on with tuning the parameter **Splitting Coefficient**:

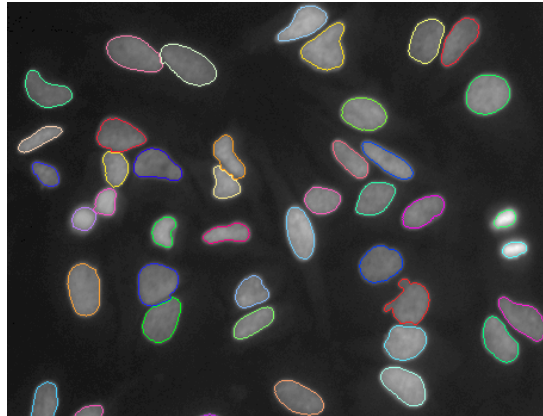
1. Right click on the parameter **Splitting Coefficient** to open the context menu.
2. Select **Tune Parameter**.



Several splitting coefficients are calculated and are displayed by moving the cursor over the values of the scale on the left side of the image.



3. With a splitting coefficient of 0.35 all nuclei are separated as required.



4. Select this value and click **OK** to transfer it to the building block.

 A screenshot of the 'Find Nuclei' software dialog box. The title bar is orange and contains the text 'Find Nuclei'. Below the title bar, there are several controls:
 

- 'Channel:' dropdown menu set to 'DRAQ5'.
- 'Method:' dropdown menu set to 'M'.
- 'Diameter:' input field with the value '13.0' and the unit 'µm'.
- 'Splitting Coefficient:' slider control with the value '0.35'.
- 'Common Threshold:' slider control with the value '0.40'.
- 'Output Population:' input field with the text 'Nuclei DRAQ5'.

#### 3.4.1.4 **Building Block: Find Cytoplasm**

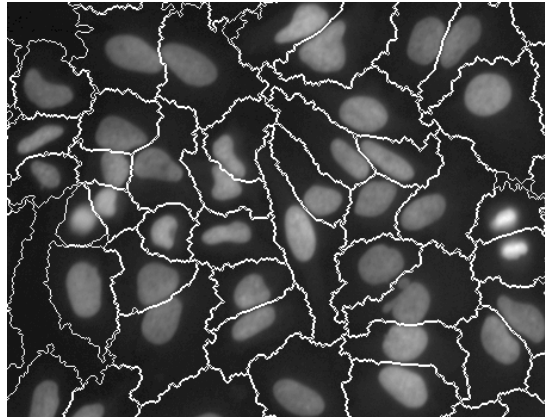
With the next analysis step you want to detect the whole cells. With a dye which stains as well the nuclei as the cytoplasm, we can differentiate the cytoplasm from the background.

1. Add a second building block: **Find Cytoplasm**

 A screenshot of the 'Find Cytoplasm' software dialog box. The title bar is orange and contains the text 'Find Cytoplasm'. Below the title bar, there are several controls:
 

- 'Channel:' dropdown menu set to 'DRAQ5'.
- 'Nuclei:' dropdown menu set to 'Nuclei'.
- 'Method:' dropdown menu set to 'A'.
- 'Individual Threshold:' slider control with the value '0.15'.

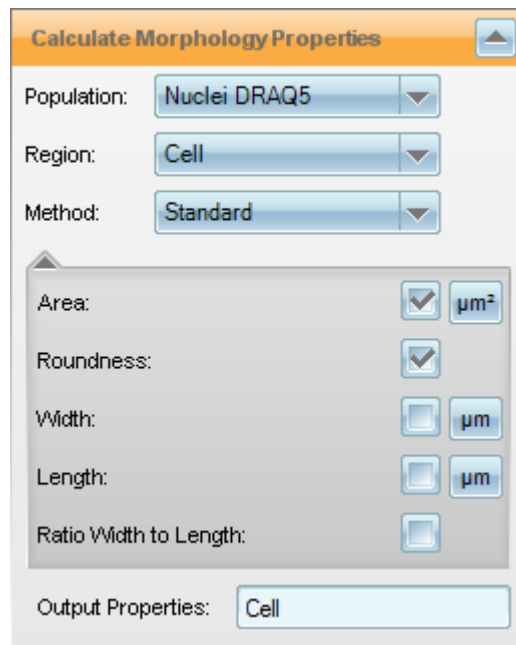
2. The cytoplasm detection should look like this:



### 3.4.1.5 Building Block: Calculate Morphology

This building block serves for calculating morphological parameters of the detected cells as basis for the final results.

1. Add the building block: **Calculate Morphology Properties**

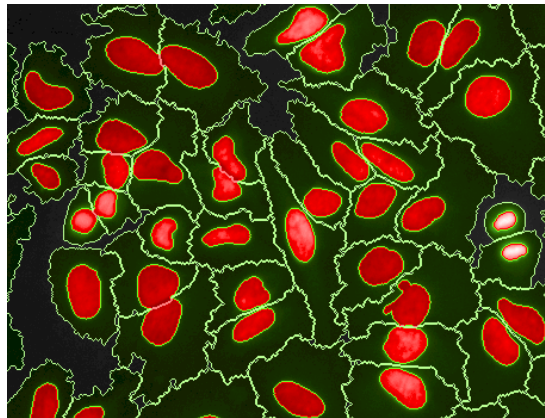
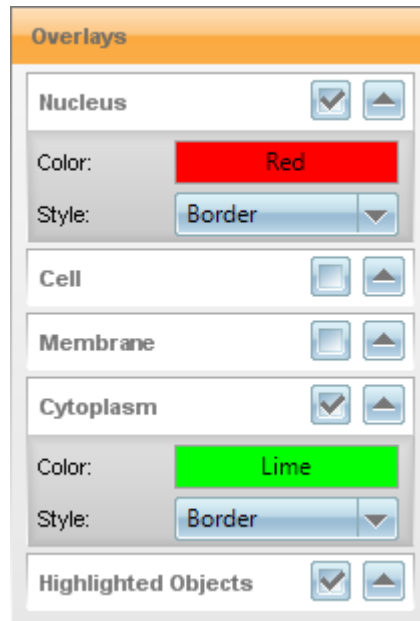


2. The standard method for the cell region calculates all cell properties, which are displayed in the private results (**Image Analysis Results**) for this building block:

Object No	Cell Area [µm <sup>2</sup> ]	Cell Roundness
1	95.9366	0.666537
2	37.7334	0.668055
3	56.7235	0.720607
4	865.156	0.586903
5	1041.99	0.54818
6	620.012	0.66149



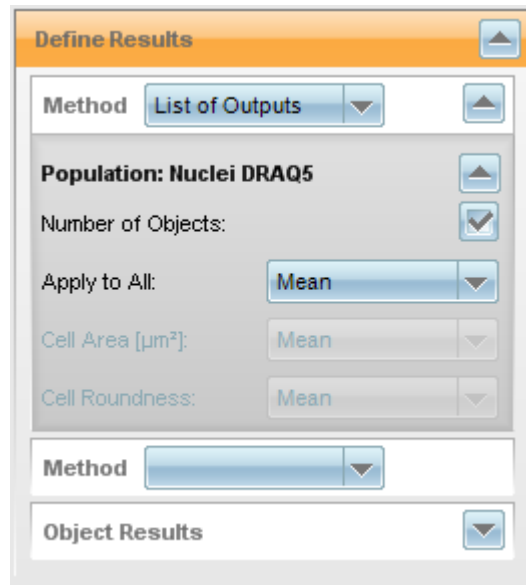
3. Displaying the detected cells in different colors may help you in distinguishing single objects: Open the **Image Control** window to select region and color for the overlay.



### 3.4.1.6 Building Block: Define Results

Now we define the readout values which will be saved as **Evaluation** in the database. **Define Results** is always the last building block in the analysis sequence.

1. Create your output as follows:



### 3.4.2 Test Analysis

The analysis sequence is now complete and you can test it with single images. Test the analysis in some other fields or wells and adapt the parameters if necessary. You will find the results under **Image Analysis Results**. These results are transient and not saved to the database.

### 3.4.3 Save Analysis

Once you are satisfied with the results you can save your analysis sequence to the database:

1. Click **Save...** in the **Global Control** section.
2. Insert an analysis name: *Quickguide Exp1*.
3. Click **OK**. The analysis sequence is saved to the database.

## 3.5 Evaluation

The **Evaluation** pane serves for applying an analysis sequence to a complete measurement (as opposed to single images on the previous screen).

1. Click **Evaluation** in the Navigation bar.



2. Make sure that **Single** mode is activated.
3. The current measurement and analysis sequence are already loaded. Otherwise you have to load your measurement and analysis sequence from the database using the [...] buttons.

Mode:  **Single**  Batch

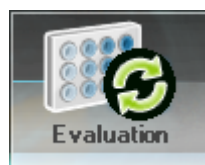
Experiment: Quickguide Exp1

Measurement: Training Plate - MEASUREMENT

Analysis: Quickguide Exp1

Evaluation:

4. Open **Navigation - Measurement**: Select wells and fields to be evaluated.
5. Click **Start**.



The evaluation starts and the results are displayed in a table (**Evaluation Results**).

Evaluation Results									
Well Results									
Display	Use for Z'	Row	Column	Plane	Timepoint	Nuclei - Number of Objects	Nuclei - Cell Area [ $\mu\text{m}^2$ ] - Mean per Well	Nuclei - Cell Roundness - Mean per Well	Number of Analyzed Fields
<input checked="" type="checkbox"/>		I	4	1	0	227	937.5	0.7361	1
<input checked="" type="checkbox"/>		I	5	1	0	192	950.9	0.7319	1
<input checked="" type="checkbox"/>		I	6	1	0	221	962.7	0.7433	1
<input checked="" type="checkbox"/>		I	7	1	0	199	1000	0.7152	1



## **4    *Complex Experiment***

---

## 4.1 Experiment Description

### Goal

In this more complex experiment you will calculate the mitotic index of a cell population. The mitotic index is a measure for the proliferation 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 the cell cycle can be identified using e.g. antibodies against the phosphorylated epitope of a core histone protein (pHH3). This marker leads to specific fluorescence in the nuclei of cells.

### Plate Layout

For this experiment we are interested in the region C4 – E13.

		HCS Application Plate																								
		OrderNo: HH12970012																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A																										
B																										
C																										
D					0	0.003	0.008	0.016	0.03	0.08	0.16	0.3	0.8	1.6												
E																										
F																										
G																										
H																										
I																										
J																										
K																										
L																										
M																										
N																										
O																										
P																										

**HeLa cells, 5000 cells/well, Demecolcine treated; conc. in [µM], anti- $\alpha$ -tubulin – AlexaFluor® 488 labeled and anti-phospho-histone H3 – AlexaFluor® 647 labeled, nuclei stained with Hoechst 33342 and actin with TRITC – Phalloidin**

**HeLa cells, [cells/well], untreated, stained with DRAQ5™ and CellMask™ Green**

**HepG2 cells, [cells/well], untreated, stained with Hoechst 33342**

**FocalCheck™ Beads, 15 µm, green ring stain, dark red throughout**

**Store at +4 °C**

### Staining

- Nuclei stained with Hoechst 33342.
- Tubulin immunostained with Alexa 488 anti-tubulin antibody (as a reference signal for observing the cell morphology).
- pHH3 immunostained with Alexa 647 anti-pHH3 antibody to mark the mitotic cells.

### Compound

In this region we have a titration of Demecolcine from left to right. Demecolcine is a “mitotic poison” due to its ability to cause microtubule depolymerisation. This arrests the cells in the M-phase and increases the number of mitotic cells in the cell population.

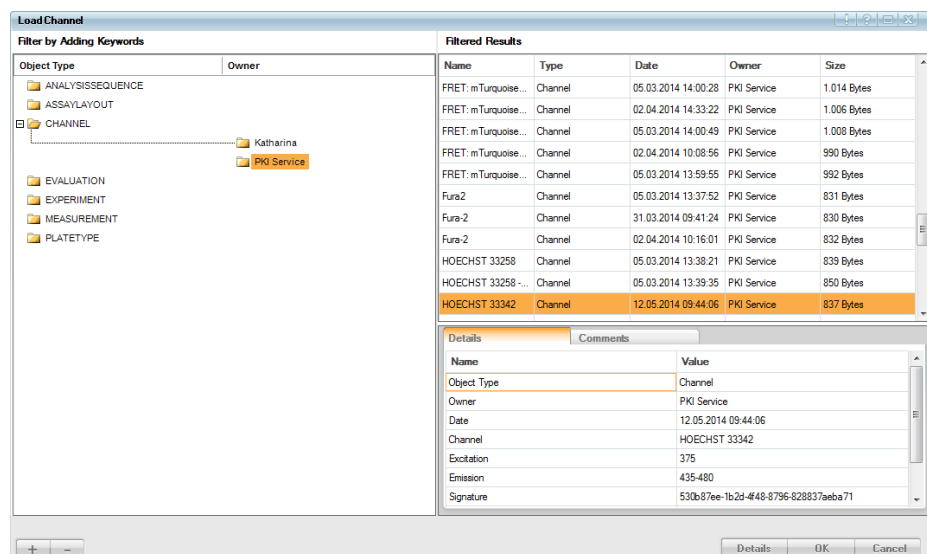
## 4.2 Setup

Now we want to create the complex experiment from scratch:

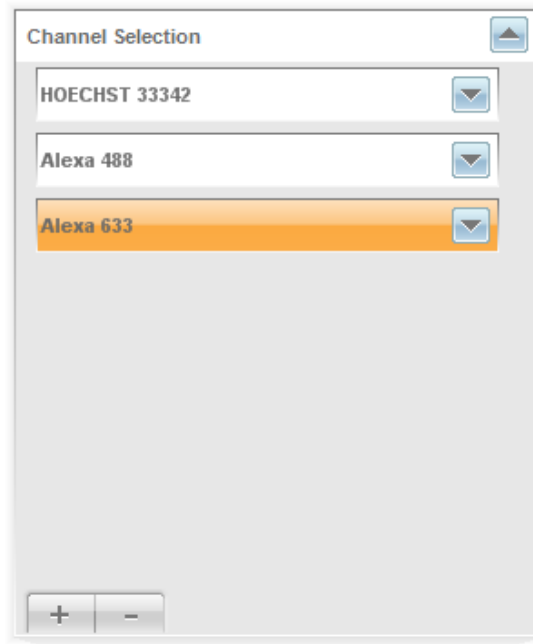
1. Click **Open Lid** in the Navigation bar and insert the HCS Application Plate.
2. Click **Setup** in the Navigation bar.
3. Select **Plate Type**: 384 PerkinElmer CellCarrier for the HCS Application Plate.
4. Select **Objective**: 20x long WD.
5. Select **Opt. Mode**: Non-Confocal (only relevant for Operetta with confocal upgrade).
6. Set **Excitation Power** (Xenon Light Source) to 50%.

### 4.2.1 Channel Selection

1. Activate the **Channel Selection** pane.
2. Click **+** to open the **Database Browser** for adding the required channels. In this experiment you need the three channels **HOECHST 33342**, **Alexa 488** and **Alexa 633**.
3. Select the predefined channel **HOECHST 33342** and click **OK**.

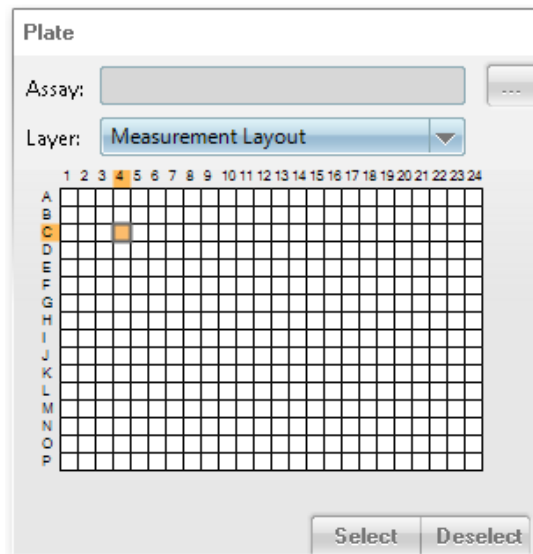


4. Repeat this for the channels **Alexa 488** and **Alexa 633** to create the whole channel sequence.

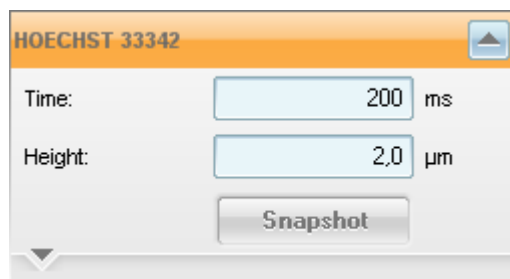


Now you may check and adapt the channel settings:



1. Select one well in the region of interest (C4 – E13) in the **Navigation – Define Layout – Plate** window.



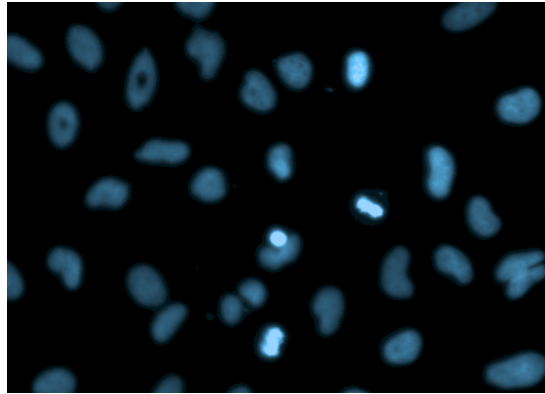
2. Click **Snapshot** to take an image with the corresponding exposure settings.



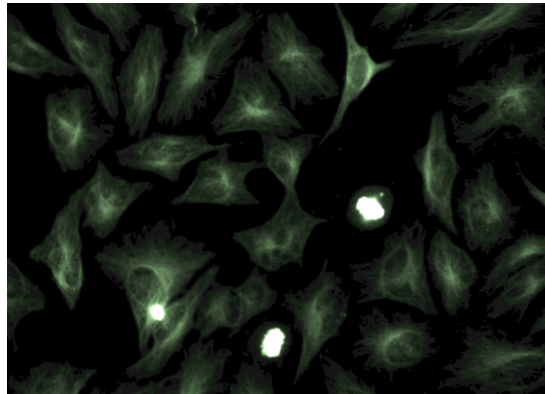


3. Check if the cells are focused. Modify the height if necessary. In this example the best height was at 3  $\mu\text{m}$ .
4. Check the brightness: Right click into the image opens the context menu where you can select **Measure Rectangle**. Select a small area within a typical object. The mean intensity of the area is displayed for each channel.
5. Please check your exposure settings in some other wells as they might have to be adapted due to variations in staining and assay signal. Regarding the **HOECHST 33342** and **Alexa 633** signal, you intend to reach a constant signal intensity .
6. Click  again to generate a new image and modify the settings if necessary.
7. Repeat these steps to check the channel settings for the remaining channels **Alexa 488** and **Alexa 633** (intensity for **Alexa 633** >4000).
8. Click  in the **Global Control** section to check the whole channel sequence.
9. If you are satisfied with your settings close the block **Channel Selection** and proceed with **Layout Selection** to create a plate layout and well layout.
10. After this procedure the images for each channel should look like this:

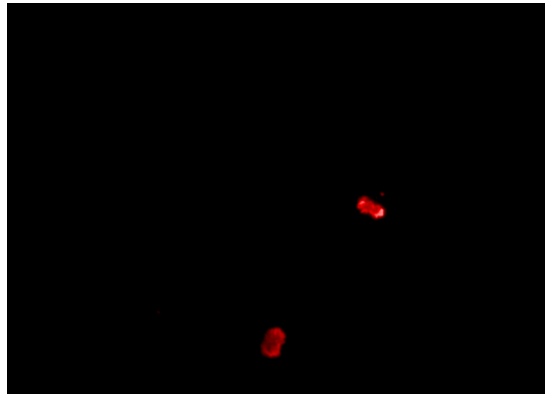
"HOECHST 33342 channel"



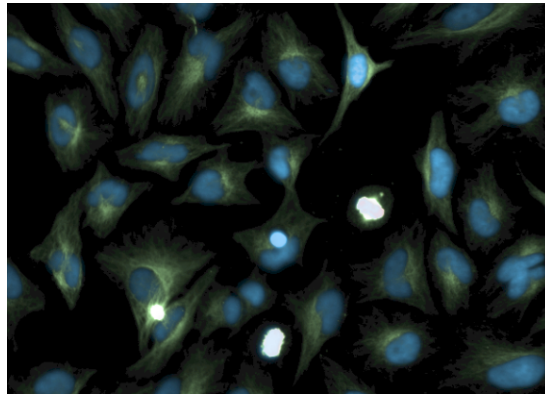
"Alexa 488 channel"



"Alexa 633 channel"



"Overlay 3 channels"



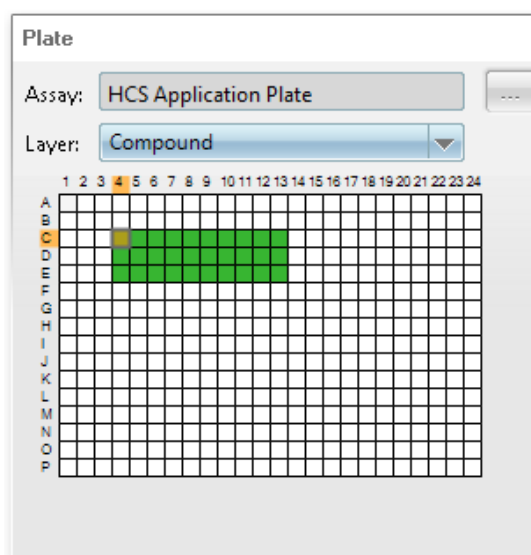
## 4.2.2 Layout Selection

In this experiment you will measure the wells C4 – E13. For a better orientation you will load a predefined assay layout first and then select the wells for the plate layout. Furthermore you will create a well layout with 5 fields, because the total amount of mitotic cells is low. Therefore a well layout with many fields is helpful for the calculation of the mitotic index.

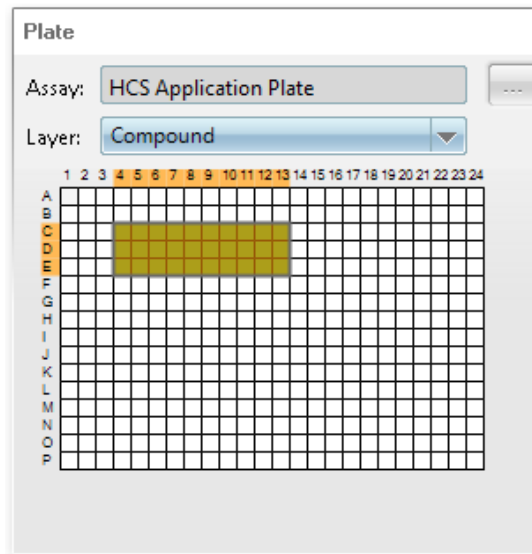
Within the Harmony software you have the opportunity to create an assay layout with information about the particular assay conditions in each well, such as compound name or concentration. This assay layout consists different layers which can be displayed during the actual measurement or analysis for a better orientation during setup, measurement and evaluation.

At this point you will open the predefined assay layout of the HCS Application Plate.

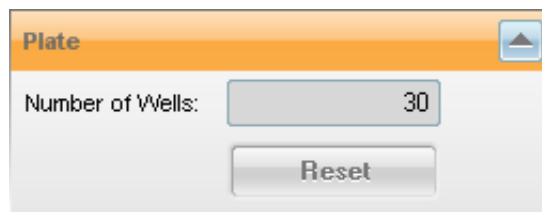
1. In **Navigation - Define Layout - Plate**: Click  to open the **Database Browser** and load the **HCS Application Plate** assay layout.
2. Now you can select a layer of this assay layout in the **Plate** window. Choose the layer **Compound**. The colors of the wells indicate how the plate was filled.



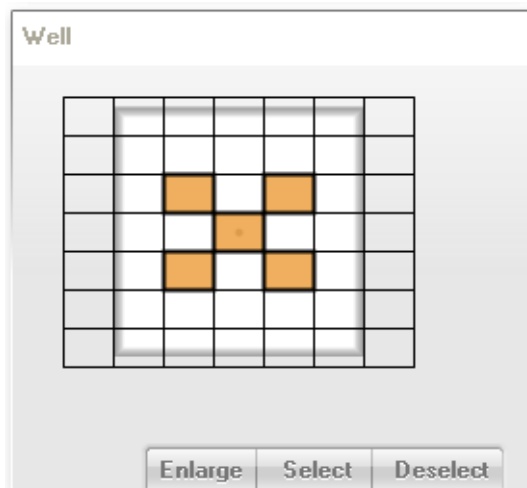
3. Mark the wells you want to measure (green area).



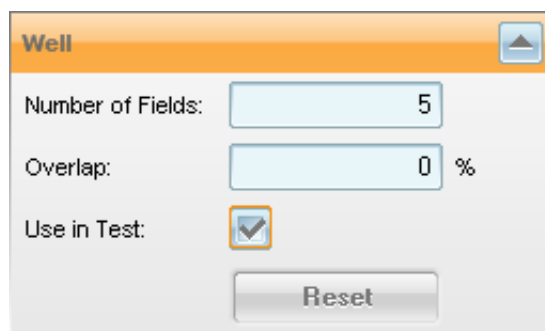
4. Click **Select**: The number of selected wells will be displayed in the **Plate** block and the wells turn gray.



5. In the **Well** pane mark 5 fields within the well.



6. Click **Select**. The number of selected fields will be displayed in the **Well** block and the fields turn gray.



7. **Use in Test** should be activated to include your well layout in the test measurement.

### 4.2.3 Test Measurement

With this function you can check the complete experiment in one well. In our example this will create a set of 5 images for the selected well.

1. Select one single well in **Navigation - Define Layout - Plate**.
2. Click **Test** in the **Global Control** section to start a test measurement in this well.
3. To view the images select the desired well and field in **Navigation - Test Images**. If you are satisfied with your image you can save your experiment.

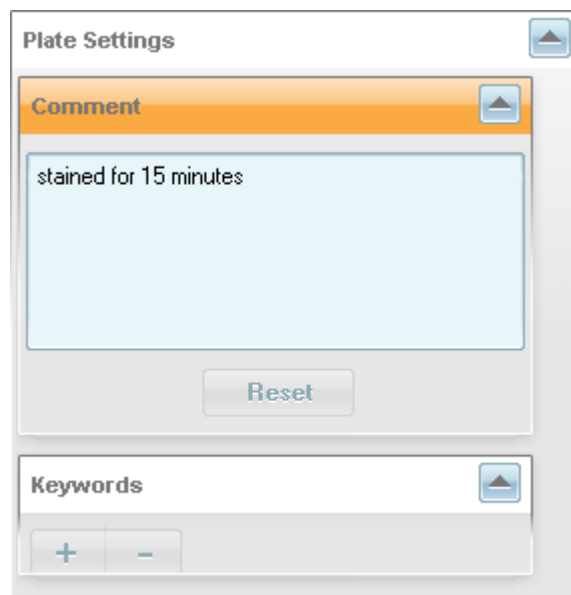
### 4.2.4 Save Experiment

1. Click **Save...** in the **Global Control** section. The **Save Experiment** dialog appears.
2. Insert an experiment name: *Quickguide Exp2*.  
Optionally you can add a comment to your experiment.
3. Click **OK**. The experiment will be saved to the database.

## 4.3 *Run Experiment*

---

1. Click **Run Experiment** in the Navigation bar.
2. The just created experiment is already loaded and the global parameters are displayed:  
**Plate Type:** 384 PerkinElmer CellCarrier  
**Objective:** 20x long WD.  
**Assay Layout:** HCS Application Plate
3. Enter **Plate Name:** Training Plate.
4. Optionally you can add some comments and keywords in the **Plate Settings** window.



5. Click **Start** .The measurement starts.

Control Mode: **Local** Remote

Experiment: Quickguide Exp2 ...

Analysis:

Plate Type: 384 PerkinElmer CellCarrier

Objective: 20x long WD

Assay Layout: HCS Application Plate ...

Plate Name: Training Plate

Shutdown

Max Duration: 0h 13min

Temperature: off

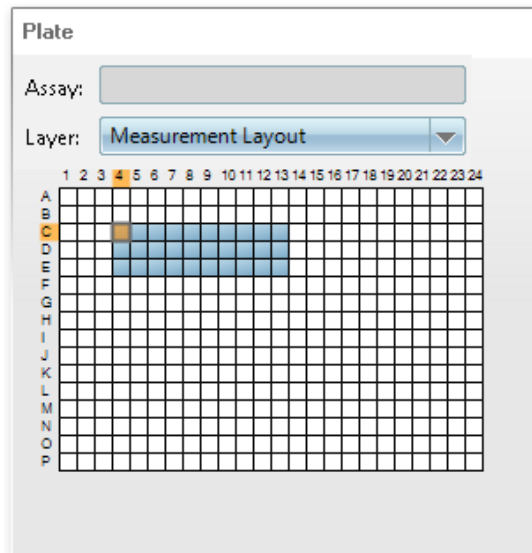
CO2: off

Start

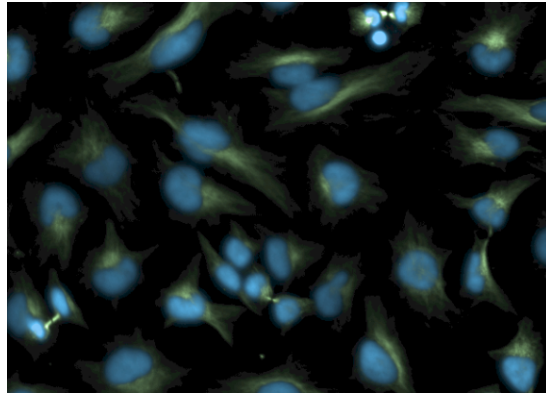
### 4.3.1 View Images

During and after the measurement you can click on the measured wells (blue) on the plate control and view the images.

1. Mark one measured well in **Navigation – Measurement – Plate**.



2. The first measured image in the well will be displayed (center field):




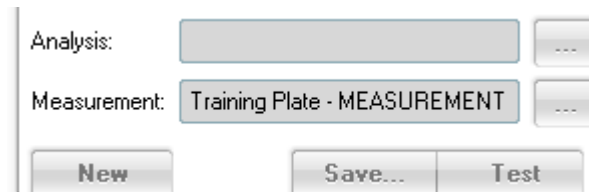
3. To view more images navigate via **Plate** and **Well** pane.




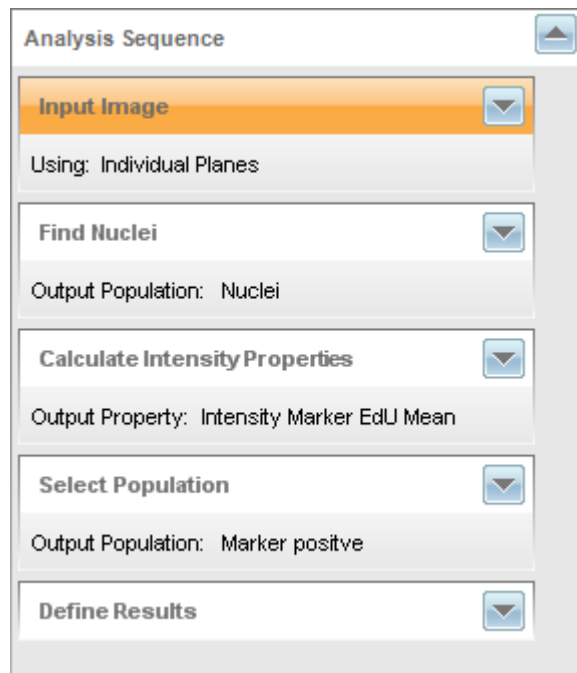
## 4.4 Image Analysis

For this experiment you need to use a Ready-Made Solution (RMS) to analyze the Mitotic Index.

1. Click **Image Analysis** in the Navigation bar.
2. Load a predefined analysis from the database: Click  to open the **Database Browser**.



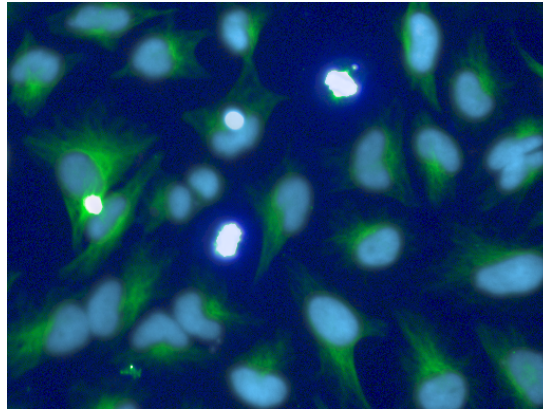
3. Select "RMS Quantification of Marker in Nucleus" and click . The predefined analysis sequence is displayed.



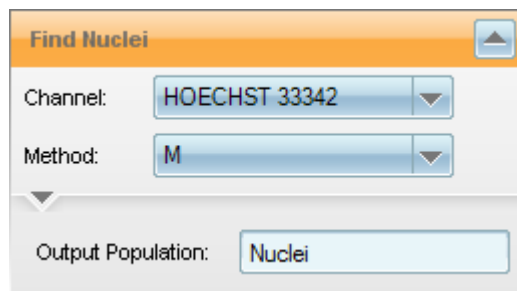
### 4.4.1 Analysis Sequence

It is now necessary to check the predefined parameters of the building blocks and adapt them to the actual images.

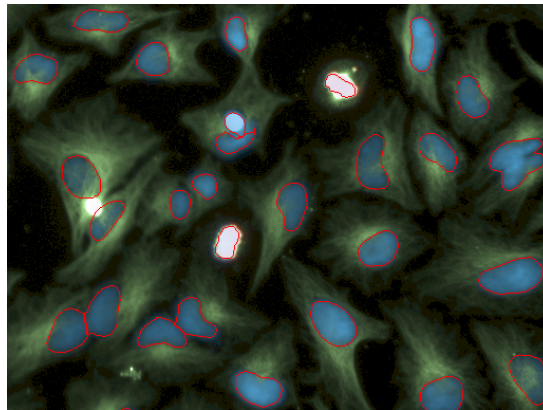
1. Select one well and field (**Navigation - Measurement**) to select the image to be evaluated.



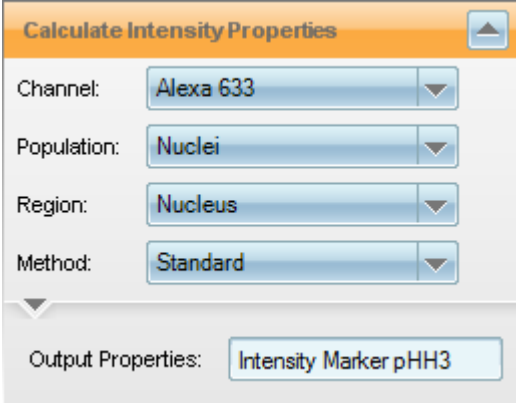
2. Open the building block **Find Nuclei**.



3. Select the Channel **HOECHST 33342** and **Method M**.
4. Expand the building block and change the splitting coefficient to 0.35 for a complete nuclei separation.  
Click .



5. Open the building block **Calculate Intensity Property** and select the specific marker channel (**Alexa 633**). You can change the output name to "Intensity Marker pHH3".  
Click .



**Calculate Intensity Properties**

Channel: Alexa 633

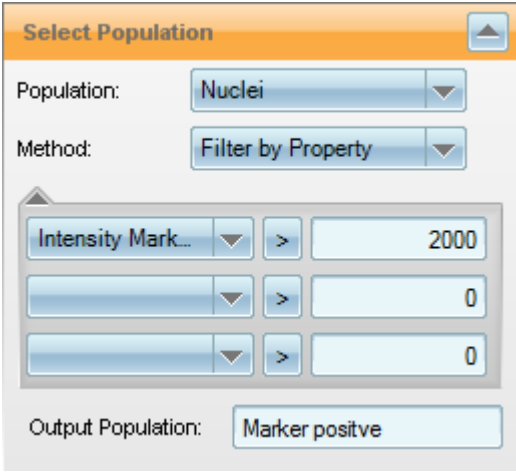
Population: Nuclei

Region: Nucleus

Method: Standard

Output Properties: Intensity Marker pHH3

6. Open the building block **Select Population** to adjust the intensity threshold for the determination of positive cells.



**Select Population**

Population: Nuclei

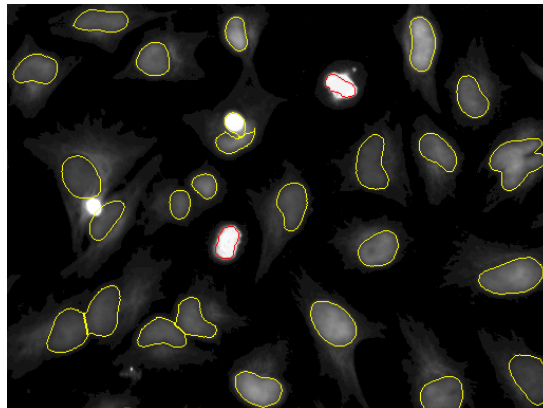
Method: Filter by Property

Intensity Mark... > 2000

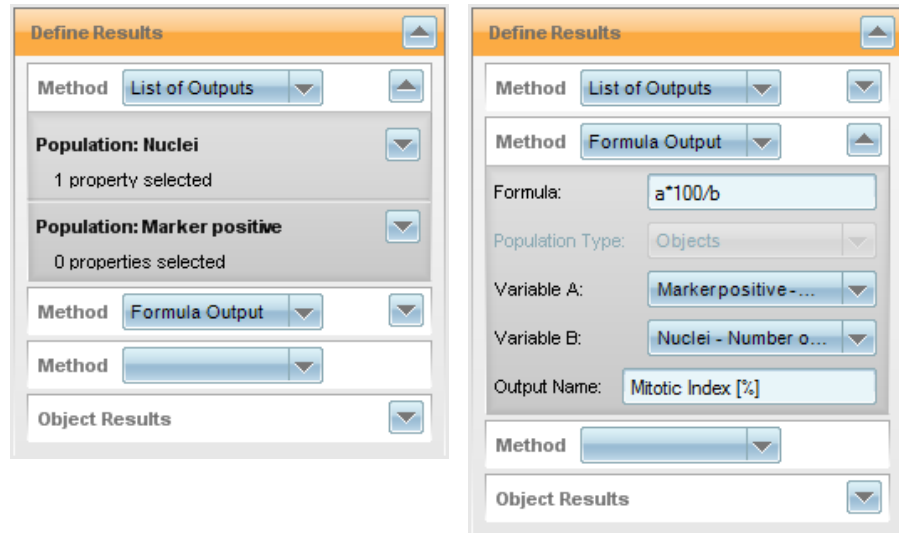
> 0

> 0

Output Population: Marker positive



7. Open the building block **Define Results** to have a look at the output. You can change the output name to "Mitotic Index".



#### 4.4.2 Test Analysis

The analysis sequence is now complete and you can apply a test analysis to one image. You will find these test results under **Image Analysis Results** and an image of “Marker Positive” in the **Image Display**.

#### 4.4.3 Save Analysis

If you are comfortable with your analysis sequence you can save it to the database:

1. Click **Save...** in the **Global Control** window.
2. Insert Analysis Name: *Mitotic Index Quickguide Exp2*.
3. Click **OK**. The analysis sequence is saved to the database.

## 4.5 Evaluation

Now the analysis sequence will be applied to all wells and fields of your measurement.

1. Click **Evaluation** in the Navigation bar.

Mode:  **Single**  Batch

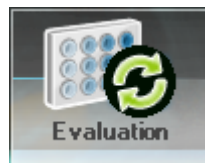
Experiment: Quickguide Exp2

Measurement: Training Plate - MEASUREMENT

Analysis: Mitotic Index Quickguide Exp2

Evaluation:

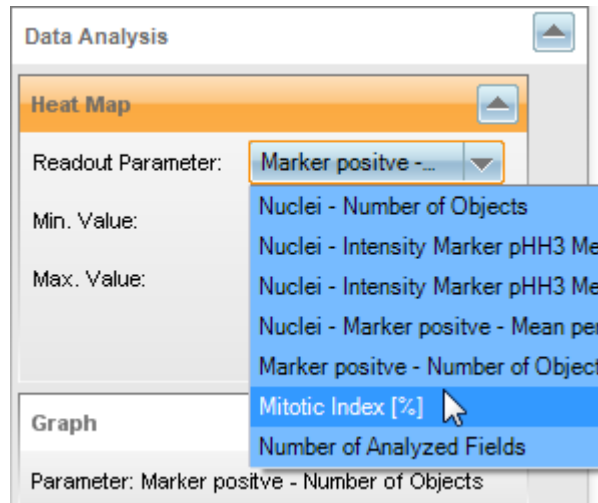
2. Make sure that **Single** mode is selected.
3. As you just developed your analysis sequence with your actual measurement both are already loaded. Otherwise you have to load a measurement and analysis for evaluation by clicking  to open the **Database Browser**.
4. Open **Navigation – Measurement**: Select wells to be evaluated.
5. Click . The evaluation starts and the results are displayed in a table.



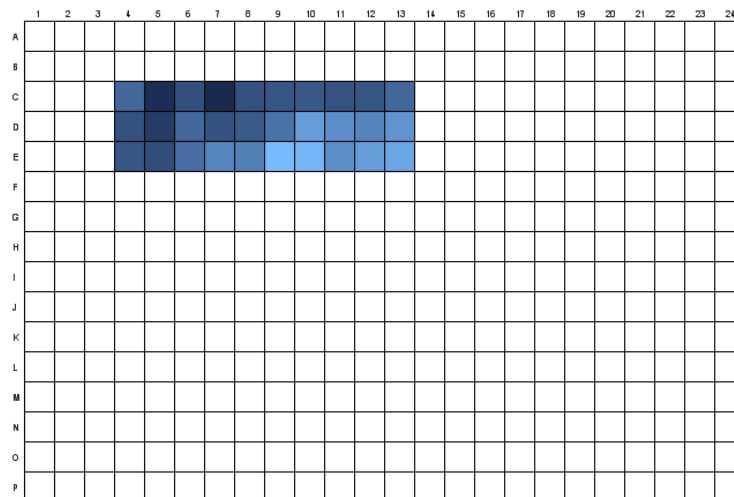
### 4.5.1 Data Analysis

Here you can select the output parameter that will be visualized as a heat map for a statistical overview.

1. Open **Data Analysis** block.
2. Open **Heat Map**.
3. Select Mitotic Index as **Output Parameter**.



- The heat map will be displayed in the **Content Area**.



In an ideal experiment the heat map would show an increasing color gradient in relation to the mitotic index. The heat map visually indicates outliers and shows also a first impression of the compound's EC50.

- For a further evaluation of the numerical results you can select data points for a graph view or export results to a spreadsheet program (**Settings – Data Management – Export Data**).

## **5 Shut Down**

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It is recommended to switch off the Operetta (instrument) to extend the life time of the components if the instrument is not required for several hours or even longer. Harmony and the Harmony PC can also be shut down if not required anymore (see exceptions below).

**Notice**

Do not shut down Harmony and the Harmony PC if ...

- an evaluation is still running.
- background jobs or scheduled tasks are used for data management.
- there are office installations of Harmony which need access to the database on the Harmony PC.

**How to shut down the whole system**

1. Close the Harmony software by closing the window in the usual way (X button).
2. Shut down the Harmony PC.
3. Close the main valve of the carbon dioxide supply (if applicable).
4. Switch off the Xenon light source.
5. Switch off Operetta using the power switch.

The on/off switch does not switch off the Operetta immediately. There will be a controlled shutdown of the electronics with a certain delay before the instrument is really switched off.



**Danger!**

**Direct electrical contact - Electrical shock.**

- *In case of emergency unplug the power cable to disconnect the Operetta from mains supply immediately.*
- *The mains connection must be accessible at any time.*







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