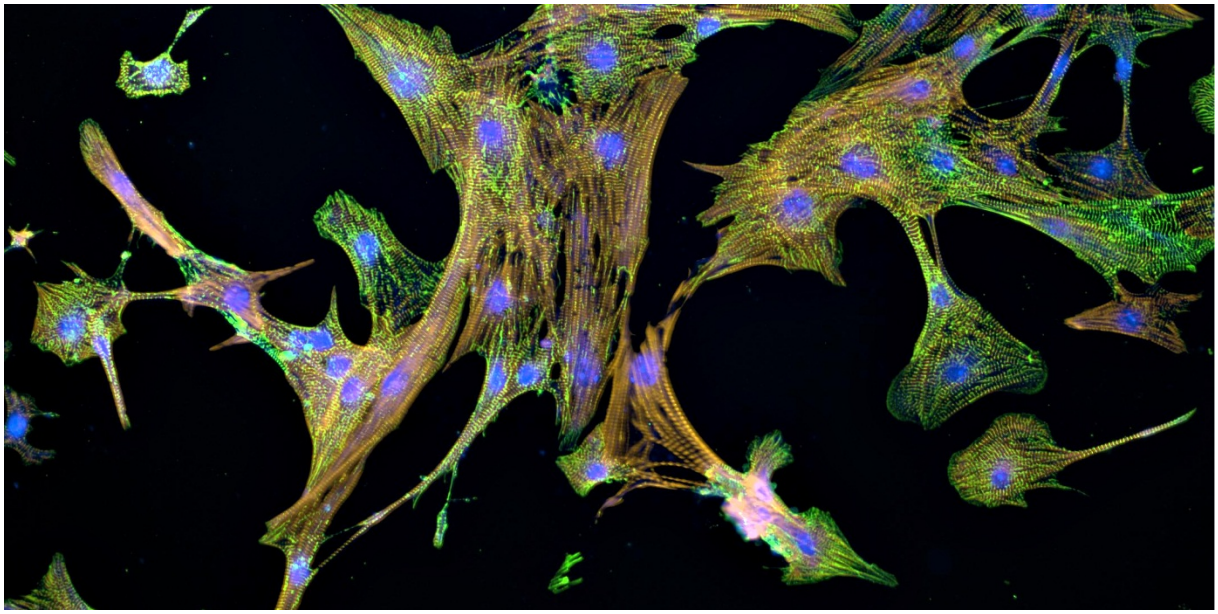
Opera Phenix™ Application Guide

3rd Edition



A Guide for High-Content Analysis (HCA)

Imaging Cell-Based Assays

PerkinElmer®
For the Better

Release History

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Service Contact

Information: http://www.perkinelmer.com
Portal: http://evoportal.perkinelmer.com
Email: opera.helpdesk@perkinelmer.com

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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.

Part I covers instrument-specific information and guidelines for successful preparation, measurement and data management of your HCS applications. These chapters might differ in their relevance to you depending on your previous experience.

Chapter 1 delivers all information you need before you design your HCS application for the Opera Phenix.

- If you have no previous experience with HCS assays general information about suitable cell lines, microplates and fluorophores can be found in chapters 1.1 to 1.3 and chapters 1.6.1 and 1.6.3 and is recommended as starting point.
  Experienced HCS scientists might skip these chapters; however we highly recommend reading the instrument-specific chapters that describe unique characteristics of the Opera Phenix like its patented Synchrony Optics™. Please note that information in chapters 1.4, 1.5, 1.6.3 and 1.6.4 also has significant implications for the assay design.

Chapter 2 provides detailed step by step guidance for the experimental setup at the instrument.

- Chapters 2.1 and 2.2 are a recommended reading for any user and guide you through the necessary steps for performing a measurement. The following chapters address more specific measurement modes like FRET or Digital phase contrast.

Chapter 3 provides complete assay protocols and experimental setup including image analysis for a Cell cycle, Apoptosis and Cytotoxicity application.

- If you want to get started right away, these example protocols will help you to set up HCS applications via a hands-on approach which familiarizes you with the whole workflow. They describe the complete process starting with the preparation of the assay plate, the experimental setup, the image analysis sequence and finally the secondary analysis of the results.

Chapter 4 introduces the basic facts of data management.

- HCS can generate huge amounts of data very quickly. Proper data management is an often neglected task and we highly recommend familiarizing yourself with the concepts presented in this chapter and with the data handling tools provided by Harmony..

Chapter 5 (Appendix) is a collection of technical data.

- Here you can find tables with specific technical detail regarding the Opera Phenix e.g. characteristics of each objective lens, descriptions of each filter, compatibility of objectives with microplates, a glossary etc. This chapter should act as a useful reference even for the experienced user.

What's new in the 3rd Edition?

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

- Page 64: Updated section "How to define a suitable well layout" – improved well layout using "Field Centered" option (from Harmony 4.5)
- Page 66: Updated section "Z-stacks and their application in 3D imaging" – all planes in a stack can be selected by pressing CTRL+A (from Harmony 4.6)
- Page 73: New section "Global Image" – new feature (from Harmony 4.5); global image creation can be canceled and progress is indicated by progress bar (from Harmony 4.6)
- Page 74: Updated section "Brightfield Imaging – When and How to Use It" – new Brightfield Correction (from Harmony 4.5)
- Page 106: New section "Automated PreScan/ReScan – PreciScan™" – new PreciScan feature (from Harmony 4.5)
- Page 161: Updated section "Objectives" (Appendix) – new objectives 20x Air high NA and 40 Air high NA supported (from Harmony 4.5)
- Page 163: Updated section "Plates" (Appendix) – specs and objective compatibility updated

Chapter 1 – Designing Cell Based Imaging Applications

This chapter describes some of the factors that need to be considered when designing image-based cell assays. The common pitfalls are discussed and recommendations are given for how to optimize your assays.

Here, you can find:

1.1 Cells

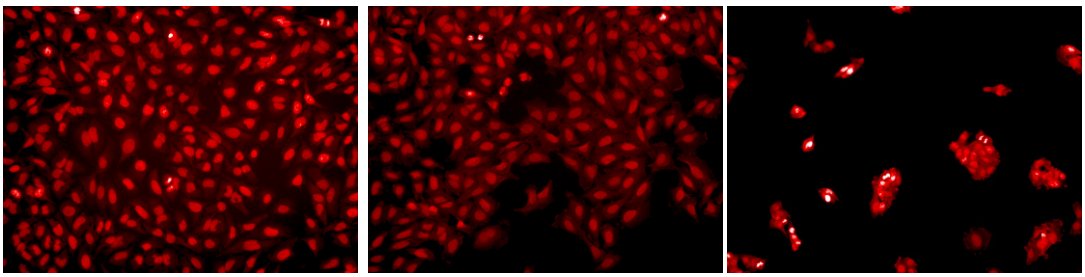
1.1.1 How to select a suitable cell line

Two criteria need to be considered when selecting a cell line for imaging applications:

- Adherence
- Growth pattern

Both parameters will influence the subsequent image analysis steps, and are dependent upon the specific cell line.

Adherent cell lines that grow as a flat monolayer and are firmly attached to the microplate are ideally suited for this purpose. See ***Figure 1-1*** and ***Table 1-1***.



A – HeLa B – U2OS C – HepG2

Figure 1-1: HeLa and U2OS cells (panels A and B respectively) show a monolayer of well-spread and well distributed cells, perfectly suited to HCS. HepG2 cells (panel C) tend to form clusters and are therefore more difficult to use. Cells were seeded at 5 x 10^3 cells / well and were incubated overnight at 37 °C. Cells were then fixed and stained with DRAQ5™ and images were recorded using an Operetta and the 20x long WD objective.

Cell line	Description	Spreading / Flatness	Attachment	Conclusion
U2OS	Human osteosarcoma cell line	++	+	Ideally suited for imaging applications.
HeLa	Human cervical cancer cell line	+	+	Suitable for imaging applications.
CHO-K1	Chinese hamster ovary cells	+	+	Suitable for imaging applications.
HepG2	Human hepatocellular liver carcinoma cell line	-	+	Difficult for imaging applications. Coating is necessary.

Table 1-1 (continued over page): Selected cell lines and their suitability for imaging applications, particularly with regard to their spreading and attachment phenotypes.

Cell line	Description	Spreading / Flatness	Attachment	Conclusion
Hek293	Human embryonic kidney cell line	-	+/-	Difficult for imaging applications. Coating is necessary.
A431	Human epithelial carcinoma cell line	-	+/-	Difficult for imaging applications. Coating is necessary.
Jurkat	Human T cell lymphoblast-like cell line	-	-	Difficult for imaging applications. Coating is necessary.

Table 1-1 (continued): Selected cell lines and their suitability for imaging applications, particularly with regard to their spreading and attachment phenotypes.

If your application requires a less favorable cell line (e.g. HepG2 or HEK293), then coating can significantly improve the growth pattern. For more details please see section 1.2.8.

We recommend limiting the number of passages prior to plating cells for assays, as passaging might change the physiology and/or the morphology of cells.

Suspension cells

Cells in suspension provide a greater challenge for imaging applications for two reasons:

- Due to their round morphology, the analysis of cell compartments is more difficult.
- They are only loosely attached to the bottom of the plate and may therefore move around during image acquisition, resulting in a blurred image.

If your application requires the use of cells in suspension, then coating can significantly improve their adherence to the plate bottom. For more details please see section 1.2.8.

1.1.2 How to determine a suitable cell density

There are two conflicting requirements for the cell density:

- High density for statistically meaningful results.
- Low density for robust cell detection.

We recommend a confluency of 70 – 90 % as a good compromise to suit both requirements (i.e. 70 – 90 % of the plate bottom is covered by a cell monolayer).

If your assay relies on the evaluation of a specific signal on the plasma membrane of cells then a lower confluency (40 – 60 %) can be advantageous for minimizing the overlap between cell membranes.

The number of cells per well to be seeded depends on:

- The growth rate.
- The growth time prior to performing the experiment.
- The cell line.
- The well area.

Of course, factors such as the age of the cell line, the medium used, any supplements provided etc. will also influence growth, but to give you a rough idea of the number of cells per well, please see the seeding numbers typically used in our lab (***Table 1-2***).

Plate type and respective well area	**Number of cells seeded per well**
ViewPlate-96 F TC, well area: 32 mm^2	1.7 x 10^4 HeLa cells
ViewPlate-96 F TC , well area: 32 mm^2	1 x 10^4 CHO-K1 cells
CellCarrier-384 well area: 11 mm^2	5 x 10^3 HeLa cells
CellCarrier-384 well area: 11 mm^2	3 x 10^3 CHO-K1 cells

Table 1-2: The number of cells seeded per well, for different cell lines and well formats, which are necessary to achieve a confluency of approximately 80 % after 18 h growth.

When cells grow in multiple layers (confluency > 100 %) it is difficult to achieve robust and reliable cell detection. Make sure that you use cultures of less than 100 % confluency.

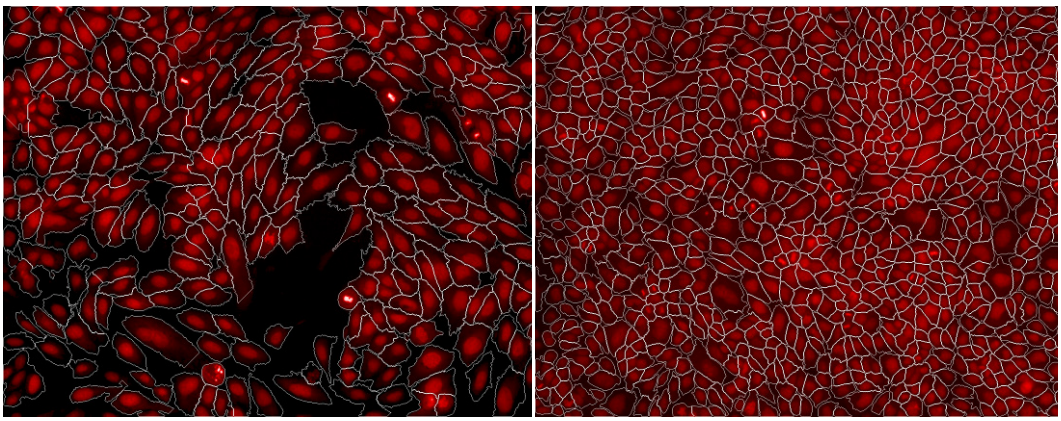


Figure 1-2: MDCK cells with a confluency of 80 % (left) and 100 % (right). Cells are stained with DRAQ5™. Cells with 100 % confluency partially overlap and also vary considerably in size, compromising robust and reliable cell detection.

1.1.3 Considerations for the assay layout

Control wells, i.e. wells containing a positive control or “maximum effect” (e.g. a high concentration of an activating ligand) and wells containing a negative control or “minimum effect” (e.g. a high concentration of an inhibitory compound), are essential for optimizing image analysis in order to achieve a good signal-to-background ratio and high Z´ value.

The control wells should be included at the beginning and the end of the measurement path (and potentially also in between) to enable identification of the following:

- Potential local variances over the plate.
- Potential time-dependent fluctuations over the plate.

The assay layout in ***Figure 1-3*** shows an example of a good distribution of the control wells.

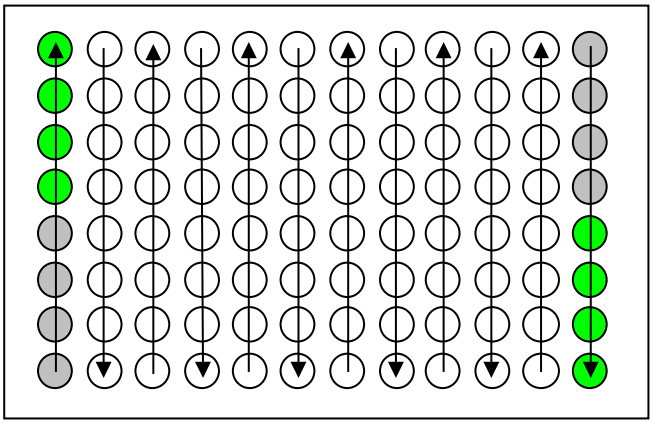


Figure 1-3: Example of a suitable distribution of control wells on a 96-well plate. Green: positive control, grey: negative control, white: sample area. Arrows indicate pathway of imaging.

Harmony performs measurements using a vertical pathway (illustrated by the arrows in ***Figure 1-3***). By arranging the wells of your dose-response treatments in columns (instead of rows), you have the advantage of being able to see the results of one complete dose-response early on in the measurement.

1.2 Microplates

High content screening plates have to fulfill several, sometimes conflicting, demands. They must:

- Provide a suitable environment for cellular growth.
- Have excellent optical quality.
- Be compatible with the objective.

These requirements are discussed in more detail in sections 1.2.1 – 1.2.8.

The plate and objective are dependent on each other and therefore must be considered in combination. See section 1.3 for more details.

For most applications the use of black pigmented plates with a clear, flat, low, polystyrene or cyclic olefin bottom for cell culture is suitable.

The choice of plate is crucial for the success of your experiments (see below). Once you have selected a plate that fulfills all your specific requirements and works well, do not change it unless you have a good reason to do so!

1.2.1 Glass versus polystyrene bottomed plates

Standard microplates are typically available with polystyrene (PS), cyclic olefin (CO) or glass bottoms:

- Tissue culture (TC) treated PS-plates and CO-plates with the ability to bind proteins and to support cell attachment are suitable for many different applications, however some PS-plates show high autofluorescence upon excitation with UV-light which is reduced in CO-plates.
  Please refer to ***Table 5-6*** for PS-plates with good optical properties suitable for use with the Opera Phenix.
- Glass exhibits excellent optical properties but is less suitable for cell culture. It usually has to be coated with proteins that enhance cell adherence and growth (for details on coating see section 1.2.8). Please refer to ***Table 5-6*** for glass bottom plates that are suitable for use with the Opera Phenix.

1.2.2 Plate bottom thickness

The thickness of the plate bottom has implications for:

- The choice of objective.
- The planarity of the plate bottom (in combination with the material that it is made from).

We generally recommend thin bottom (< 200 µm) plates. These plates can be used with high numerical aperture (high NA) air objectives and water immersion objectives as they typically have a high NA but a short working distance (WD).

Water immersion and high NA air objectives provide better resolution and collect more light from the specimen than long WD air objectives with a relatively low numerical aperture. Thick bottom plates (> 250 µm) require the use of long WD air objectives. For more details on objectives see section 1.4.

Table 5-5 gives an overview of the available objectives and the compatible plate bottom thicknesses.

Please note that, independently of the objective, the minimum suitable plate bottom thickness is 110 µm, as focusing of plates with thinner bottoms is not supported by the laser-based autofocus system.

It is not recommended to use plates with a plate bottom thickness higher than 1 mm as the automatic image alignment procedure requires the correction collar to be set to ≤ 1 mm. A wrong correction collar setting might impact this.

1.2.3 Plate geometry

Microplates for HCS imaging have a sample area with wells on a transparent plastic or glass bottom, which is surrounded by a plate rim positioning the sample area in a certain height. Most plates vary in their bottom thickness (see ***Figure 1-4***, value I) and plate bottom height (see ***Figure 1-4***, value H). In many cases microplates have ribs on the plate rim, protruding towards the wells (see ***Figure 1-5***). These ribs come in all forms and sizes and are not standardized, i.e. not part of the SBS (Society for Biomolecular Screening) standard description.

So called high bottom plates (H value > 300 µm) have a high probability of measurement area limitations when water immersion or high NA air objectives are used (see section 1.2.4 “Measurement restrictions”). This is caused by the short working distance of these objectives which can lead to collisions with the scan table or the plate’s ribs (see ***Table 5-6*** and ***Table 5-8*** for the restriction behavior of selected plates for use with water immersion objectives).

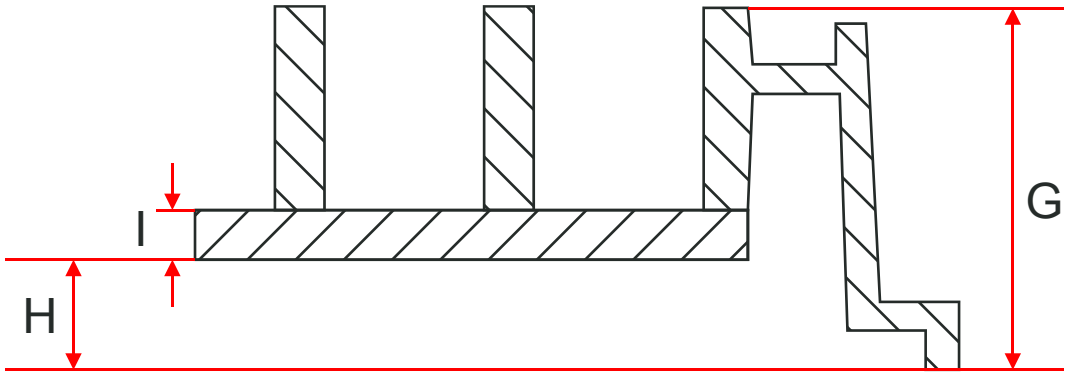


Figure 1-4: Sketch of plate geometry, showing the values that are important to consider. These are the thickness of the plate bottom (I) and the distance between the plate bottom and the rim of the plate, bottom height (H).

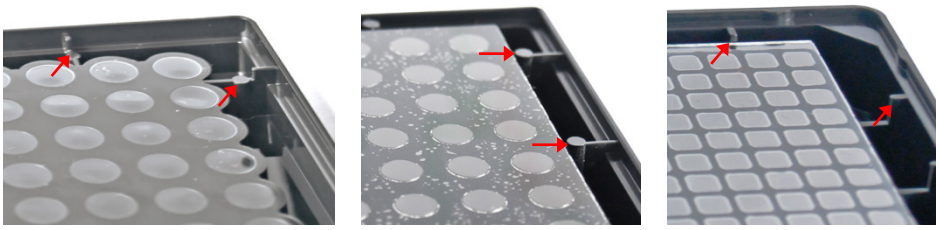


Figure 1-5: Examples of “ribs” (red arrows) at the bottom of three different plates. The shape of the ribs is not standardized and is not part of the standard SBS recommendations.

The values of parameters H and I are crucial in terms of objective choice and the corresponding possible measurement area of a plate. Certain combinations of plate type and objective can lead to non-measurable areas on the plate, because the objective would collide with the scan table of the instrument.

The probability of measurement restrictions is increased by the following factors:

- Objective with short working distance (e.g. water immersion objectives)
- Plate type with high plate bottom or very thick plate bottom
- High focus height

Note that there is a slight difference between the physical thickness and the optical thickness of the plate bottom. In the plate type definition wizard the physical thickness is required, but for focusing you might want to think about the optical thickness which is calculated as follows:

Optical Thickness = (physical thickness) / (refractive index)

The refractive index is dependent on the material. Glass has a refractive index of 1.51, PS of 1.58. Therefore, at the same physical thickness, the optical thickness differs if a different material is used for the bottom.

The overall plate height is restricted to 22 mm including the lid. Plates that exceed this cannot be loaded into the Opera Phenix as they will collide with the instrument.

1.2.4 Measurement restrictions

If your plate has a high bottom design and you want to measure it with an objective lens with a short working distance or in an unusually high focus height, you need to consider that the objectives can get very close to the well bottom. Due to the large physical dimensions of water immersion objectives in particular, these would be more prone to collide with the plate rim or scan table when focusing into outer wells of a microplate (***Figure 1-6***).

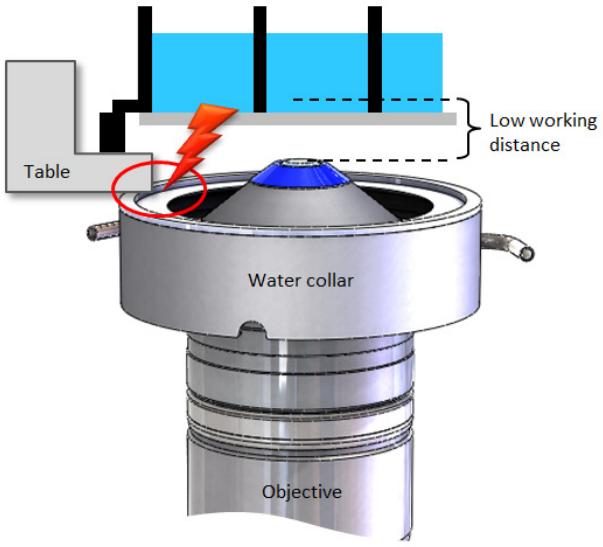


Figure 1-6: Illustration of the collision risk between objectives with a low working distance, and the table.

However, Harmony will prevent collisions between any objective and the scan table, and as a result you might see non-measurable wells marked on the plate layout. The probability of such measurement restrictions is increased when high bottom plates (parameter H > 300 µm) are used as the objective has to move up even more.

To visualize restrictions in Harmony, the plate is divided into three different areas indicating the measurability (see ***Figure 1-7***):

- **"safe area"** – no collision of objective with scan table or plate. All wells can be measured safely.
- **"caution area"** – collision of objective with plate ribs is possible but not fatal. All wells can be measured. However, it is the responsibility of the user to check for potential issues, see below.
- **"restricted area"** – collision of objective with scan table could be possible and would be fatal. These wells cannot be measured.

To avoid any collisions with the scan table, the respective wells are automatically excluded from the plate layout in Harmony. The wells of the "restricted area" are marked with an "x" and cannot be selected (outer wells in ***Figure 1-7***).

As mentioned in section 1.2.3 as well, many microplates have ribs on the plate rim, protruding towards the wells. The shapes of these ribs are not standardized, i.e. not part of the SBS standard description. As no reliable information is available about the rib geometry it is not possible to exactly calculate which areas of the plate can be measured successfully. That is why the “caution area” has been introduced in the Harmony plate layout (wells with red border in ***Figure 1-7***). Wells within this area are measurable but if ribs are present potential collisions of objective and plate rib can occur (based on the worst case scenario). The collision would lift the plate and the visible result might be either focus failures or unfocused sample images. The caution area should be tested before the actual measurement to confirm which part is reliably measurable.

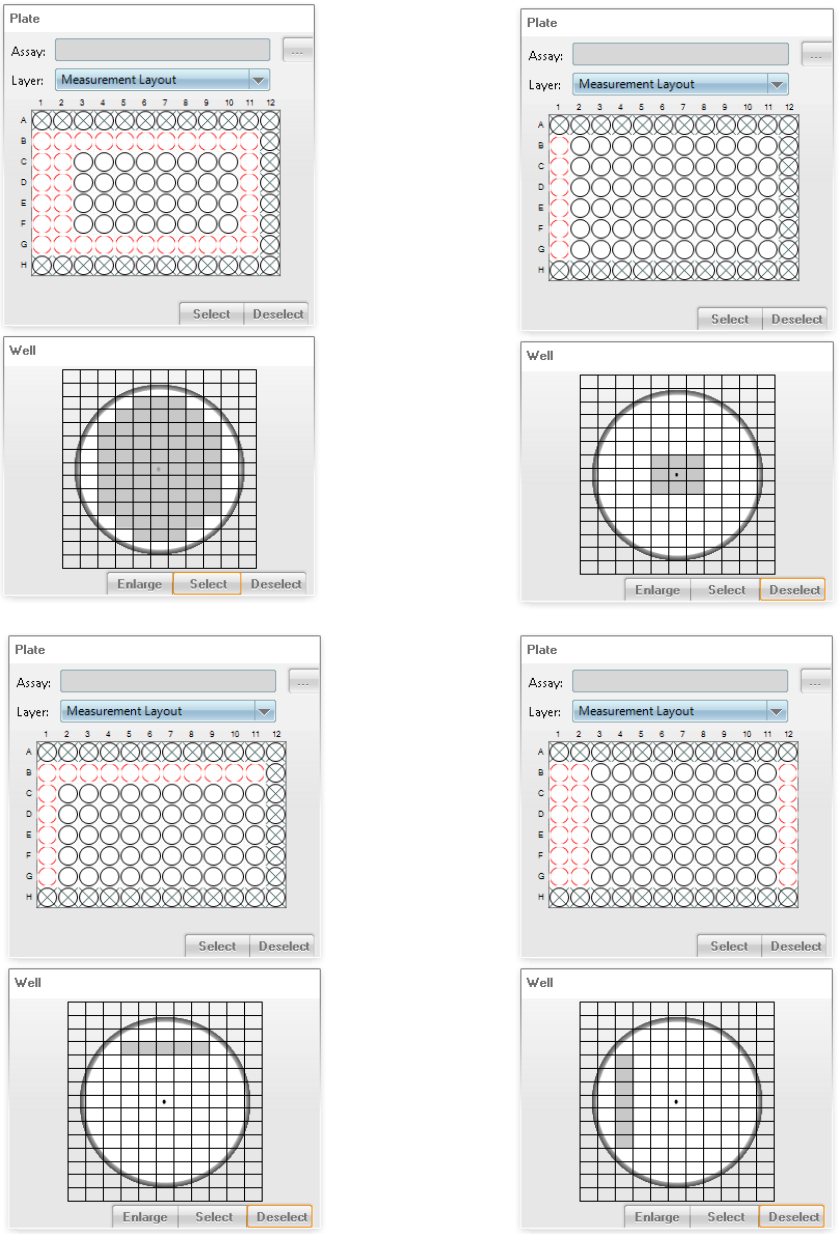


Figure 1-7: The Harmony measurement layout is separated into different areas. The “restricted area” (x-wells) is the non-measurable area of the plate. These wells cannot be measured because the objective would collide with the scan table. The “caution area” (wells are marked red) can be selected and measured completely. But here the objective could potentially collide with ribs of the plate rim which could lead to focus failures or unfocused images. The “safe area” (white wells) can be measured without any limitations. No contact between plate and objective is expected. The position of the different areas can change depending on the selected subfields in the well (see examples above).

In your office version of Harmony or directly at the Opera Phenix, select the objective you want to use and your intended microplate as well as the (estimated) maximum focus height and the field layout. Restrictions can occur from a certain focus height upwards. Then check which wells are measurable before filling these wells with your assay ingredients in the lab. See the Harmony Help or User Manual for further details.

If you want to remember the measurable area for a certain plate type we recommend saving the information as a layer into the assay layout.

1.2.5 Planarity

The planarity of a plate bottom is influenced by its thickness and also by the material used.

Glass is a very rigid and inflexible material therefore both thick and thin glass bottoms are highly planar and well suited for confocal imaging.

Thick plastic bottoms are usually planar and therefore suitable for imaging, although this may prevent the usage of high NA objectives (see section 1.4 “Objectives” for more detail).

Thin foil bottoms are flexible and can be bent, especially in 96-well plates with a relatively large well area. Depending on the plate quality, it can be possible that samples can only be focused accurately in the middle of the well.

The well area of 384-well plates (like the CellCarrier-384) is small enough to exhibit good planarity even with a thin PS- or CO-bottom. As a result they are suitable for HCS applications.

Table 5-6 in the Appendix lists the plates that are available from PerkinElmer, with a summary of their properties.

1.2.6 Plate format

The Opera Phenix accepts any plates that comply with the Society for Biomolecular Screening (SBS) standards for microplate footprint. The overall height is limited to 22 mm including the lid. Plates exceeding this limit will collide with the instrument and cannot be loaded. Typically, 96-well or 384-well plates are used for HCA applications. Both plates have their advantages and disadvantages and your choice will depend on the intended application.

- **96-well plates**
  - Advantages: Easier to use in manual mode. Better suited to long-term live cell applications as the cells are less affected by evaporation.
  - Disadvantages: Requires a higher working volume which equals higher costs. 96-well plates with thin foil bottoms can have an insufficient planarity (see section 1.2.5).

- **384-well plates**
  - Advantages: The working volume is small, resulting in a cost efficient solution for high throughput assays. Good planarity even with a thin foil bottom.
  - Disadvantages: Handling several plates in a manual mode, even using 16-channel pipettes, can be tedious and less accurate. Automated liquid handling devices such as the FlexDrop™ Reagent Dispenser or JANUS® Automated Workstation are recommended for dispensing and aspirating to facilitate the use of 384-well plates.
- **1536-well plates**
  - Advantages: Further savings of media, reagents and compounds (well diameter < 2 mm, working volume 3-10 µl). Providing optimal performance and cost reduction for high-throughput screening.
  - Disadvantages: Automation is essential using these plates. Automated liquid handling devices such as the FlexDrop™ Reagent Dispenser or JANUS® Automated Workstation are recommended for dispensing and aspirating to facilitate the use of 1536-well plates. Not recommended for live cell assays due to high evaporation. Neighboring wells are within the field of view when imaged with a low magnification objective (1.25x), which will complicate image analysis.
- **Microscopy slides**
  - Advantage: Suited for tissue samples.
  - Disadvantages: Slides can only be measured when placed into a slide holder (we recommend PerkinElmer's HCS Slide Holder for 1 slide or for 4 slides). Slide samples have to be prepared carefully when scanned with autofocus laser systems (see section 1.3 and 2.9).

1.2.7 Adding new plates

If your preferred plate type is not included in the Harmony database, you may add it using the Plate Type Definition Wizard. You will be asked to fill in several values regarding the dimensions of the respective plate, which can usually be found in the manufacturer's notes or on their website. However, sometimes the values for the plate parameters H (plate bottom height) and I (plate bottom thickness) are not specified. The plate wizard offers a possibility to scan for these two values, which are otherwise difficult to determine. This scan is implemented in the Plate Wizard (see ***Figure 1-8***).

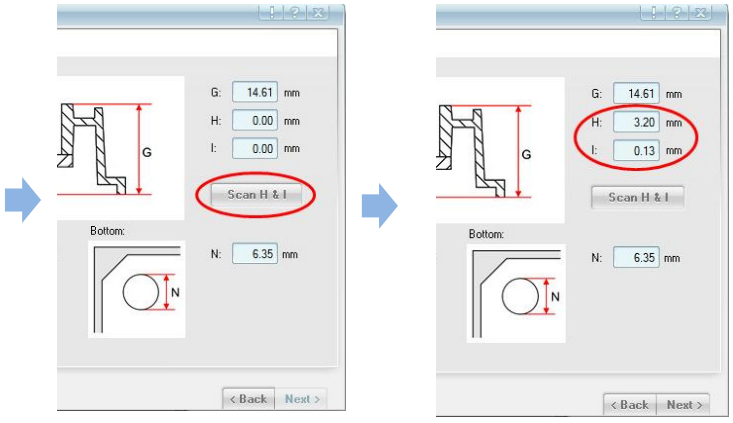


Figure 1-8: Adding a new plate type in the database. The "Define Plate Type" wizard can be found under Harmony settings or by clicking with the right mouse button near the Plate Type dropdown-field (Setup screen) and selecting "Plate Type Details" from the context menu. If the parameters H and I of a plate are unknown, an H & I scan can be performed. The values for H and I are determined by scanning several wells. The median of these results is calculated and can be inserted automatically into the plate definition.

Please note: If the plate bottom thickness is completely unknown, set the correction collar to 0.5 for a first scan. Then set the correction collar to the value of I that resulted from that scan and perform a second scan to finally determine the precise values for H and I.

Please consider the criteria mentioned above and double-check the compatibility of a new plate with the objectives needed for your assay using the following procedure:

Select the newly defined plate type on the Setup screen (you don't need to insert the plate into the Opera Phenix). With the selected objective, Harmony will perform a security check to test the compatibility of the plate with the objective. This security check also calculates the restrictions of the measurable plate area at the given focus height. For more information about these restrictions, please see the Harmony Help or User Manual.

Please note that incorrect plate dimension values may lead to focus failures or even hardware damage.

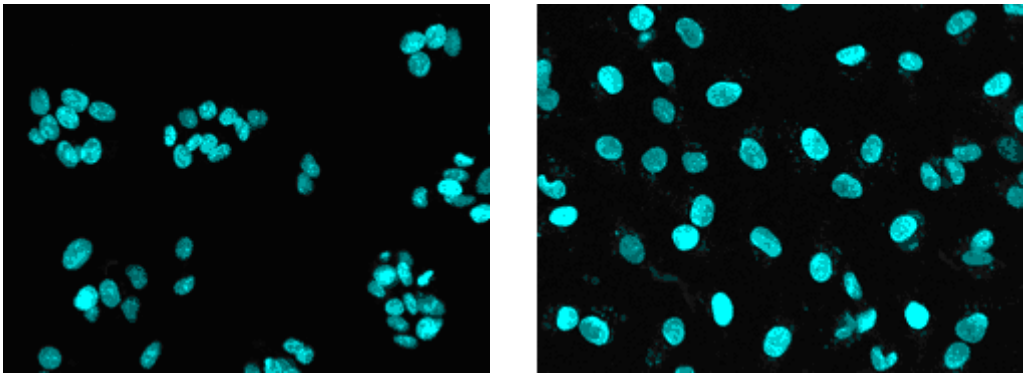
In addition we recommend application tests of new plates before their routine use. Potentially disruptive factors identified during tests could be insufficient planarity of the plate bottom or an unsuitable environment for cell growth (e.g. insufficient support of adherence or even toxic effects).

1.2.8 Coating

Both glass and plastic bottoms can be coated with various biomolecules to improve the confluency and adherence of cells.

A variety of extracellular matrix molecules, natural proteins and synthetic polypeptides are available for this purpose.

A comparison of non-coated and Collagen I coated microplates is shown in ***Figure 1-9*** and ***Figure 1-10***. ***Figure 1-9*** clearly shows that the Collagen I coated surface promotes **even spreading** of cells.



Non-Coated Collagen I Coated

Figure 1-9: Comparison of cell growth on non-coated (left) and Collagen I coated (right) CellCarrier plates. HepG2 cells were seeded at equal densities and were incubated overnight at 37 °C, 5 % CO_2. Following nuclear staining the plates were imaged using a 20x objective. Left: cells on the non-coated surface grow in agglomerates and in multilayers. Right: cells are evenly spread when grown on the Collagen I coated surface.

Figure 1-10 shows the improved adherence of cells growing on Collagen I coated surfaces compared to non-coated plates. After the fixation and washing steps, more than half of the cells growing on non-coated surfaces had been lost, whereas most cells growing on Collagen I coated plates were retained.

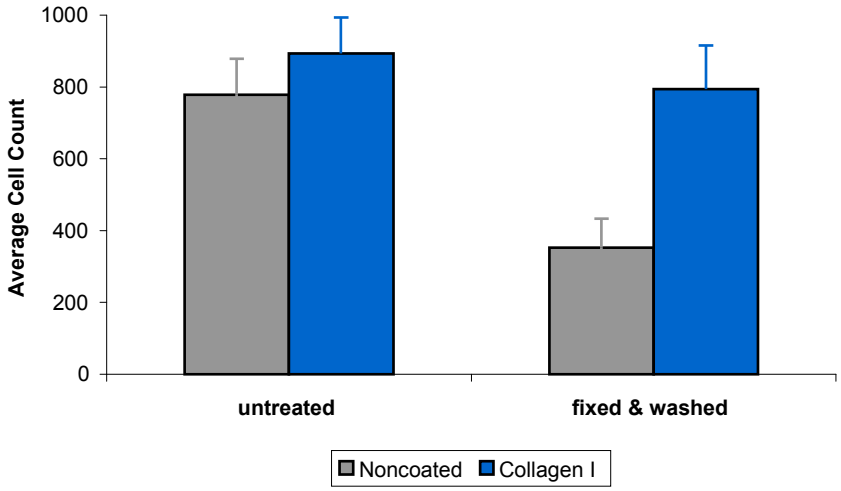


Figure 1-10: Comparison of the cell count on non-coated (grey) and Collagen I coated (blue) CellCarrier-384 plates before and after the fixation and washing steps (Error bars indicate the standard deviation for N = 40 wells). HepG2 cells were seeded into non-coated and Collagen I coated CellCarrier 384-well plates, and were incubated overnight at 37 °C, 5 % CO_2. Cells were stained and both plates were measured. The cells were then fixed with 4 % FA, washed twice with PBS, and both plates were measured again. The average cell count on non-coated and Collagen I coated plates before the fixation and washing steps is similar, but after fixation and washing nearly half of all cells on non-coated plates had been lost, whereas on Collagen I coated plates most cells were retained.

Commonly used coatings are:

- **Poly-Lysine:** a synthetic positively charged polymer, existing as two enantiomers, Poly-D-Lysine (PDL) and Poly-L-Lysine (PLL). Both are commonly used, however, PDL is not degraded by cellular proteases and is therefore often the preferred choice. As Poly-Lysine is a synthetic protein, it does not influence the signaling pathways of cells and is completely free of any animal contaminants. Almost all cell types will adhere to Poly-Lysine coated plate bottoms.
- **Collagen:** the most abundant protein in mammals that is found throughout the body and a major component of the Extracellular matrix (ECM). The most frequently used types of Collagen for coating are Collagen type I and IV. Collagen I is suitable for endothelial and epithelial cells, muscle cells and hepatocytes. Collagen IV is the major constituent of basement membranes and offers more physiologically relevant conditions for cells as well as improvement of the adherence of specific cell types, e.g. PC-12 (rat adrenal pheochromocytoma cell line). A protocol for the preparation of Collagen I coating can be found in the standard protocols section in the Appendix.
- **Laminin:** the major non-collagenous component of the basement membrane. Laminin is used for epithelial cells, leukocytes and myoblasts and has also been shown to stimulate neurite outgrowth.
- **Fibronectin:** an extracellular matrix glycoprotein. Fibronectin is mainly used for the culture of endothelial cells and fibroblasts.
- **Ultra Low Attachment (ULA) coating:** Coating prevents the attachment of cells to the well surface. ULA coating in round-bottom wells facilitates formation of round spheroids after incubating seeded cells.

1.2.9 Round (U) bottom plates

For some cell-based microplate assays round (U-shaped) bottom plates can be used supporting specific applications. For example round bottom plates can be provided with an Ultra-Low Attachment (ULA) surface enabling the formation of spheroids, three-dimensional (3D) cell culture models for cell-based microplate assays. However, the round bottom can cause focusing problems. The laser-based autofocus system relies on the detection of reflections of the laser beam. Usually these occur at the interface between air and plate bottom and plate bottom and buffer. If the plate bottom is curved, the autofocus laser is not passing perpendicularly and less light is reflected back, making reliable focusing difficult. That is why round bottom plates can be measured in the well center only as the plate bottom is not curved at this position (see ***Figure 1-11***).

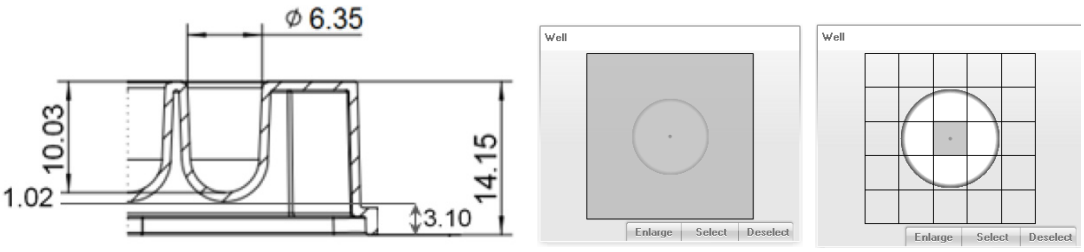


Figure 1-11: Drawing of a U-bottom plate. A: Side view of a plate section showing the U-shaped well bottom. Only the center of the Well can be measured as the auto focus just works in the flat area B: Well view in Harmony using a 10x air objective (left) or a 40x air objective (right) showing the center field in grey. The flat area in the center of the well bottom is not curved indicated by the circle. Fields within that circle can be measured. Fields outside that circle cannot be measured.

The following objectives will work robustly with 96 well U-bottom plates (tested with the PerkinElmer CellCarrier Spheroid ULA 96-well Microplate):

- 10x Air
- 20x Air long WD
- 40x Air long WD

1.3 Slides

Microscopy slides can be measured if placed into a suitable slide holder. The respective plate type will assume the slide holder being the plate and the slide(s) being the well(s). In the Plate Type Definition Wizard, you will be asked to select the respective template and the slide's orientation within the slide holder.

There are multiple methods for preparing slide samples. Some will work in combination with a laser-based autofocus system, others will not. Therefore we highly recommend reading section 2.7 "Basic facts about Laser-based Autofocus systems" and 2.9 "An introduction to the measurement of tissue samples on slides" before preparing your samples. Understanding the underlying principles of laser-based autofocus systems in general and our approach for focusing slides will enable you to make better choices for your slide sample preparation.

The following slide sample preparations have worked well in our lab:

- Position the sample on the cover slip, not on the slide.
- The one peak autofocus can be easily thrown off by the use of a mountant with a refractive index similar to water, especially when using a water immersion objective. This leads to defocused images as an incorrect peak has been used for fitting. No autofocus error messages are seen (For more details see section 2.9).
- Cover the sample with a mounting medium that has a similar refractive index to glass.
- Avoid enclosing air bubbles within the region of interest.
- Only use cover slips of good optical quality which have a minimum thickness tolerance.
- Place the cover slip in the middle of the slide. When preparing your sample, keep in mind that the slide's edge may not be in the measurable plate area (see the section "Restrictions of Measurable Plate Area" in the Harmony Help or User Manual).
- Think about the working distance of the objectives you plan to use when choosing the cover slip (particularly its thickness). Further details can be found in section 1.4.3.
- When working with tissue sections, larger objects or patterns on slides it might be very useful to make use of the automated PreciScan (optional plug-in) which was introduced with Harmony 4.5. For more details please refer to chapter 2.11.
- Another feature introduced in Harmony 4.5 is called global image and allows region-based rather than field-based evaluations by creating a new image assembled from many input images (preferrably recorded with overlap). Please find more information about global image in chapters 2.2 "Experimental Setup – Optimization and Finalizing Steps" and 2.10 "Manual PreScan/ReScan option".

However, we still recommend using microplates instead of slides whenever it is feasible. High-content screening instruments are optimized for measurements of microplates and, in general, slide measurements are more prone to artifacts. In addition, cell samples covered with buffer can be prepared more efficiently in a microplate. Please refer to sections 1.1 and 1.2 and the standard protocols in the Appendix for further information and help with the sample preparation in microplates.

The one peak autofocus can be easily thrown off by the use of a mountant with a refractive index similar to water, especially when using a water immersion objective. This leads to defocused images as an incorrect peak has been used for fitting. No autofocus error messages are seen (For more details see section 2.9).

1.4 Objectives

All currently available objectives for the Opera Phenix are listed in ***Table 5-5*** with their innate features like the resolution, working distance (WD) and focal depth.

Before you can select the appropriate objective you must identify certain key requirements of your application:

- What size are the objects to be analyzed?
- How many cells per well will you need for a significant statistical analysis?
- How important is the imaging speed and how prone is your sample to photobleaching?
- What microplate are you using?

1.4.1 Resolution and magnification

The size of the objects that you intend to analyze will determine the resolution that you need.

Resolution is defined as the smallest distance between two distinguishable spots. In the Opera Phenix this depends on:

- The magnification and numerical aperture (NA) of the objective.
- Use of water immersion, high NA or low NA air objective.
- The pixel size of the camera.
- The binning of your image.

In practice, as the pixel size of the camera is fixed, the determining factor will be the binning and the objective.

If you choose the 2x2 binning this will quarter the image size and thus improve the acquisition speed but at the expense of image resolution.The resulting image will have half the resolution of an unbinned image.

For objectives the general rule applies that a high NA, usually coming with a high magnification, leads to a high resolution. The use of water as the immersion medium further increases the NA and thus the resolution. ***Table 5-5*** shows how the effective resolutions of the Opera Phenix depend on the objective used.

We recommend using a 20x objective as a starting point.

For analysis of whole cells or large compartments such as nuclei and cytoplasm, a 10x objective will be sufficient.

For detailed analysis of smaller organelles, or high resolution images for publication, the high magnification objectives (40x and 63x) are more suitable.

Use no binning if you need the highest resolution, but be aware that the size of an image without binning is four times increased compared to an image with 2x2 binning (the default). This slows down image acquisition, analysis and display significantly.

Example:

If you choose the ready-made solution (RMS) for the receptor internalization assay (described in the Image Analysis Guide, section 2.11 “Receptor Internalization”) you will notice that it generates a very robust read-out based only on the analysis of intensity in a region near the nucleus. This region of higher intensity is caused by the receptor being trafficked to endosomal compartments. At a magnification of 20x these appear more or less as one spot near the nucleus (***Figure 1-12***).

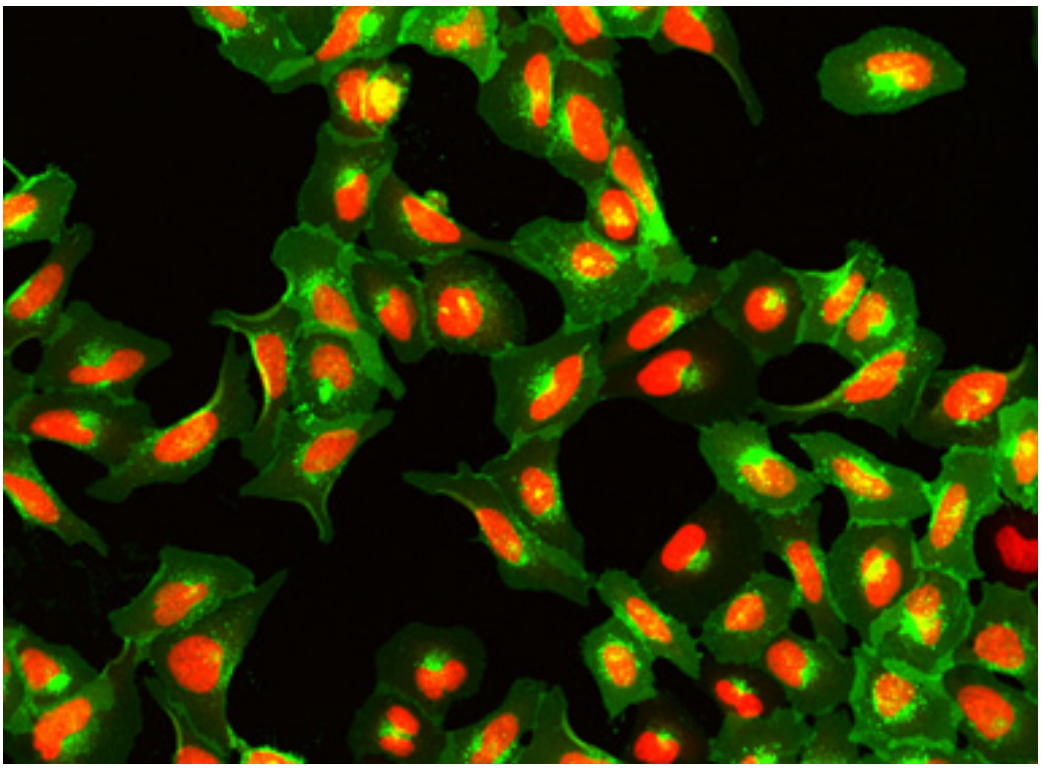


Figure 1-12: An example of an image from a receptor internalization assay. The internalized receptor appears as a spot-like structure near the nucleus. The image was acquired on the Operetta using a 20x objective.

With a higher resolution, for example using the 60x objective, you can see that each spot is actually an aggregation of endosomes (***Figure 1-13***).

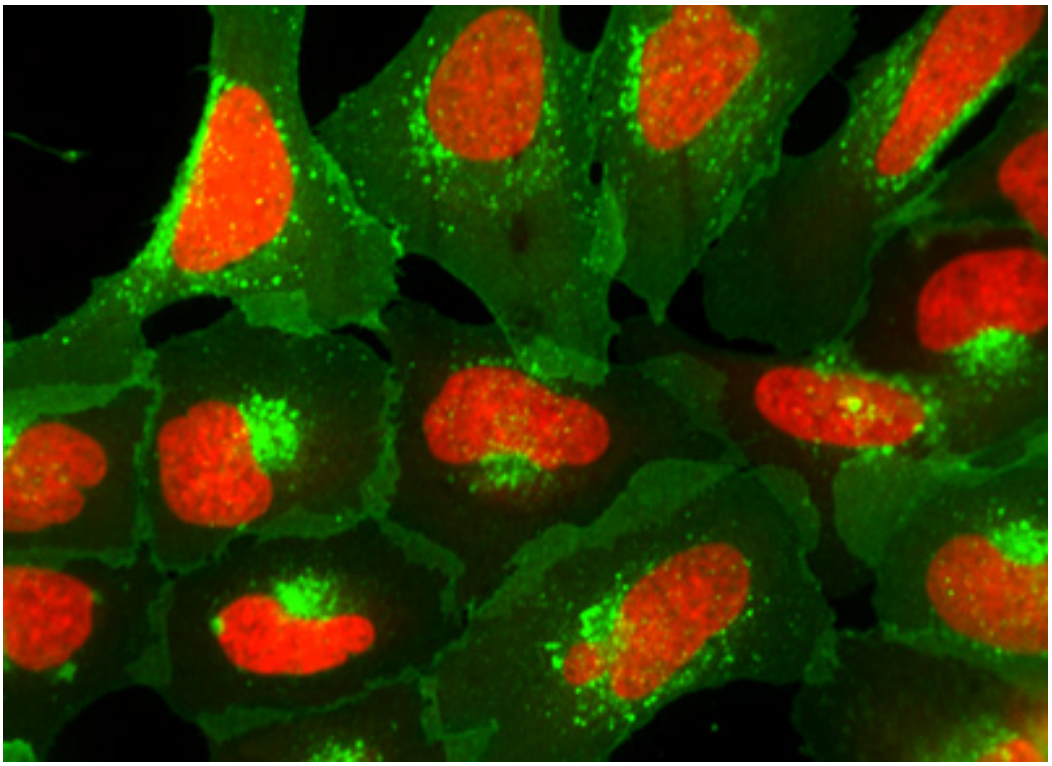


Figure 1-13: An example of an image from a receptor internalization assay, acquired on the Operetta using the 60x objective. Note the groups of endosomal spots near the nucleus.

The disadvantage with this high resolution image is that the number of cells/field is approximately 9 fold lower, i.e. to get comparable statistics your measurement time will need to be 9 times longer (and will generate 9 times more data to be stored). In addition, this high resolution does not lead to any improvement in the result obtained using the analysis sequence described in the Image Analysis Guide, section 2.11 “Receptor Internalization”. The only effect would be an increase in the evaluation time as more images have to be analyzed. Only if additional information about the process can be derived from the analysis of, for example, the number of individual endosomes and their size (see Image Analysis Guide, section 2.8 “Spot Analysis” as an example), a higher resolution is useful.

However, often a simple read-out is the most robust and in the case of the receptor internalization assay described above, we have found that the simple read-out based on the measurement using a 20x objective gives the best result.

Don’t fall for “the higher the resolution the better”! A high resolution is not desirable in every case and the acquisition of irrelevant details may even complicate the subsequent analysis of the effect that you are examining. The optimal resolution for your application depends strictly on the amount of detail that you want to analyze.

In general, we strongly recommend selecting the lowest magnification possible for your application for two reasons:

- Faster measurement and analysis.
- Significant statistics with a low number of fields.

For more details on statistics please see section 2.1.4 “How to define a suitable well layout”.

1.4.2 Numerical Aperture

The numerical aperture (NA) of an objective is a dimensionless number that determines the light-collecting abilities and the resolution of an objective.

The higher the NA, the higher is the resolution and the light-collecting efficiency. E.g. a 20x air objective with a NA of 0.8 is 4 times more efficient at collecting light than a 20x air objective with an NA of 0.4.

As a general rule it is desirable to use high NA objectives because:

- The necessary excitation power / exposure time is lower / shorter respectively and therefore photobleaching is reduced.
- The resolution is improved.

The NA values of all available objectives can be found in ***Table 5-5***.

Opera Phenix features three water immersion objectives (20x, 40x and 63x). Water is used as an immersion medium to increase the NA and the light transmission of an objective. The NA of an objective thus depends on the angle of aperture and the refractive index of the medium the light passes. Compared to air the refractive index of water is about 30% higher, which means that the achievable NA for water immersion objectives increases proportionally.

Please note that you can **either** have a high NA **or** a long WD distance (see below). Both are mutually exclusive due to the general working principle of objectives.

Your individual application will dictate what is more important, high NA or long WD. If you have already adapted your cells to a thick bottom plate then the trade-off in NA that you have to accept when using a long WD objective might be more acceptable than the additional effort involved in establishing the assay on a thin bottom plate. On the other hand, if you have very delicate samples with low fluorescence intensity which are prone to photobleaching, it might be worth switching to a thin bottom plate to enable the use of a high NA objective.

1.4.3 Working distance

The WD of an objective (see ***Figure 1-14***) is the distance from the lens to its focal point.

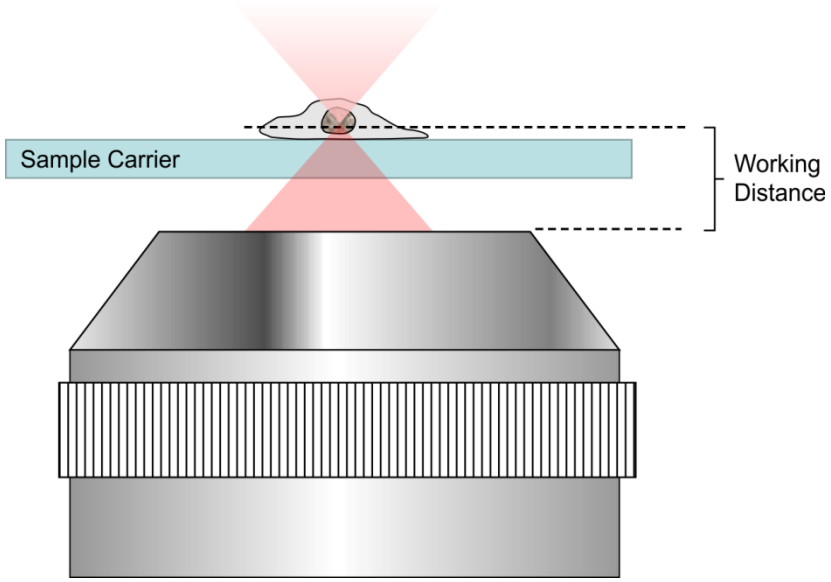


Figure 1-14: Illustration of WD. The working distance is defined as the distance between the front end of the objective and its focal plane.

The WD of an objective determines which type of plate can be used (with regard to plate bottom thickness and plate geometry, see sections 1.2.2 and 1.2.3).

As a general rule, high NA objectives have a lower WD and require plates with a thin (< 200 µm) and low bottom (small distance between plate bottom and rim of the plate). If plates with a thicker or higher bottom are used, the measurement area will be restricted or they will not work at all. On the contrary, long WD objectives work with a large variety of plate types, e.g. plates with high bottoms and/or bottoms thicker than 200 µm. Therefore, you will encounter plate restrictions with **water immersion objectives more often than with air objectives in Opera Phenix.** In ***Table 5-6*** to ***Table 5-8*** in the Appendix the compatibility of different plates with the available objectives is shown.

1.4.4 Correction Collar

Objectives are either inherently optically corrected for a specific plate bottom thickness, or they have a correction collar to adjust the optical correction to a certain plate bottom thickness. This will prevent spherical aberrations caused by differences in plate bottom thickness. For details on setting the correction collar please consult the Harmony Help or User Manual.

In Opera Phenix the long WD air objectives 20x and 40x have such a correction collar and are therefore suited for a wider range of plates. All the other objectives are corrected for a plate bottom thickness of 0.17 mm.

Please see ***Table 5-5*** for more details.

Before you start a measurement using the long WD 20x (NA 0.4) or 40x (NA 0.6) air objective, please set the correction collar to the value of the plate bottom thickness! Otherwise you will not achieve optimal results for the acquired images. Depending on how wrong the correction collar is set, the images will be blurred and distorted or in more extreme cases you will encounter problems to focus the plate bottom.

Please note that a high NA 20x (NA 0.8) and 40x (NA 0.75) air objective are available as well. They don't have a correction collar and should only be used with thin bottom (< 200µm) plates.

Please do not set the correction collar to values higher than 1 mm as automatic image alignment is only possible for correction collar settings ≤ 1 mm.

1.4.5 Water immersion vs. air objectives

While the basic properties of the objectives are explained above, we here explain in detail the functional differences between water immersion and long WD air objectives with special emphasis on confocal imaging.

Compared to air objectives water immersion objectives allow for a higher resolution and better in depth imaging of thick objects. Water as immersion medium allows better light collecting capabilities and matches the refractive index of water-based cell samples. By this, light scattering at the immersion medium and plate bottom interface and thus loss of transmission is minimized.

As a general reminder for this section: Water immersion and high NA air objectives have a high NA, but a short WD, and the long WD air objectives used in the Opera Phenix have a low NA but a high WD.

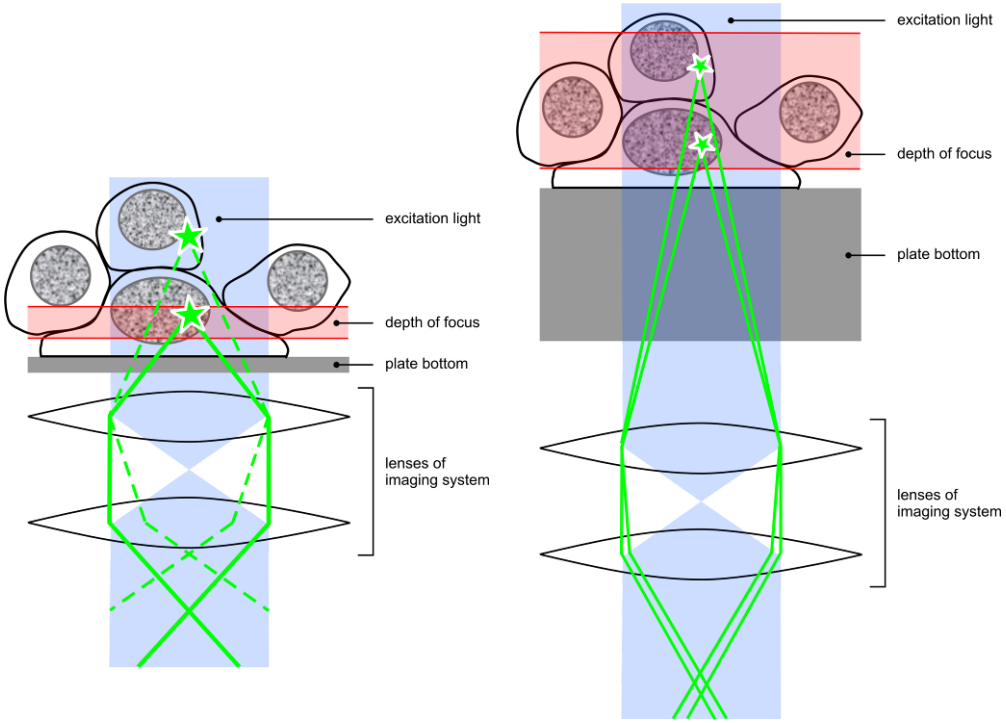


Figure 1-15: Comparison of a high NA objective (left) and a long WD objective (right); both are shown in non-confocal mode. The different sized stars symbolize the amount of fluorescence emission collected.

High NA objectives have a **better light collection efficiency** than long WD (= low NA) objectives. In addition, they **have a thinner depth of focus**, i.e. fluorescence emission generated outside of this plane is projected less sharply onto the camera (indicated by the dotted line in the left of ***Figure 1-15*** which ends in a different image plane to the solid green line).

In contrast, long WD objectives have a **lower light collection efficiency** (represented by the smaller stars and thinner lines in the right of ***Figure 1-15***). All of the emission fluorescence from within their **larger depth of focus** is indiscriminately projected onto the camera (indicated by the fact that both lines in the right of ***Figure 1-15*** end in approximately the same image plane).

The objects of interest will be optimally captured if their thickness is equal to the depth of focus of the employed objective (for a list of the depth of focus of all available objectives, see ***Table 5-5***).

The thin depth of focus and the high NA of PerkinElmer's water immersion objectives are especially advantageous in combination with confocal imaging, as these characteristics add to the suppression of out-of-focus light, thus improving the contrast. This enables the highest achievable axial resolution of z-stacks for 3D applications (see ***Figure 1-16***).

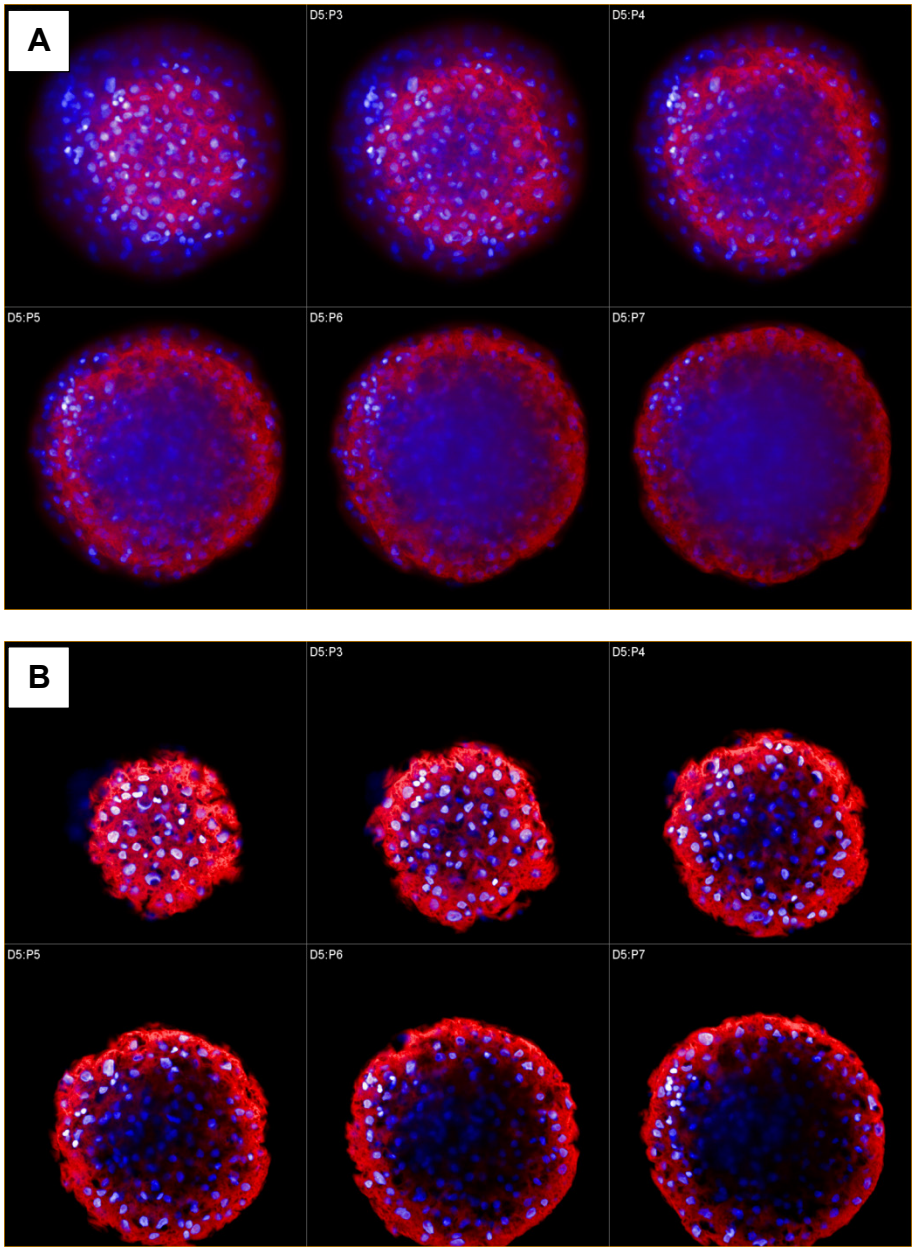


Figure 1-16: Same 3D microtissue imaged with either 40xhNA (NA 0.75) (panel A) or 40xW (NA 1.1) objective (panel B) . A confocal z-stack of a human liver microtissue (from Insphero) stained with Hoechst and CellMask™ Deep Red was acquired with a step size of 5µm and identical exposure settings. Two effects contribute to the overall sharper and brighter appearance of nuclei and cytoplasm in panel B: the higher numerical aperture of the water lens and it`s smaller depth of focus.

In summary, water immersion objectives are useful when:

- You need a high resolution.
- The structure you want to analyze is as thick as the depth of focus of the respective high NA objective.
- Your sample has low fluorescence intensity.
- You want to reduce the acquisition time.
- Photobleaching is critical: using water immersion objectives the necessary laser power / exposure time is lower / shorter respectively.

The main drawback of water immersion objectives is their short WD of about 0.6 mm which requires the objective to move very close to the plate bottom when focusing into a well. Especially the water collar with its large physical dimensions has a high risk to collide with the table when focusing into the outer wells of a microplate.

The exception is the 20x water immersion objective which has a working distance of 1.7 mm.

Therefore, your sample needs to be placed in a high quality microplate with a low, thin bottom. This limits the number of suitable microplates and / or the usable area of the microplates. For further information on plate restrictions please refer to section 1.2.4.

In summary, long WD objectives are useful when:

- The structure of interest is as thick as the depth of focus of the respective long WD objective.
- You want to use a microplate with a high and / or a thick bottom.
- The bottom of the microplate is slightly bent (in this case the higher depth of focus means that the cells are nevertheless reasonably sharp over the whole image field).

However, the necessary prerequisite for using a long WD objective is that your sample has a high fluorescence intensity and / or you are willing to accept longer exposure times.

The remaining water at the plate bottom from measurements with water objectives can lead to focus failures or image distortions when using air objectives at the same position. Please dry your plate when changing from water to air objectives.

1.5 Optical Modes

1.5.1 Introduction to Confocal Imaging

The confocal optics of the Opera Phenix are designed for high resolution; high transmission efficiency and optimized suppression of out-of-focus light. Furthermore, the proprietary optical setup called Synchrony Optics minimizes crosstalk during simultaneous confocal measurements. The following chapters describe these features, their technical background and consequences for an optimal experimental setup.

Advantages of confocality

The main advantage of confocality is to strongly improve image contrast in 3D samples by rejection of out-of-focus-light. Two effects contribute to this (see ***Figure 1-17***).

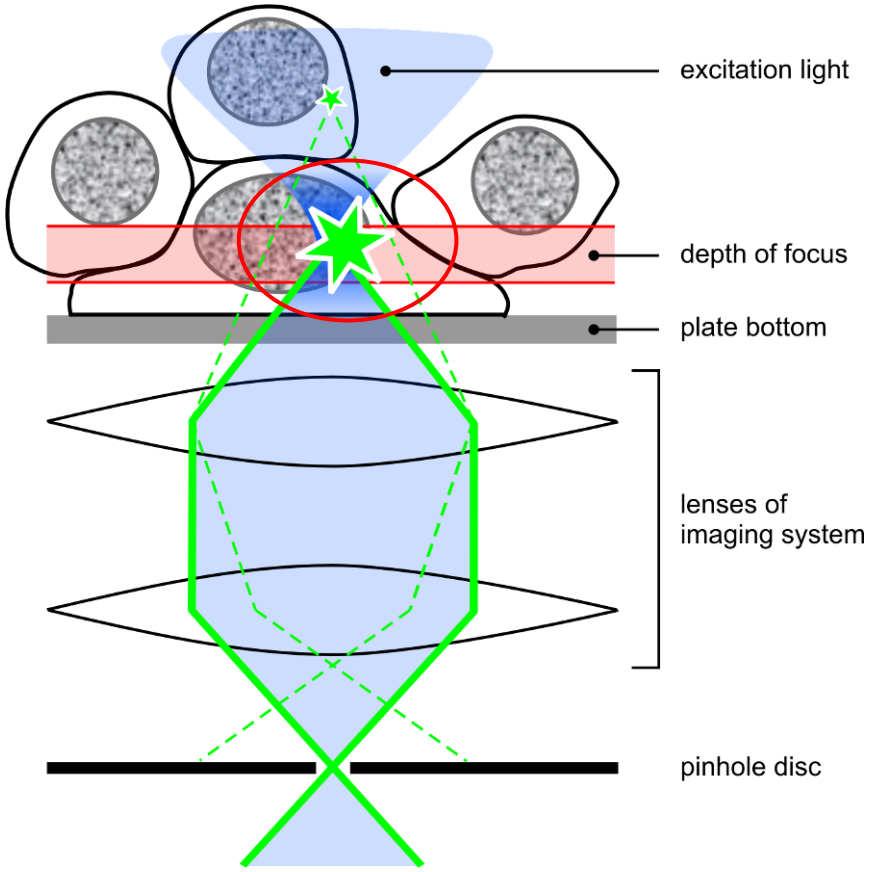


Figure 1-17: Illustration of the principles of the confocal acquisition mode.

The first contribution results from the fact that excitation light (solid green line) that is focused onto the sample by the objective has to pass through a small aperture called a pinhole. This concentrates the main excitation intensity within the focal plane of the objective (illustrated by the darker blue zone of the excitation light in ***Figure 1-17***). Consequently, cells are optimally excited at this point (indicated by the large green star in ***Figure 1-17*** which symbolizes the amount of fluorescence emission) whereas cells outside this zone are excited less (indicated by the smaller green star). This minimizes the amount of out-of-focus fluorescence that is generated. However, the contribution of this effect depends on the specific objective and magnification that is used.

The second (and most commonly known) factor is that remaining out-of-focus fluorescence emission is blocked by the pinhole disk on its way back from the sample (dotted green line in ***Figure 1-17***) to the detector. Only a very small portion of this out-of-focus fluorescence will pass through the pinhole and reach the camera, while the majority of light is reflected at the disk itself.

In non-confocal mode (i.e. widefield mode), all areas of the sample are excited equally strong (illustrated by the evenly distributed blue excitation light in ***Figure 1-18***), and thus will emit equal amounts of fluorescence (illustrated by the green emission stars). In addition, the out-of-focus light (dotted green line) is not blocked from reaching the camera. Especially in thicker samples, this will reduce the resolution of features that are located in the focal plane of the objective and any fine detail will be lost.

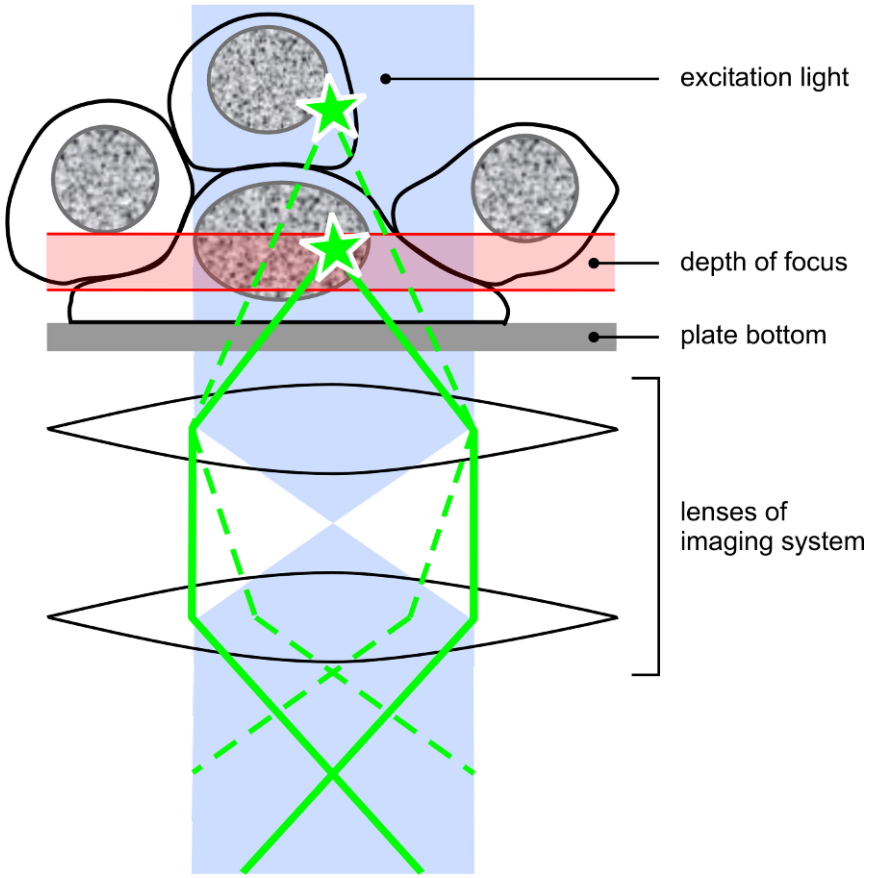


Figure 1-18: Illustration of the principles of the non-confocal acquisition mode.

A direct comparison of images from a multilayered cell sample, acquired in confocal and non-confocal modes, is shown in ***Figure 1-19*** and ***Figure 1-20*** respectively.

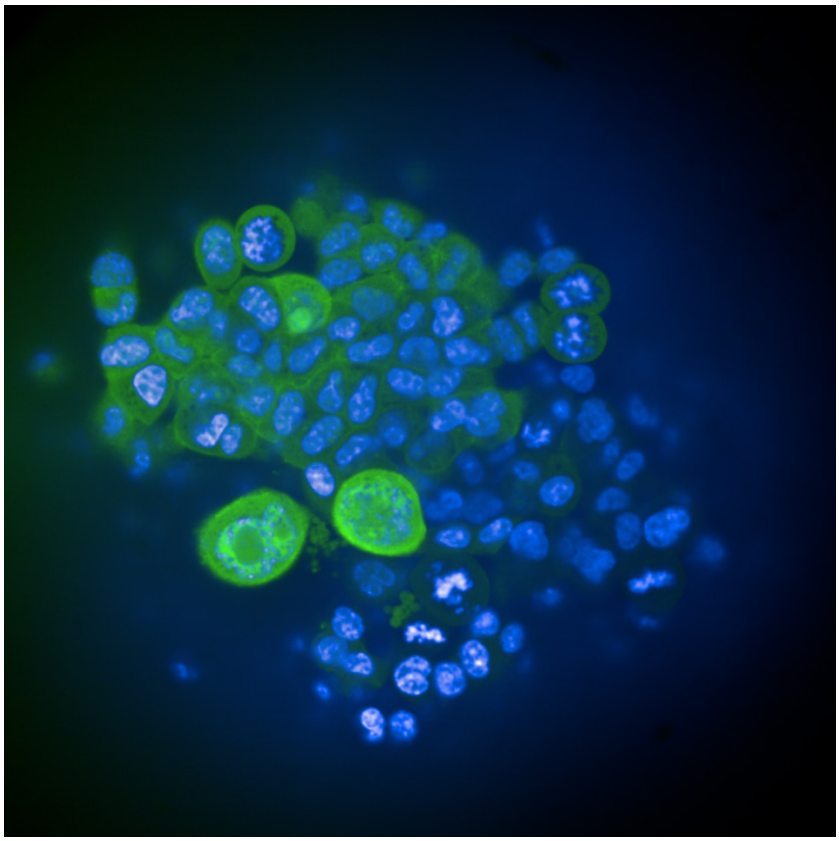


Figure 1-19: Confocal image of a cancer microtissue (HCT116) stably expressing cytosolic GFP (shown in green), nuclei are stained with Hoechst 33342 (shown in blue). The image was acquired with a 40x water immersion objective (NA 1.1).

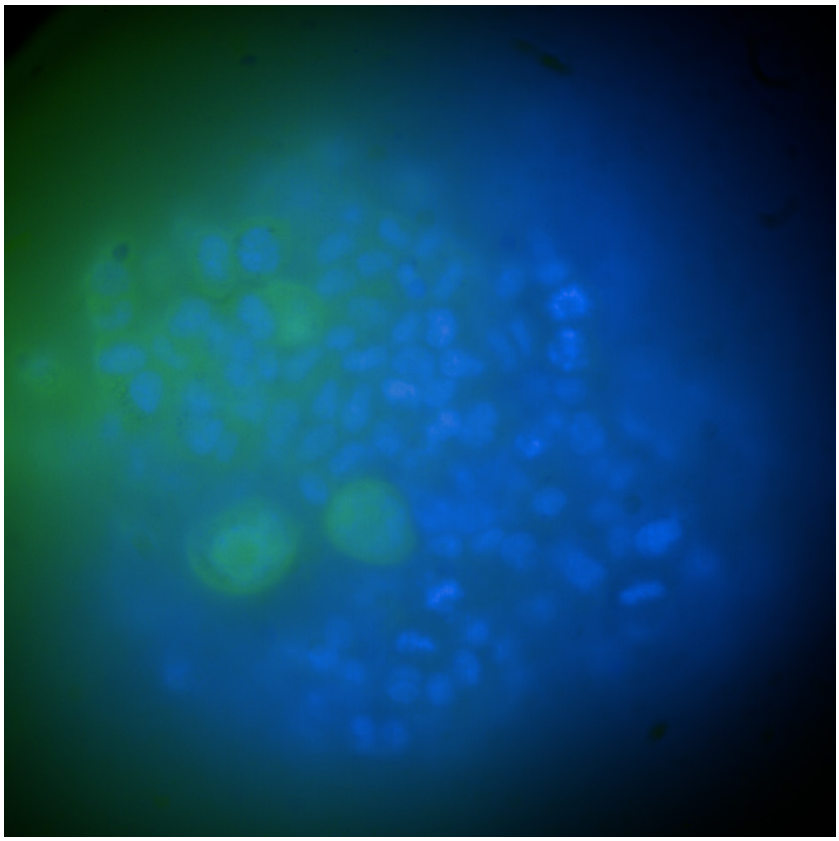


Figure 1-20: Non-Confocal image of a cancer microtissue (HCT116) stably expressing cytosolic GFP (shown in green), nuclei are stained with Hoechst 33342 (shown in blue). The image was acquired with a 40x water immersion objective (NA 1.1).

Confocality in the Opera Phenix

The Opera Phenix is a microlens-enhanced spinning disk confocal microscope. The sample is simultaneously excited and scanned with multiple pinholes, enabling the combination of confocality AND high speed acquisition.

The confocal unit comprises two spinning discs; a microlens and a pinhole disk (see ***Figure 1-21***).

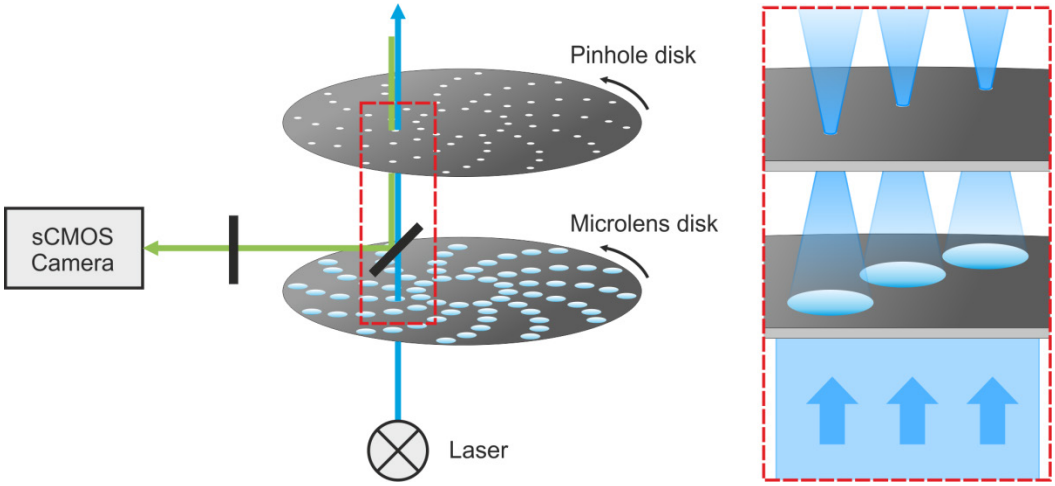


Figure 1-21: Confocality in the Opera Phenix. Insert: Each microlens captures the laser excitation from larger area and focuses it onto its pinhole counterpart.

The pinhole disk is responsible for creating the confocality (as explained previously), whereas the microlens disk increases the transmission of excitation light (and thus reduces exposure times). Each microlens captures excitation light from a large area and focuses it onto the corresponding pinhole, so that 55% of the incident light is transmitted. Without the microlens disk only 0.74% of the total area of the pinhole disk would transmit light thus the microlens disk is improving the transmission efficiency by a factor of 75 (see insert in ***Figure 1-21***).

The pinholes of the confocal disc have a diameter of 50 µm, the same diameter as used in the Operetta system or the former Opera QEHS.

However, the distance between the pinholes has been increased compared to the Operetta system and the Opera QEHS thus reducing out-of-focus crosstalk between the pinholes. This translates into sharper images with less background when using **thick** samples such as cell-multilayers, microbodies and tissue samples, see ***Figure 1-22***.

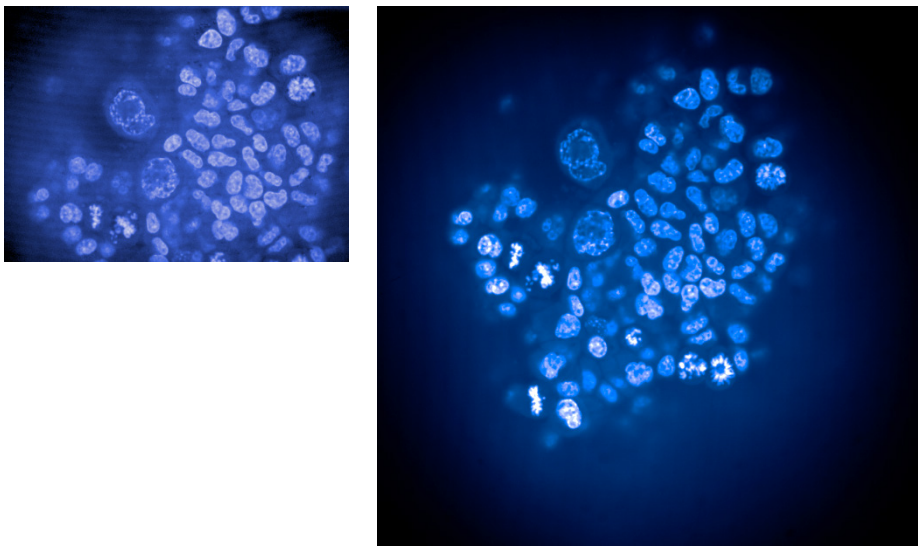


Figure 1-22: Confocal image of a cancer microtissue (HCT116), nuclei are stained with Hoechst 33342. The left image was acquired with an Opera® QEHS and the right image with an Opera Phenix™ with increased pinhole distance which reduces background caused by out-of-focus crosstalk. Both images were acquired with a 40x water immersion objectives in a z-height of 15µm.

An excellent resource with interactive animations illustrating the principles of spinning disk confocal microscopy can be found at http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/index.html

1.5.2 Considerations for confocal measurements

The microlens enhanced pinhole disc of the Opera Phenix with its improved pinhole distance effectively rejects out-of-focus light resulting in enhanced optical sectioning. Although the microlenses dramatically increase the light throughput there is still a loss of light at the spinning disc.

Therefore, the improvements in contrast and axial resolution come at the expense of the overall sample brightness and therefore longer exposure times are necessary. Depending on the sample and the analysis requirements, the exposure time has to be increased when using the confocal mode. For example, a multilayered, highly fluorescent cell sample or a 3D microtissue as shown in ***Figure 1-19*** benefits from confocality. The contrast is dramatically improved and a better resolution is achieved compared to the non-confocal image (***Figure 1-20***).

Thin samples with low fluorescence intensity may yield a better result when imaged with a high NA objective in non-confocal mode.

The choice of objective also needs to be considered when determining the optimal acquisition mode for a sample. The topic of high NA vs. long WD objectives is discussed in section 1.4.5. Combining confocal acquisition with automated water immersion objectives will help to further decrease acquisition time and additionally improve image resolution compared to high NA air objectives (see ***Figure 1-16*** in section 1.4.5)

Detailed recommendations for the experimental setup are given in chapter 2.

1.5.3 Synchrony Optics™

The ability of a multi-camera Opera Phenix to acquire images of different fluorophores in parallel considerably increases speed compared to one-camera systems. However, a potential drawback is the spectral **crosstalk** between simultaneously excited channels. If you are not familiar with the phenomenon of crosstalk (often also called spill-over) please refer to chapter 1.6.3.

The novel proprietary Synchrony Optics™ can significantly reduce this crosstalk during simultaneous confocal acquisition.

Working principle of Synchrony Optics

In this chapter we describe in detail the technical background of Synchrony Optics. The following chapter "Practical implications of Synchrony Optics" will then help you to optimize the usage of your instrument and avoid potential pitfalls.

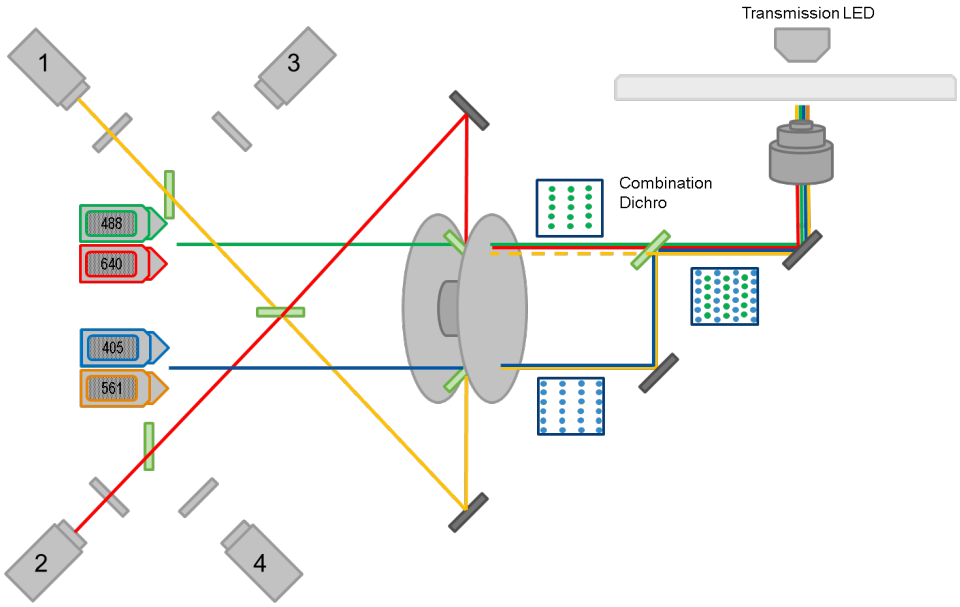


Figure 1-23: Simplified schema of the Synchrony Optics light path of an Opera Phenix with four laser lines and four cameras

Excitation lasers (at the left side in ***Figure 1-23***) are grouped into two pairs so that neighboring laser lines – which usually create the most crosstalk – are in different groups. In this example we will focus on the 405nm (depicted in blue) and 488nm laser (depicted in green) for the excitation of Hoechst and GFP. The Hoechst emission spectrum shows a broad overlap with GFP thus measuring these dyes simultaneously with a traditional approach like e.g. the Opera QEHS gives rise to a high amount of crosstalk (see ***Figure 1-24***).

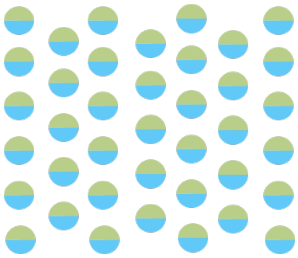
Light from each laser group (blue and green line) is delivered through a separate fiber to two opposing quadrants of the spinning microlens and pinhole disk. **This results in two non-overlapping excitation pinhole patterns for the two excitation lasers (the blue and green pattern shown in *Figure 1-23*).** These are then combined at the combination dichroic mirror resulting in a non-overlapping excitation pattern at the right side of the combination dichroic. This is focused into the sample, where consequently **distinct** locations are excited with the 405nm and 488nm lasers (see blue and green dots in ***Figure 1-24***).

The light emitted by the fluorophores is collected by the objective and split according to wavelength at the combination dichroic mirror. Light from the fluorophore with shorter emission wavelength (orange, emission of Hoechst in our example) will then pass through the lower quadrant where it is then reflected by the primary dichroic

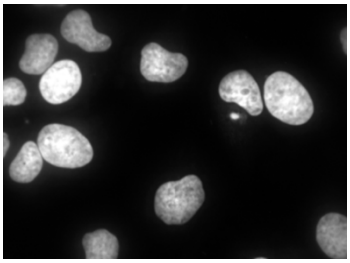
mirror towards camera 1. Light from the other fluorophore (red, emission of GFP in our example) passes through the combination dichroic mirror and will then pass through the upper quadrant where it is then reflected by the primary dichroic mirror towards camera 2).

For spectral characteristics of the combination dichroic see ***Figure 1-25*** on the next page.

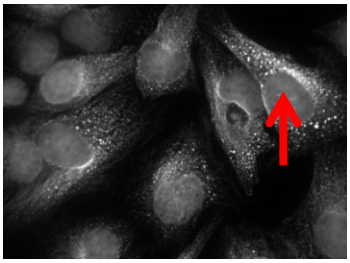
The key point is that the crosstalk signal – in our example fluorescence emission of Hoechst in the spectral range of GFP emission (indicated by an **orange dotted** line) – will be directed towards the upper pinhole window and not to the pinhole window where its excitation light passed through. But as the pinhole patterns are non-overlapping the vast majority of this **crosstalk signal does not hit any pinholes and is reflected**. So the majority of crosstalk from Hoechst never gets further than the pinhole disk and does not reach camera 2, where it would show up in the GFP channel image. In ***Figure 1-24*** this is illustrated within the sample:



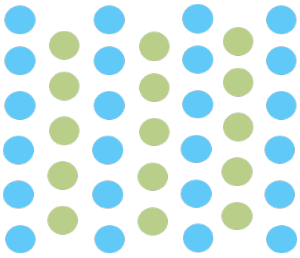
In a convential Nipkow disk system **the same** location is excited simultaneously with 405nm and 488nm.



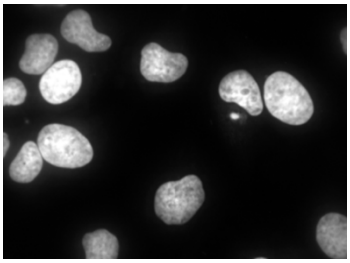
Hoechst image



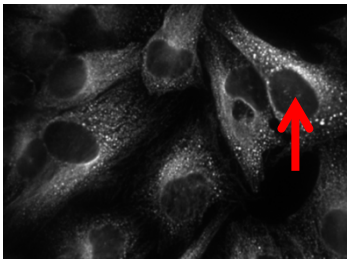
GFP image with Hoechst crosstalk in the nucleus.



Opera Phenix with Synchrony Optics™ excites **different** locations simultaneously with 405nm and 488nm.



Hoechst image



GFP image **without** Hoechst crosstalk in the nucleus.

Figure 1-24: Comparison of crosstalk between conventional Nipkow disk systems and the Opera Phenix during simultaneous acquisition of Hoechst and GFP channel. Images were recorded with a 60x water immersion lens.

In summary confocal Synchrony Optics separate excitation of adjacent fluorescent channels in time and space to reduce spectral crosstalk during simultaneous acquisition on average by 98%.

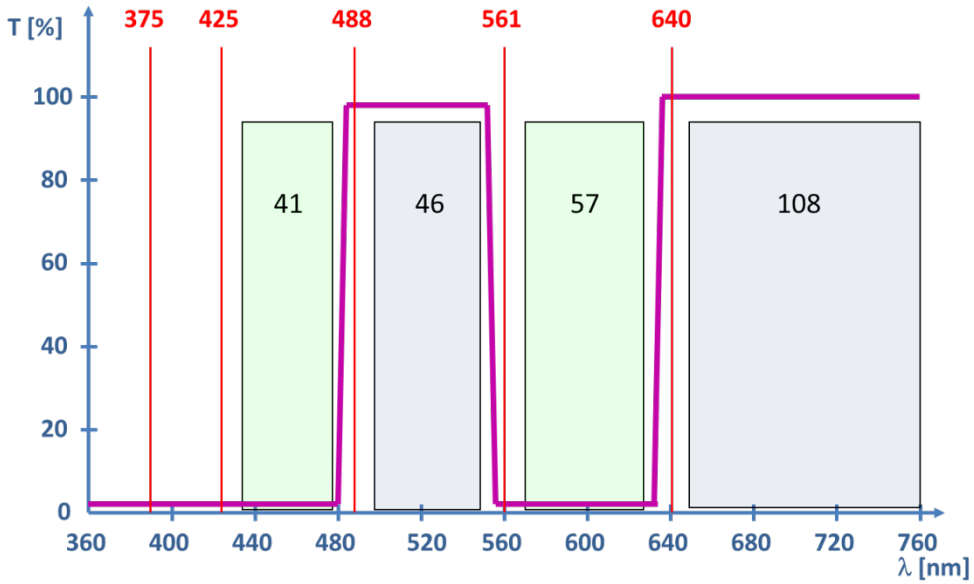


Figure 1-25: The standard combination dichroic is transmissive (magenta line) between 500-550nm and above 650nm and reflects in the wavelengths bands below 500nm and between 550-650nm. Thus neighboring emission bands (green and grey) are sent into different lightpaths back to the pinhole disk. Thus crosstalk into a neighboring emission band will be sent into the "wrong" path and be reflected at the disk as it does not hit any pinholes.

Practical implications of Synchrony Optics

Residual crosstalk

Synchrony Optics minimizes the crosstalk significantly between adjacent emission bands, but there is some residual emission crosstalk and you have to confirm whether this is acceptable for your application.

This is especially important for the nuclear dyes Hoechst and DAPI which a) are exceptionally bright and b) their emission spectra show a very broad overlap with the neighboring EGPF channel (500-550nm).

Please refer to the chapter about crosstalk (1.6.3) for further instructions and information about crosstalk in multi-camera devices in general and its implications.

Implications for FRET and Fluorophores with long Stokes Shift

The same mechanism that minimizes crosstalk will prevent measuring fluorophores with long Stokes shift or FRET pairs in confocal mode.

Please note that this applies to all Opera Phenix devices, including 1, 2 and 4 camera models.

For a given excitation any light that is emitted at wavelenths that fall into the emission band adjacent to the normal emission band will be sent by the standard combination dichroic (see ***Figure 1-25***) into the "wrong" lightpath and thus be rejected at the pinhole disk. E.g. if your fluorophore or FRET pair donor has to be excited with 488nm and the (acceptor) emission is in the 570-630nm range this cannot be measured in the confocal mode.

Harmony will automatically prevent impossible combinations of excitation and emission in confocal mode and you will be informed accordingly.

However, to enable the most common FRET pairs such as CFP-YFP FRET in confocal mode, we added an alternative combination dichroic which combines the adjacent bands in this range, please refer to chapter 2.4. So, in fact such FRET pairs as well as dyes (with long Stokes shift) which are excited with a UV laser and emit in the 500-550 nm range can be measured in confocal mode.

Another possibility is to chose a Long-Stokes Shift fluorophore or an acceptor of your FRET pair that is even further shifted into the **third band** (in the example above this would be the 650-760 nm band). In this case you will be able to measure them again in confocal mode.

Please note that this effect also applies to crosstalk, if emission extends across three bands, crosstalk will not be reduced in the third band (which is e.g. the case for Hoechst, which also extends into the overnext channel, see ***Figure 1-26*** below).

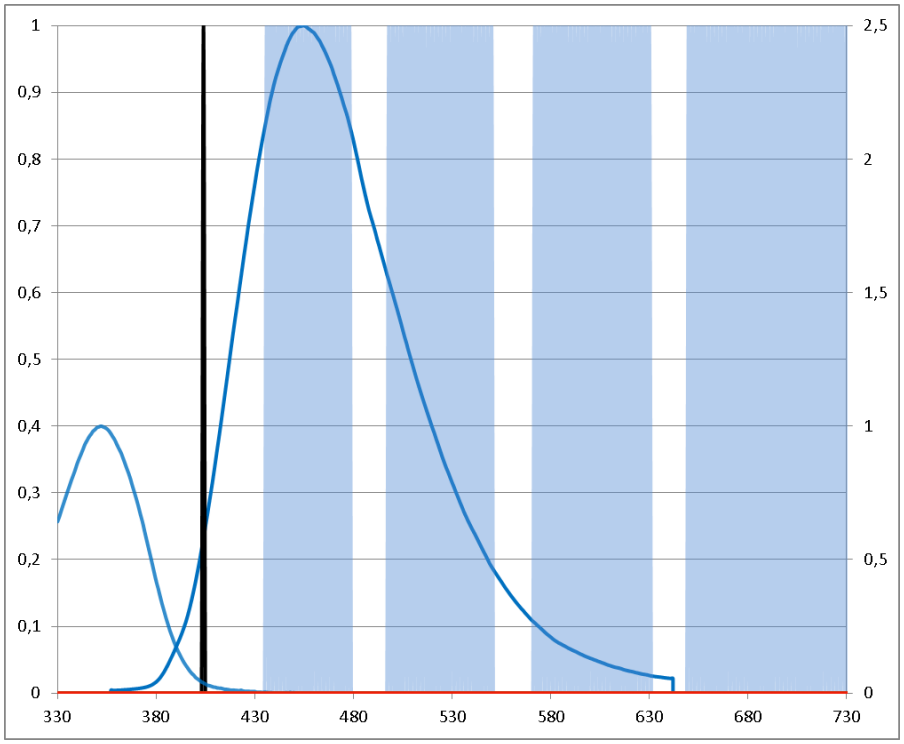


Figure 1-26: The emission of Hoechst extends across three emission bands of the Opera Phenix. In non-cofocal mode ~40% of the Hoechst signal captured in the first emission band reaches the adjacent second band (e.g. GFP, 500-550nm) and ~ 8% is detected in the third band e.g. TRITC, 570-630nm). Synchrony Optics significantly reduces crosstalk into the adjacent band, but not crosstalk into the third band.

1.6 Fluorophores

In this section we will discuss some of the fundamental principles of using fluorophores for imaging applications, to enable you to make an educated choice of fluorophore when planning your assay. The correct choice of fluorophore is crucial for simple and fast assay development.

This section discusses:

- Signal versus reference stains (section 1.6.1)
- Whether the fluorophore fits with the filters (section 1.6.2)
- How to select a fluorophore combination and avoid crosstalk (section 1.6.3)
- How to avoid photobleaching (section 1.6.4)

Please note: we only provide recommendations in this chapter for the use of fluorophores which have worked well in our lab. This chapter is neither a comprehensive discussion of the available fluorophores on the market, nor a detailed discussion of the basic principles of fluorescence. For the latter, we recommend the following for further reading:
http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorescenceintro.html

1.6.1 Signal versus reference stain

Signal stain

When you start planning your assay you have a certain read-out in mind. For example you may want to determine the ratio of concentrations for a given protein in the cytoplasm compared to the nucleus. In order to measure this you will need a fluorescent label for the protein, the so-called signal stain.

This fluorescent label can be attached to the protein by various methods. It could be a fluorophore coupled to an antibody, you could tag the protein directly with a fluorescent protein such as GFP (Green Fluorescent Protein), or there might be a specific fluorescent stain available which exclusively stains your target protein.

The method that is applicable will depend entirely on your target and will require thorough research during the assay planning stages.

Good starting points for your research are:

- www.lifetechnologies.com
- www.cellsignal.com
- www.rndsystems.com
- www.sigmaaldrich.com
- www.abcam.com

Examples of well suited methods for assays in the areas of Cell Cycle, Apoptosis and Cytotoxicity can be found in chapter 3.

Reference stain

- The first step of the analysis is always a robust identification of your cells (see Image Analysis Guide, section 1.3 “Setting up an Image Analysis Sequence” for details). In this context, robust means that it always works reliably, independently of your assay conditions (e.g. both for positive and negative control).
- A nuclear reference stain can be the basis for robust cell detection. It is crucial that the nuclei are well stained in any given assay condition.

A typical beginner’s error is to include ONLY the signal stain(s) in the assay planning. The intensity of the signal stain varies with the experimental conditions and in most cases this is unsuitable for object detection.

Example:

If you attempt to perform the nuclear detection using your signal stain, when performing a cytoplasm-to-nucleus translocation assay, then you would get a result similar to that shown in ***Figure 1-27***.

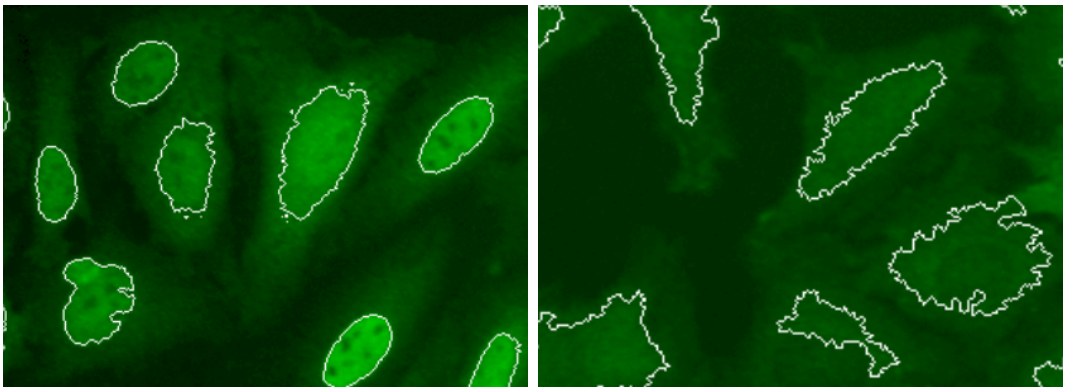


Figure 1-27: The images show positive (left) and negative (right) controls in a cytosol-to-nucleus translocation assay (see section 4.9 “Cytosol to Nucleus Translocation”). The nuclear detection is based on the signal stain channel.

In the positive control, shown on the left in ***Figure 1-27***, the nuclei are detected sufficiently well (except in non-responding cells without nuclear translocation). However, in the negative control (right) nuclei are not sufficiently contrasted and thus detection is inadequate and insufficient for analysis. Any further detection (e.g. of the cytoplasm) based on this would lead to artifacts and cause unnecessary variations in your final results.

By using a **reference stain** (sometimes also called a counter stain) for nuclei in addition to a signal stain, the quality of nuclear detection is always good in both positive (***Figure 1-28***, left) and negative (***Figure 1-28***, right) controls.

For another example of the use of the signal stain for detection, see Image Analysis Guide, chapter 2.15 “Cell Shape – Cell Rounding”.

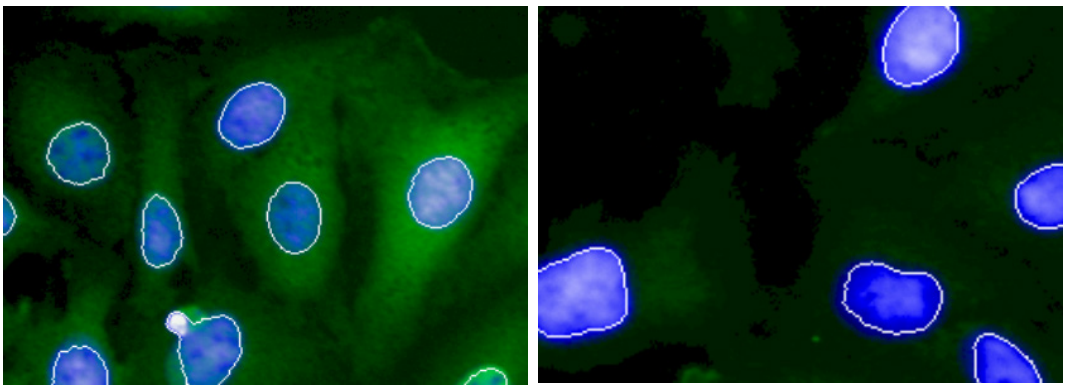


Figure 1-28: The images show positive (left) and negative (right) controls in a cytosol-to-nucleus translocation assay (see section 4.9 “Cytosol to Nucleus Translocation”). The nuclear detection is based on the reference stain channel.

It is often useful to include a reference stain for the nucleus in your assay setup.

Some nuclear reference-stains also weakly stain the cytoplasm and are therefore sufficient for robust detection of cytoplasm (see below).

Membrane and organelle reference staining is rarely necessary (only for specific applications, e.g. colocalisation). For an example of a membrane analysis that uses the cytoplasmic reference stain without the need for a specific membrane reference stain, see Image Analysis Guide, chapter 2.7 “Quantification of Membrane Marker”.

When using a reference stain:

- First select your signal stain(s), then identify a free wavelength and pick your reference stain accordingly. Usually you will be restricted in the choice of your signal stain(s), whereas there is more choice for reference stains.
- Especially the most common nuclear reference dyes Hoechst 33342 and DAPI have emission spectra that extend far into the green range thus giving rise to crosstalk. Please refer to chapter 1.6.3 “How to avoid crosstalk in a fluorophore combination” for further information.
- Make sure that the reference stain is roughly in the same range of brightness as the signal stain (see section 2.1.2 for further explanation).

Reference stains for nucleus and/or cytoplasm

Many nuclear stains also allow the detection of cytoplasm due to incidental fluorescent labeling of cytoplasmic components such as RNA or proteins. There are numerous reference stains available (see ***Table 1-3***), but the preferred and most commonly used nuclear and cytoplasmic reference stains in our lab are Hoechst 33342 and DRAQ5™.

Dye	Staining protocol suited for…	Excitation/ Emission Wave-length [nm]	Tips for use	Examples (described in the Appendix, starting on page 171)
Stained compartment: **NUCLEUS**				
Hoechst 33342	live and fixed cells	355/465	Preferred if the robustness of the nuclear detection is crucial, and if cytoplasmic detection via the nuclear stain is not necessary. Brighter than most other fluorophores, use at low concentration.	Example Datasets 1, 2, 4, 5, 6
DAPI	fixed cells	345/458	Preferred if the robustness of the nuclear detection is crucial, and if cytoplasmic detection via the nuclear stain is not necessary. Brighter than most other fluorophores, use at low concentration.	
SYTO® 10	live cells	484/505		
BOBO™ -3	fixed cells (dead cells)	570/602	Also stains the nuclei of non-fixed cells that have compromised membranes. As a result, it is suitable as a "dead" stain (see the application "Cytotoxicity-1" in section 5.2).	Example Dataset 1
Propidium-Iodide	fixed cells	534/620		
Stained compartment: **NUCLEUS AND CYTOPLASM** (the latter being more weakly stained)				
Cell Mask™ Blue (often in combination with **Hoechst 33342)**	fixed cells	346/450	Preferred if both nuclear and cytoplasmic detection are necessary. Please note that CMB can only be used with the 375nm laser line. Depending on the cell line, the contrast between the nucleus and cytoplasm may be too low for robust nuclear detection: - Use either in addition to Hoechst 33342, to increase the nuclear intensity, or - Use an alternative pair of nuclear and cytoplasmic stains with different wavelengths.	

Dye	Staining protocol suited for…	Excitation/ Emission Wave-length [nm]	Tips for use	Examples (described in the Appendix, starting on page 171)
DRAQ5 ™	live and fixed cells	647/670	Preferred if both nuclear and cytoplasmic detection is necessary. Works with most cell lines, however, for some, the contrast between the nucleus and cytoplasm may be too low for robust nuclear detection. In this case, use nuclear and cytoplasmic stains with different wavelengths.	Example Datasets 3, 5, 7
Stained compartment: **CYTOPLASM**				
Calcein	live cells	492/514	Very bright and bleaches easily.	
CMFDA	live cells	490/517	Very bright and bleaches easily.	

Table 1-3: Reference stains for nuclei and/or cytoplasm.

In live cell applications any nuclear (DNA) stain is toxic at high concentrations. Please use the lowest possible concentration, exposure time and laser power to get just enough signal intensity for robust image analysis.

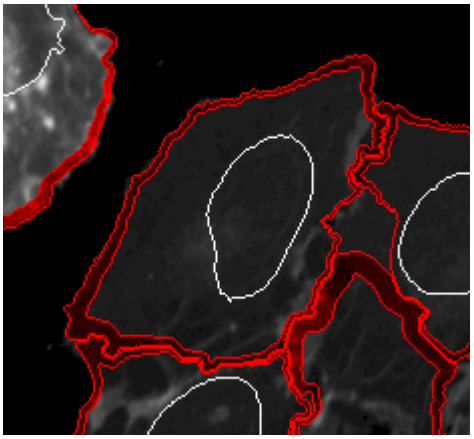
Reference:

Martin RM, Leonhardt H, Cardoso MC (2005) "DNA Labeling in Living Cells". Cytometry Part A: The Journal of the International Society for Analytical Cytology, 67 (1), pp 45-52.

Instead of applying a whole cell reference stain you might consider using label-free digital phase imaging. Digital phase images are recorded in brightfield mode at the lowest phototoxic level possible. Whole cell segmentation can be applied easily as the DPC images are contrast optimized. For more information please refer to section 2.3.2.

Reference stains for Membrane and Organelles

From an image analysis perspective, the quantification of a marker located in the plasma membrane is very similar to the quantification of a marker located in the cytoplasm, except that it uses a different region of interest. This is described in detail in the Image Analysis Guide, chapter 2.7 “Quantification of Membrane Marker”. The only prerequisite is a good cytoplasmic stain as a specific membrane reference stain, in our experience, does not enhance the result. See ***Figure 1-29*** for an example of a membrane detection based on a cytoplasmic stain.



Dynamic plasma membrane region of ± 5 % around the detected cytoplasmic outlines.

Figure 1-29: Example of a user-defined plasma membrane region. The definition is based on a good detection of the cytoplasmic region by the analysis software. This is in turn dependent on an excellent cytoplasmic reference stain, in this case DRAQ5™. For further details, see Image Analysis Guide, chapter 2.7 “Quantification of Membrane Marker”.

The detection of small organelles (mitochondria, lysosomes, peroxisomes, etc.) is usually performed as described in the Image Analysis Guide, chapter 2.8 “Spot Analysis”. The prerequisites for this, as previously seen for detection of the membrane, are a good detection of the nucleus and cytoplasm, and a subsequent user-defined region of interest as a basis for the spot detection. In our experience, a reference stain for the organelles in addition to a functional organelle stain (e.g. a stain for mitochondrial membrane potential) is not required.

1.6.2 Spectral Characteristics of Fluorophores

The most important optical characteristics of a fluorophore are its excitation and emission spectra, i.e. the excitation and emission intensities as functions of wavelength.

When using the Opera Phenix, knowledge of the spectral characteristics of fluorophores is not necessary, as the filter settings for a large number of fluorophores are pre-defined according to their spectra. Simply choose the fluorophore's name and the filters are set accordingly (see the User Manual for details). Lists of fluorophores and their filter settings can be found in the Appendix in ***Table 5-1*** to ***Table 5-3***.

However, there are two cases when you will need to consider fluorophore spectra:

- You want to determine if a fluorophore combination will show crosstalk when acquired simultaneously (see section 1.6.3 for details).
- You want to create filter settings for a new fluorophore that is not yet listed in the pre-configured settings (see Harmony Help or User Manual, section "How to add and configure a channel" for details").

Usually, the fluorophore manufacturer will supply the spectra; however, there are also many free web-based applications available like e.g. the "Fluorescence SpectraViewer" at www.lifetechnologies.com which allows to plot and compare spectra, and to check spectral compatibilities and issues such as crosstalk (here called spillover).

1.6.3 How to avoid crosstalk in a fluorophore combination

Understanding the underlying principles of crosstalk is especially important if you own a **multi-camera Opera Phenix**.

Crosstalk can occur if the spectra of several fluorophores in one sample have a significant overlap. In a multichannel application this can lead to situations whereby a signal is recorded in one channel (e.g. GFP channel) that is in fact partially emitted by a dye of another channel (e.g. Hoechst), this emission crosstalk can potentially reduce your assay window and even falsify your read-out and thus must be taken into account.

In this chapter we will discuss both types of crosstalk (excitation and emission crosstalk), however, the emission crosstalk is especially relevant as it has implications for the selection of measurement mode (sequential or simultaneous channel acquisition). Application examples and step-by-step guidance for the experimental setup can then be found in chapter 2 "Experimental Setup".

Emission crosstalk

This type of crosstalk occurs in general during simultaneous acquisition with a multicamera system. In Opera Phenix emission crosstalk is primarily relevant in non-confocal mode and only to a low extent in confocal mode. This is due to Synchrony optics.

Figure 1-30 illustrates emission crosstalk. Dye1 is only excited by laser 1, and dye 2 is only excited by laser 2, but their emission spectra overlap and thus cannot be separated completely by emission filters. If now both dyes are excited **simultaneously**, emission crosstalk will occur and the emission of dye 1 will be captured partially in the emission band of dye2.

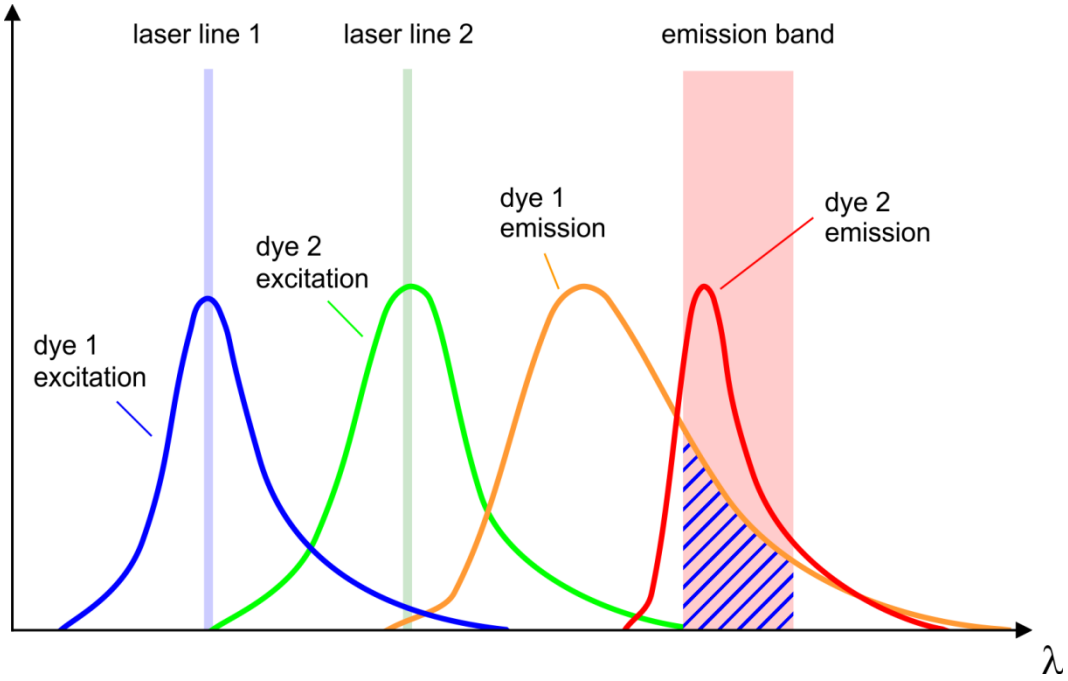


Figure 1-30: The figure shows the fluorophore spectra of two dyes and their excitation and emission bands, illustrating the effects of **emission crosstalk**.

How to check for emission crosstalk

One classic example of crosstalk was already mentioned in chapter 1.5.3 “Synchrony Optics™” and will also be used in this chapter, i.e. crosstalk from the nuclear stain Hoechst 33342 (represented by dye 1 in ***Figure 1-30***) into the neighboring spectral band where e.g. EGFP or Alexa Fluor 488 (represented by dye 2) is recorded.

To check for crosstalk **excite the crosstalking fluorophore (Hoechst) and record the neighboring channel (GFP) with laser power set to 0 %.** In this case you will see the pure crosstalk in the GFP channel and can judge if it would disturb your application. In non-confocal mode this is highly likely as the crosstalk is very prominent (see ***Figure 1-31 A***), whereas in confocal mode there is only residual crosstalk (see ***Figure 1-31 B***). However, please note that this will show up in a **patterned form** (see ***Figure 1-31 C***).

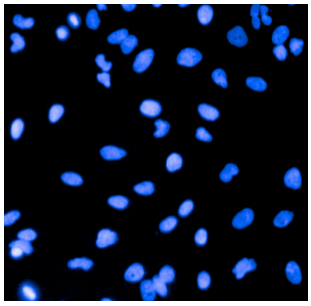
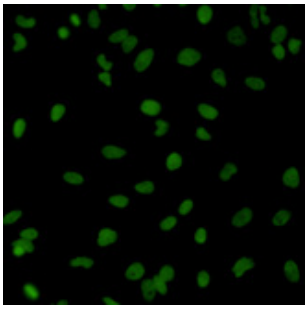
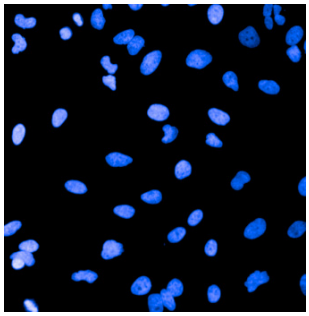
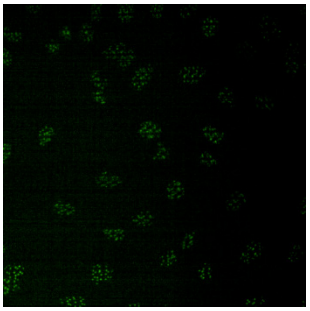
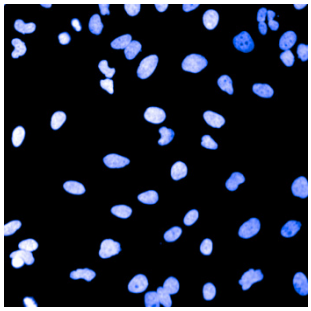
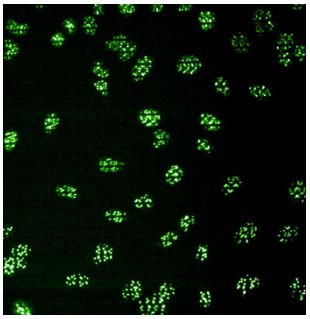
Condition	Hoechst channel	GFP channel
A) Non-confocal mode. Mean Hoechst intensity adjusted to 1000 counts. The Hoechst nuclei are clearly visible in the GFP channel.		
B) Confocal mode. Mean Hoechst intensity adjusted to 1000 counts. The Hoechst nuclei are only faintly visible in the GFP channel due to Synchrony Optics.		
C) Confocal mode. Mean Hoechst intensity adjusted to 5000 counts. An unnecessarily high excitation power for Hoechst was chosen to clearly illustrate the pattern effect you have to check for.		

Figure 1-31: Sample with Hoechst and GFP, the images of the Hoechst and GFP channel were acquired simultaneously with a 40x water immersion objective. The GFP channel was not excited (the 488nm laser power was set to 0%), so any fluorescence visible in the GFP channel is pure Hoechst crosstalk.

Please refer to chapter 2.2.2 for step-by-step recommendations to minimize the crosstalk and/or to avoid it completely by using sequential measurement mode.

Excitation crosstalk

Excitation crosstalk is usually not an issue when using the Opera Phenix, but explained here to complete the crosstalk chapter.

Figure 1-32 illustrates the effects of excitation crosstalk. Dye 1 is only excited by laser line 1, whereas dye 2 is optimally excited by laser line 2, but also (to a lesser extent) by laser line 1. Therefore there will be emission from dye 2 when either of the laser lines is used. The excitation of dye 2 with laser line 1 is independent of the measurement mode, i.e. it will occur in simultaneous and also in a sequential mode.

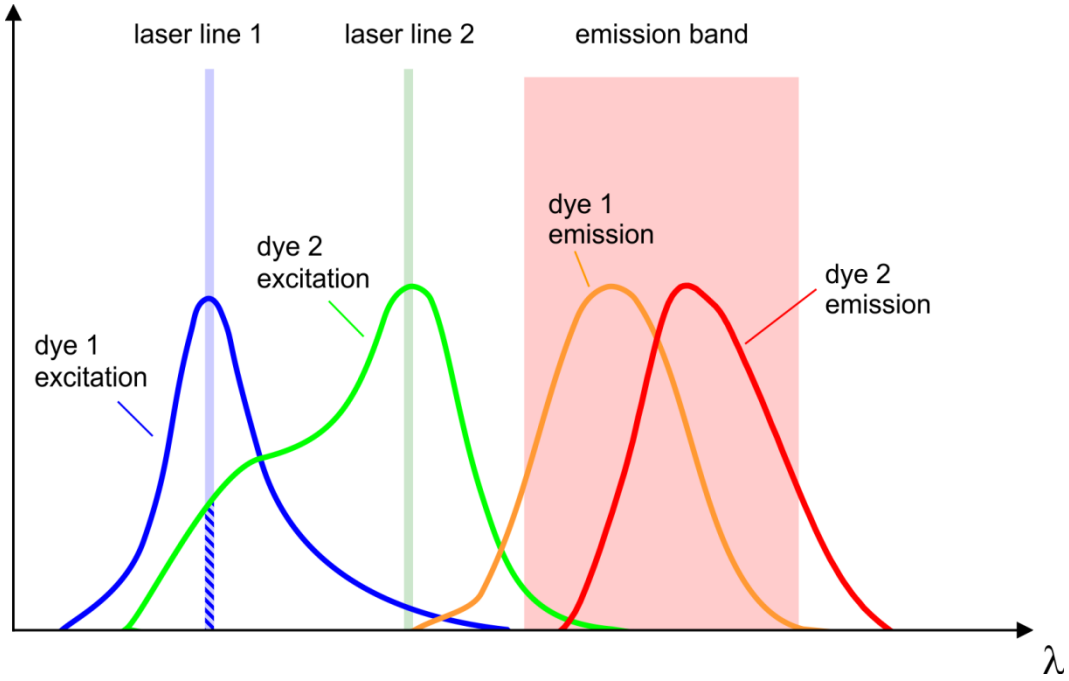


Figure 1-32: The figure shows the fluorophore spectra of two dyes and their excitation and emission bands, illustrating the effects of **excitation crosstalk**.

The laser lines and emission bands in Opera Phenix have been selected to avoid excitation crosstalk for commonly used fluorophores (the fluorophores that are available as preconfigured channels). If you wish to use new / not covered fluorophores please ensure they are not excited by the laser line that belongs to another fluorophore in your assay.

1.6.4 How to avoid photobleaching

One of the major potential pitfalls when working with fluorophores is photobleaching (often also termed fading).

Fluorophores are susceptible to degradation and structural changes due to high intensity illumination (high excitation power in combination with a long exposure time), leaving them eventually unable to fluoresce. The effect is irreversible and is especially pronounced in cellular applications due to the limited number of fluorophores available.

In general, photobleaching is minimized by using the lowest possible excitation power and the shortest possible exposure time.

This is supported by maximization of the fluorescence detection yield which can be done by using high-numerical aperture objectives, e.g. water immersion objectives (see section 1.4.2 “Numerical Aperture” for details).

Photobleaching during manual testing for a suitable excitation power / exposure time in the setup phase of the experiment can be even more deleterious to your data, as this usually affects some of your control wells. Please refer to section 2.1.2 “Setting the focus height, excitation power and exposure time” for recommendations of how to protect your sample against photobleaching during the experiment setup.

Chapter 2 – Experimental Setup

This chapter guides you through many aspects of performing HCS measurements.

Initial Steps

Finalizing Steps and Special Measurements

Special Topics

2.1 Experimental Setup – Initial Steps

This section is intended to support you in the process of setting up an experiment for a new application, and includes some tips and recommendations.

Once you have worked with your Opera Phenix for some time, you will have a set of experimental protocols available in your database. The recommended way of setting up an experiment for a new application is to load a similar, existing experimental protocol and then modify it. This is usually faster than setting up a new experiment from scratch as many variables, such as the microplate, preferred reference dyes etc., do not usually change between experiments.

2.1.1 Selection of microplate and objective lens

The decision about a specific microplate and a suitable corresponding objective will have been made long before the actual setup of the experiment (see chapter 1).

Therefore, the only actions you need to perform at the beginning are:

- Verify that your chosen objective is installed in the instrument. If an air objective is missing, use the Objective Wizard to insert it (please refer to the Harmony Help or User Manual for details). Water immersion objectives can only be inserted or changed by a service engineer.
- If the air objective has a correction collar, set it to the value of the plate bottom thickness (please refer to the Harmony Help or User Manual for details).
- Put the plate in the instrument.
- Select the plate and objective from the respective list on the Harmony Setup Screen. If your plate is not available in this list, please refer to section 1.2.7 “Adding new plates”.
- Check whether the combination of your plate and field layout and your (estimated) maximum focus height causes any restrictions in the measurability of your plate (see section 1.2.4 “Measurement restrictions”).

2.1.2 Setting the focus height, excitation power and exposure time

The three parameters focus height, excitation power and exposure time are interdependent and the adjustment of these is performed in iterative cycles. For this reason, they are discussed together in this section. Some general guidelines for adjusting each parameter are described, and a suggested workflow is presented.

These adjustments are critical for the quality of a measurement. Please take your time and perform them carefully and accurately.

Focus height

A focused object appears to be “**sharp**” and “**bright”.** Therefore, the following two criteria have to be considered when adjusting the focus height:

- Sharpness
- Object intensity

Sharpness has to be judged visually and will be familiar to microscope users.

Not all objects within a cell are in exactly the same focal plane. If these objects are stained differently (and thus appear in different channels), you may get better results by adjusting the focus height slightly for each channel.

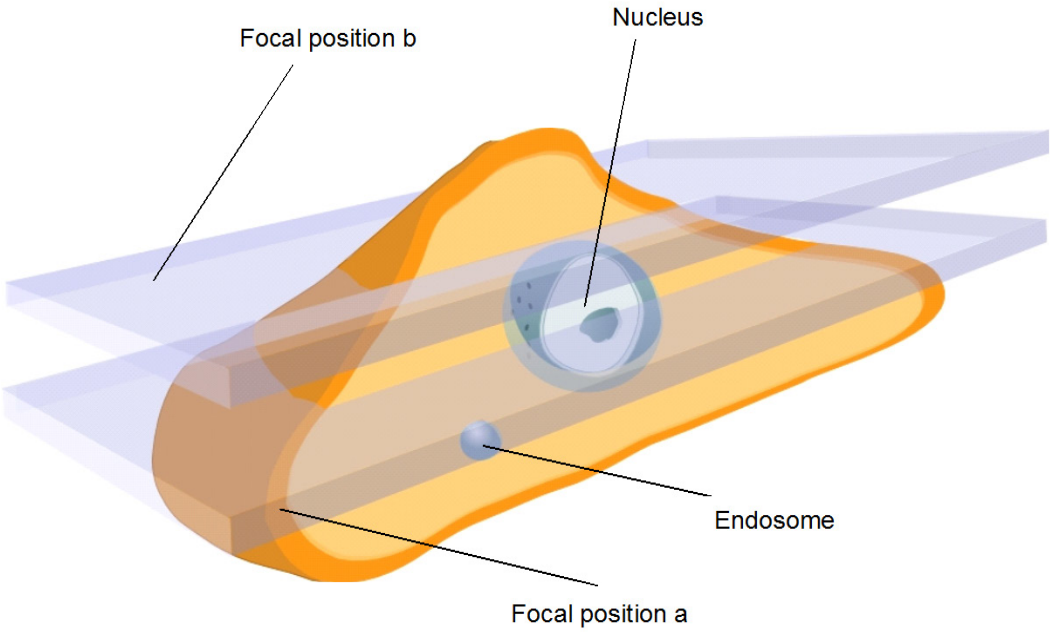


Figure 2-1: Illustration of subcellular structures in different focus heights. The endosome is optimally captured by the lower focal plane (a), whereas for the nucleus a slightly higher focal plane (b) is optimal.

To adjust the focus height for each channel individually might be a necessity for your readout. However, you may want to consider this consequence: setting different focus heights in different channels can cause changes in the channel sequence in a multi-camera Opera Phenix. They can only be acquired in (the fastest) simultaneous mode if all channels have the same focus height (please also refer to description of channel sequence editor later in this chapter).

It can be advisable to compromise in one channel and accept less well focused objects here (e.g. the nucleus channel) in order to measure several channels simultaneously. In our experience, this approach does not usually reduce the quality of the analysis, but this should be checked for your assay.

Please note that the µm values of the focus height are internally calculated using an offset. Don't be alarmed if you determine the best focus height for your cell layer at, for example, -6 µm as this can be a perfectly acceptable value to use. A negative value does not implicitly mean that you are measuring within the plate bottom (a frequently asked question).

Background information: the internal offset depends on the objective and the optical properties of the plate bottom material. Thus, the focus height will vary for different combinations.

These deviations depend on the optical properties of the plate bottom material.

Object intensities peak around the sharpest plane but are not necessarily at exactly the same plane. A good way to judge the intensity of an object is to right-click on the image and select “Measure Rectangle” from the context menu to determine the mean intensity within a typical object. See Harmony Help or User Manual for details.

For visual judgment: please make sure that you de-activate the auto contrast feature in the Image Control, otherwise the brightness will be automatically adapted to optimize the display of every single image. This will not allow you to observe the differences between images (e.g. “does it get brighter or dimmer?”).

For a detailed explanation of the differences between the measured intensity of an object and the displayed brightness on the Image View, we recommend that you see Image Analysis Guide, section 1.2.1 “Image Display Hints”.

“Show Intensity” only displays the intensity of one single pixel, which might not be a representative value of the overall object intensity; therefore the use of “Measure Rectangle” is recommended in this context.

To avoid problems with focusing, always make sure that the plate is at room temperature.

A plate taken from the fridge or the incubator and immediately put into the device will:

- Change its geometry (including bottom thickness) while its temperature equilibrates with the room temperature.
- Potentially show condensation at the lower plate bottom.

Both these issues could interfere with robust focusing if you start defining the focus height before equilibration is finished.

Exposure time/laser power

Apart from being dependent on the focus height the object intensity is mainly affected by:

- The power of the excitation laser
- The length of the exposure time

The aim of this adjustment is to get a good signal-to-noise ratio for all channels. As a result, a high emission intensity is preferred, but a saturation of the camera chip should also be avoided. As the Opera Phenix is equipped with 16 bit cameras, the maximum intensity value will be 2^{16} = 65536 counts/pixel.

At 65536 counts/pixel the camera is saturated. If the sample is brighter than this then information is lost (this is also called “clipping”, see Image Analysis Guide, section 1.2.1 “Image Display Hints” for more details) and consequently any intensity-based results are falsified. Usually an average intensity below 10000 counts is fully sufficient in our experience.

For hints on how to judge the intensity, see the previous subsection on focus height.

If you have never measured your samples before and have no idea about suitable values, please see the following suggested adjustment procedure for new samples.

If you only want to verify an existing experiment, please refer to section 2.1.6 “How to verify settings for an existing experiment”.

Example adjustment procedure for a mammalian cell monolayer stained with Hoechst 33342 and Alexa Fluor® 488 on a CellCarrier-384 Ultra plate and imaged using 20x air objective lens (for detailed explanations please refer to the User Manual):

- Start to set up the experiment by choosing non-confocal mode and binning 2.
- Select your channels (e.g. “HOECHST 33342” and “Alexa 488”) – do not change their default values for the time being.
- Select a well and a field (e.g. a positive control well).
- Click on the “Snapshot” button in each channel to get a rough estimate for sharpness and object intensity.
- You should now be able to see objects (albeit unfocused): first, roughly adjust the laser power / exposure time so that the intensity of the brightest objects is below ~10000 counts / pixel in each channel. (You are only trying to find a suitable focus height at this stage, and will optimize the other exposure settings later. You want to avoid bleaching at this point. The image will get brighter when you find the optimal focus height).
- Perform a Test measurement with a z-stack (e.g. 10 planes, starting from -4 µm, with a distance between the planes of 2 µm). Once the Test measurement is started, it will trigger the automatic image alignment procedure before displaying the two channels (then correctly aligned). Next examine each channel individually for the sharpest / brightest plane (for details see section “Focus Height” before. Don’t forget to deactivate the image displays intensity Auto Contrast). A good way supporting the search for the best plane is to have a look at the “Overview-Stack” listed under Navigation’s context menu (select all planes, right click and select Overview – Stack). Insert the best values in the focus height field of the respective channel.
- During this step you might experience difficulties in visually judging the best plane for a channel due to crosstalk of e.g. the Hoechst channel into Alexa 488. If this is the case, use “Keep these channels separated” (in the Channel Sequence function) and run a second Test measurement with the stack, this time one channel after the other (sequentially).
- If you now notice saturation (pixels with a count of 65536) in the sharpest plane, reduce the excitation power / exposure time drastically, and repeat the test measurement one more time.
- For refinement, repeat the previous steps with a narrower z-stack around the previously determined focus height (e.g. if the focus height is 2 µm use 10 planes, starting from -3 µm, with a distance between the planes of 1 µm). For information regarding recommended plane distances please see ***Table 5-5***.
- Perform the same z-stack measurement in a negative control well (or even a sample well), and verify the optimal focus height and exposure condition values. Re-adjust slightly if necessary.
- Once you have determined the optimal focus height for each channel, you need to give some thought to the channel sequence: if you want to optimize for speed then you should consider a parallel measurement of the two channels, which would be the faster option. This would only be feasible if the focus heights are identical and if existing crosstalk is not interfering with the final analysis step. You may want to compromise on sharpness of one channel (e.g. blurred nuclei) which will not affect the quality of the image segmentation (for example).

Channel Sequence Editor

You came across the new *Channel Sequence Editor* functionality several times by now and there is plenty of information to be found about it in the Harmony Help / User manual. Please read here a summary of the most important facts to familiarize yourself with it:

- Harmony automatically measures the selected channels simultaneously, if possible, to minimize measuring times.
- The number of channels which can be measured simultaneously depends on the number of cameras your instrument is equipped with (2 or 4).
- Channels can only be measured simultaneously if they have the same focus height, and if the selected combination can technically be realized (e.g. DPC and Draq5 channels would be recorded on the same camera).
- The final channel sequence also takes into account how to organize filter / dichro / z- movements in order to speed up the experiment time.
- The channel sequence will be automatically recalculated as soon a channel is added, removed or edited. Any change in the sequence will always be acknowledged by a blue frame or an information message, so that it can not be missed.
- If a stack is defined, the focus heights of all channels will be ignored. This can lead to an automatic modification of the channel sequence due to channels which then can be measured simultaneously.
- You can also force Harmony to keep certain channels separated so that they are exposed sequentially to avoid crosstalk. Harmony will then decide which remaining channels can be measured in parallel.
- If your Phenix is a one camera device, the Channel Sequence Editor is also available but it is then only displaying the sequence the channels are sorted in, considering z- and filter movements to reach the fastest sequence.

- After you have determined the optimum focus height, do not forget to reset the z-stack.
- Now perform the final adjustment of the exposure time / laser power; depending on your chosen channel sequence you can either use Snapshot (sequential mode) or Test (simultaneous channel measurement) to adjust each channel. If you perform a live cell measurement, a lower laser power is favored over a short exposure time. If you measure in simultaneous mode, keep the exposure time of crosstalking channel short and the intensity as low as possible. Please refer to chapter 2.2 “Experimental Setup – Optimization and Finalizing Steps” for more tips.
- Repeat this step in positive and negative control wells and refine the adjustment as necessary. Take your time with this as it is a critical step – some assays have a huge dynamic range and it is important to capture the intensity correctly.
- Keep in mind that with every image capture, the dyes might be slightly bleached. You may want to consider determining the exposure settings for delicate samples outside of the measurement layout (i.e. in other fields or wells).

- Typical values for this example assay are 2500 – 5000 counts/pixel for the nuclear stain (Hoechst 33342), and 2000 – 20000 counts/pixel for the signal stain (Alexa Fluor® 488). Please note that these values will depend on the stain used and on your individual assay, and are only given here as an approximation of what to aim for.

TROUBLESHOOTING

"The images from my new assay look bad (unfocused, blurred, shaded with crosstalk etc.).

Why? What can I do?"

- If the objective in use has a correction collar, check that it is set to the right value, i.e. the bottom thickness of the plate in use. See section 1.4.4 for details.
- What plates are you using? Thick plastic bottoms will typically give poorer image quality than thin glass bottoms. See section 1.2 "Microplates" for details.
- Again: What plates are you using? Some manufacturers' tolerances with regard to bending of the plate bottom (over the whole plate and especially within wells) are so high that over the distance of one image field these deviations becomes visible as "out of focus" objects. For example, a typical effect is that cells are focused at the left side of the image field and unfocused at the right side. Try another location in the well and see if this changes, if so then it is most likely due to a bend in the plate bottom. Consider using microplates specified for imaging applications with a high planarity if the effect influences your results (quite often it does not). For details see section 1.2.5 "Planarity".
- Where in the well was the image acquired? Please note that at the edge of wells, glue (that attaches the plate bottom to the dividing walls) may have leaked into the well. The glue is usually transparent and not visible in the image, but it may lead to unfocused cells. If slightly unfocused cells are visible at the well edges, we recommend excluding the respective well layout fields from the measurement.
- What cell type are you using? Flatter and well-spread cells are better for non-confocal imaging, whereas thick cells will appear more blurry. For recommended cell types, see section 1.1.1 "How to select a suitable cell line".
- What is the growth pattern of your cells and / or the degree of confluency? If the cells grow in several layers (see ***Figure 2-2***), they cannot be in focus all at once. See recommendations for cell type and density in section 1.1 "Cells".
- Please consider that the impression of "sharpness" also depends on the assay, i.e. on the amount of detail in the image. If the whole cell is stained with an unspecific dye that does not bind to subcellular structures, then the overall sharpness of the image will appear to be lower than, for example, with a tubulin stain. In this case, you might not have a problem with the focusing at all; it is just the way your assay is.
- If you are not satisfied with the analysis results using an air objective and non-confocal mode, you can continue trying confocal mode and water immersion objectives. Both will increase resolution and sharpness.

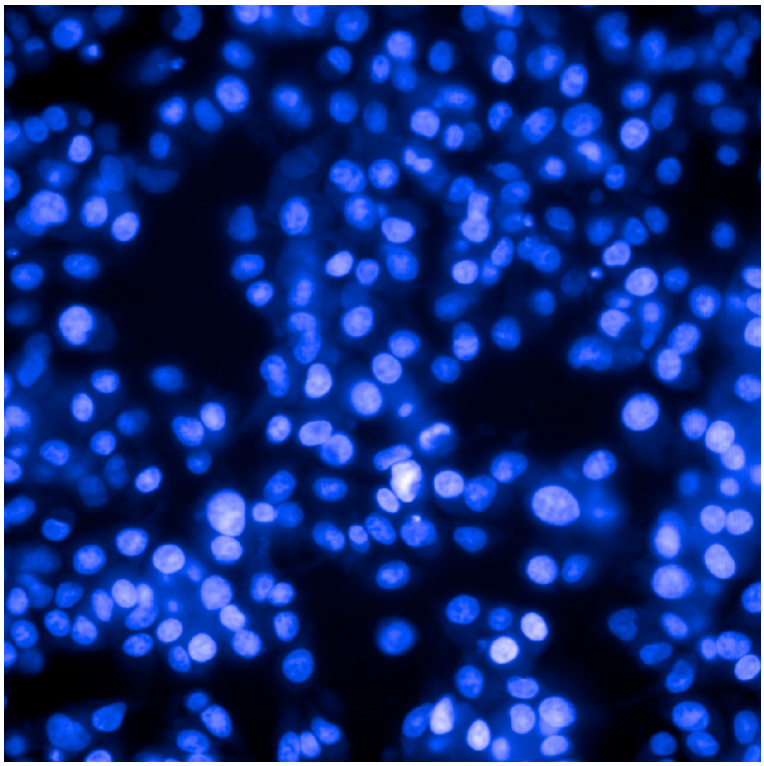


Figure 2-2: When looking at HepG2 cells growing in several layers, the focused layer appears sharper and brighter.

2.1.3 How to select fluorophore filter settings

Harmony offers a selection of pre-configured channels for the most common fluorophores, which comprise a combination of the appropriate excitation laser and emission filter (see ***Table 5-3***). By selecting the fluorophore name lasers and filters are set automatically.

In principle, there are three different emission filter settings: standard, extended and extra (see also ***Figure 2-3***):

- Standard: the usual filter setting for the fluorophore with an average width emission band, and a good starting point for most applications.
- Extended: optimized for yield: a wider emission band that captures more of the emission spectrum of UV fluorophores; disadvantage: cannot be used in combination with 488 nm laser excitation and is more prone to crosstalk.
- Extra: extra channels enable special applications such as ratiometric measurements or FRET experiments.

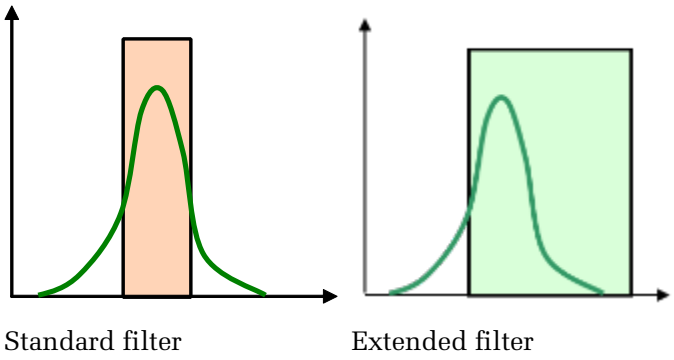


***Figure 2-3*:** Illustration of standard and extended emission filter settings. Left: standard emission filter, e.g. channel CFP, capturing between 435 and 480 nm. Right: extended emission filter that enables to yield more of the spectrum of the fluorophore, here CFP from 435 to 550 nm.

An elegant way to give a pre-defined channel a more meaningful name is to save it under a new name which is related to the actual application or dye: e.g. instead of channel name Alexa 488 one could name it “anti-P21-Alexa 488” or instead of Alexa 647 one could say “Mitochondria-647”. That way, the channels become more meaningful during later evaluation or presentation.

The default settings for focus height and exposure time in the channels are arbitrary starting points for the adjustment. The focus height, exposure time and excitation power depend mostly on your specific sample and not on the fluorophore, so these always have to be adjusted. Please also see section 2.1.2 “Setting the focus height, excitation power and exposure time”.

If you open an experiment or measurement from another device containing a channel which is using lasers or filters that are not in your Opera Phenix, the channel will display a red frame with an appropriate error message. If you intend to use the channel, replace the excitation source or filter with one present in your instrument.

2.1.4 How to define a suitable well layout

The well layout determines how many image fields are measured in one well (number of image fields) and where they are measured in the well (image field pattern).

Number of image fields

Table 2-1 gives examples of cell numbers per image field, determined using HeLa cells at ~80 % confluency. This gives a rough idea of how the magnification influences the number of cells visible in each image field, and allows a rough estimate of the number of image fields that have to be acquired in order to provide robust statistics at a given magnification.

Please note that the cell number is strongly dependent upon the cell type and that the example in ***Table 2-1*** is only meant to illustrate the effect of magnification on the number of cells visible per field.

	5x [cells / image field]	**10x [cells / image field]**	**20x [cells / image field]**	**40x [cells / image field]**	**63x [cells / image field]**
Number of HeLa cells at ~80 % confluency	**2017**	**471**	**133**	**35**	**16**

Table 2-1: Illustration of how the number of cells per image field is dependent on the magnification. $5x10^3$ HeLa cells were seeded in each well of a 384-well plate and were incubated at 37 °C for 24 h, resulting in ~80 % confluency. The cell number is the mean value of 1 to 60 image fields per well.

For a robust assay a measurement using one image field per well at 20x magnification may be sufficient for good statistics, whereas for a rare event assay several image fields per well may be required.

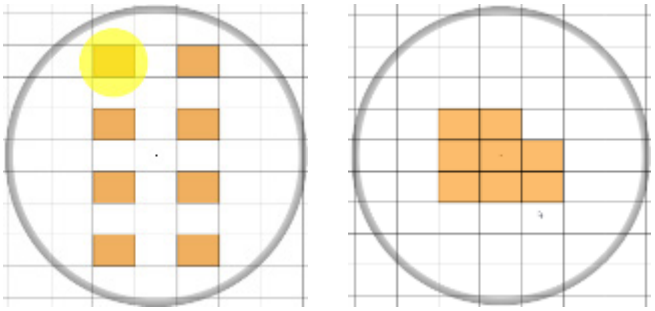
Image field pattern

The area of the well that is illuminated during image acquisition is slightly larger than the image field (yellow highlighted area in ***Figure 2-4 A***) therefore areas of neighboring fields may be bleached slightly.

If photobleaching is <u>not</u> a problem for your samples then both well layouts shown in ***Figure 2-4*** will be suitable.

For very delicate samples which are prone to photobleaching (see section 1.6.4 “How to avoid photobleaching”) we recommend

- not position fields directly side by side. A recommended image field pattern is shown in ***Figure 2-4 A***, whereas ***Figure 2-4 B*** shows an unfavorable pattern.
- selecting the pattern for the automated measurement before the experimental setup, and only using image fields that are not part of the pattern for the adjustments.



A B

Figure 2-4: A – Favorable image field pattern, particularly for delicate samples. The yellow circle indicates the actual illuminated area during image acquisition. B – Unfavorable image field pattern, particularly for delicate samples since parts of neighboring fields may be bleached.

If objects are spanning more than one field of view it is recommended to measure neighboring fields with a certain percentage of overlap (try 5% as starting point) in order to create a "Global Image" (for more details please see chapter 2.2.3).

When creating a well layout pattern, avoid the area of the well where fluids have been added or removed. Even in manual mode, there is usually one specific area where the cell layer has been more stressed or sometimes even scraped off. (For right-handed people this is usually in the left half of the well and when using automated liquid addition instruments this area is often in a more central position). In order to find the stressed areas within the wells simply perform a test measurement of an example-well covering the whole area with fields. Then use the "realistic overview" function to visualize the well coverage.

Please note that the grid shown for the image fields are only approximate positions. The exact positions, especially of the well walls, will depend on the plate type and tolerances. The difference in such positions can vary from plate to plate by a value of 250 µm which equals roughly the size of an image field when e.g. a 40x objective is used. An option to test the accuracy of the imaged positions is shown in ***Figure 2-5***:

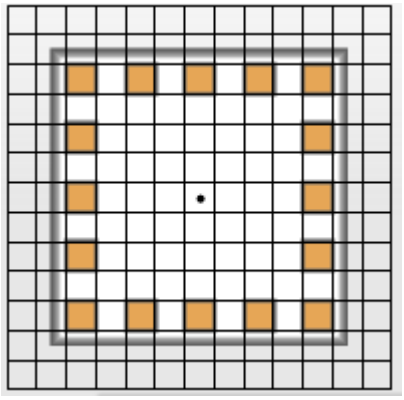


Figure 2-5: Well layout for a test measurement to verify that the image fields next to the well borders do not include parts of the well border (due to plate type and tolerances).

In case well border effects are impairing the imaging quality, the grid positioning can be changed by using the context menu option **Field Centered** (***Figure 2-6***).

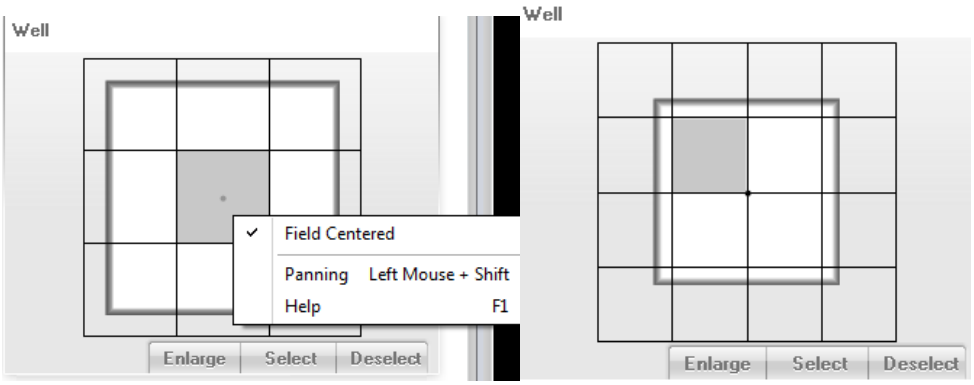


Figure 2-6: Well layout option "Field Centered". The arrangement of fields within a well can be changed (here 10x objective in combination with 384 well plate): either the fields are arranged around a center field (default) or the fields are aligned to the center of the well without a center field. The latter option here allows for covering almost one complete well without imaging well borders.

2.1.5 Z-stacks and their application in 3D imaging

Apart from z-stacks being a very useful tuning aid for the setup of the optimal focus height for channels, they are predominantly used to obtain 3D information from a cellular sample and are most useful in conjunction with the confocal acquisition mode.

The Opera Phenix is equipped with a combination of technical innovations which make it especially well-suited for measuring and evaluating thick specimens (i.e. tissue sections, microtissues, organoids, spheroids).

These technical features include:

- an increased distance between the pinholes on the microlens-enhanced confocal spinning disc, effectively reducing spatial crosstalk (see chapter 1.5.1 "Introduction to Confocal Imaging"), and
- water immersion lenses with a higher NA and a smaller depth of focus (see chapter 1.4.5 "Water immersion vs. air objectives") allowing higher resolutions deeper inside tissues.

Note that the addition of a z-stack to an existing multichannel experiment can alter the resulting channel sequence: e.g. channels measured sequentially due to different focus heights before, might then be measured simultaneously as an identical z-stack is applied to every channel (see also chapter 2.1.2 "Setting the focus height, excitation power and exposure time").

To mathematically increase the depth of focus you can capture images at several focus heights and apply the "Maximum Projection" mode in the Input Image building block to project the images into one image, which contains the features from all image planes (see Image Analysis Guide, chapter 1.17 "3D Analysis").

In ***Table 5-5*** in the Appendix you will find the recommended minimum plane distance for every objective. Please note that higher sampling (i.e. a lower distance between planes) will not improve the z-resolution. See Image Analysis Guide, section 1.17 "3D Analysis" for additional guidance on identifying suitable distances between Z planes for your application. To speed up your measurement, the inter-plane distance can be increased; however, information will be lost for distances larger than the depth of focus of the respective objective.

Visualization in Harmony: the navigate control can be used to easily browse through z-stacks. However, be careful when comparing image intensities as the auto contrast is always adjusted for the active plane. To judge the differences in brightness between planes, select an average plane (usually in the middle of the z-stack) and then deactivate the auto contrast so that the settings are kept and used for every plane.

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 a xy-, xz- and yz-section through the image stack. Section planes can be interactively moved by mouse clicks.

All planes of a z-stack can be selected by pressing **Ctrl+A**. First click on one plane and then press Ctrl+A.

For z-stack analysis using Harmony analysis sequences, please see Image Analysis Guide, section 1.17 "3D Analysis". Z-map based analysis of individual planes or analysis of the collapsed stack (maximum projection) is available. Different types of stack analysis sequences can be used for online evaluation, simultaneous to image acquisition.

Advanced 3D-Visualization: we recommend using Volocity® 3D Image Analysis Software which allows sophisticated navigation through your 3D image (for an example see Image Analysis Guide, section 1.17 "3D Analysis"). The images can be directly transferred from Harmony to Volocity by Drag & Drop of a well in the Harmony Navigation Control into an open Volocity library. In addition, Volocity includes restoration algorithms for deconvolution of your image data, and an interactive "Quantitation" module for advanced measurements.

For online analysis, all planes are used by default; therefore, please ensure that you have valid images in every plane. If the planes are blurred, they might not be suitable for the analysis sequence and this could result in artifacts or longer calculation times. If you are uncertain about that, do not use the online analysis, but rather evaluate and select only the useful planes.

Please note that it is not possible to acquire a z-stack of phase images. However, stacks of fluorescent images can be used in combination with digital phase imaging.

If a z-stack is included in a PreScan experiment (see section 2.11 "Automated PreScan/ReScan – PreciScan™") the analysis sequence associated to it must be set to maximum projection.

2.1.6 How to verify settings for an existing experiment

If you have already performed an experiment successfully several times then you will have well-tested values available for the focus height, laser power and exposure time, which can (in principle) be used.

However, consider the following situation: since the last time you performed this experiment your cell line has aged three weeks, whereas the nuclear reference stain (e.g. Hoechst 33342) has just been freshly diluted from stock. Changes like these are daily occurrences in assay preparations and might necessitate a slight adaptation of the settings, since the morphology of the cells (which influences focus height) and the staining intensity (which influences excitation power and exposure time of the Hoechst 33342 channel) may have changed.

We recommend verifying the settings for focus height, exposure time and laser power according to section 2.1.2 “Setting the focus height, excitation power and exposure time” before you start the first measurement (e.g. the first plate of a screening run or the first plate of an assay that was last performed six weeks ago). Usually, any changes that need to be made will only be minor (and often not even necessary), but particularly in the case of samples that are not suitable for repeated measurements (e.g. live cell measurements and/or delicate samples that are prone to photobleaching) this verification should not be skipped.

2.2 Experimental Setup – Optimization and Finalizing Steps

An integral focus of the Opera Phenix’s Harmony software is on high throughput. One main software tool supporting this aim is the channel sequence editor which is sorting all exposures automatically (see section 2.1.2 “Setting the focus height, excitation power and exposure time” – description of the Channel Sequence Editor).

To tap the full potential of the Opera Phenix, it is beneficial to understand the device’s technological and software options and to combine them in the best way to reach the final aim of fast high quality imaging.

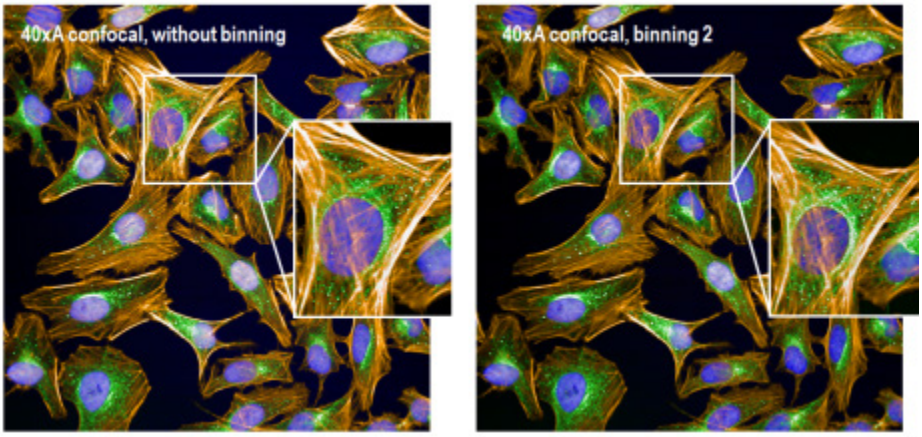
2.2.1 Influence of measurement options on experiments

Optimizing Opera Phenix experiments is an iterative process and many aspects which mutually influence each other can be actively influenced by the user. It is close to impossible to recommend one standard answer to the multitude of assay variations. But we can give you an overview of your choices when setting up your image acquisitions (and their impact):

	Pro	Contra	Chapter
Air objective	► Enables measurement of high bottom plates (due to larger working distance) ► Larger depth of focus to capture objects in slightly different heights	► Low numerical aperture (NA) thus lower light efficiency / longer exposure times, higher excitation power ► Require correction collar adjustment (20xA and 40xA) ► Lower resolution	**1.4**
Water immersion objective	► High NA, i.e. better light collecting efficiency, thus shorter exposure times, less photobleaching ► Higher resolution ► Optimal for 3 D applications in combination with confocal mode (highest achievable axial resolution) ► Close match of refractive index is optimal for in depth imaging of thick samples	► Not compatible with high bottom plates ► Smaller range of possible focus heights	
Binning 1	► Highest resolution possible – recommended for very fine structures, mostly in combination with high magnifying objective lenses	► Longer exposure times / higher laser power, more photobleaching ► 4 fold bigger memory consumption ► Slows down image acquisition, analysis and image display	
Binning 2	► Saves data space by 4-fold reduction in image size ► Shorter exposure times / less laser power, less photobleaching ► Faster image analysis ► Binning 2 sufficient for 90 % of all imaging tasks	► Half the resolution of unbinned images	
Non confocal mode	► Focussed layer is thicker resulting in shorter exposure times ► Enables more FRET fluorophore combinations	► No crosstalk reduction ► Lower SNR (more background) ► Less resolution	**1.5**
Confocal mode	► Synchrony Optics is suppressing crosstalk ► Improved image contrast, better SNR, sharper objects ► Higher resolution for most objectives ► Optimal for 3 D applications in combination with water immersion objectives (highest axial resolution)	► Longer exposure times/ higher laser power ► Less options for FRET pairs	
Simultaneous acquisition	► Fast acquistion of several channels at a time	► One focus height for all channels required	**1.5 / 2.2**
Sequential acquisition	► Avoidance of crosstalk ► Enables usage of different focus heights in different channels	► Measurement will take longer	
High laser power/ Short exposure time	► High excitation power (and short exposure time) is faster and recommended in fixed cell assays ► In simultaneous measurements, a further option to reduce (residual) crosstalk is to shorten the exposure time of the crosstalk-affected channels (and to increase their laser power accordingly) (please see Table 2-4)	► With higher laser power, non-linear photobleaching might be an issue for certain dim dyes	
Low laser power/ Long exposure time	► For live cell assays dimmed laser power is recommended	► Longer exposure times result in longer measurement times	

Table 2-2: Overview of image acquisition options for and their impact on Opera Phenix experiments.

To illustrate the effect of air vs. water immersion objectives in combination with binning on the duration of Opera Phenix experiments, we compared the exposure times of the Hoechst channel in 40x magnification with and without binning. In order to remain comparative, we kept the mean intensity of the nuclei constant in all four settings.



Lens	Bin	Exposure time x-fold shorter
40xA	1	1x
	2	4x
40xW	1	11.8x
	2	50x

Figure 2–6: Influence of binning and air vs. water immersion objectives on imaging speed: triple stained HeLa cells were imaged confocally with 40x magnification and varying acquisition parameters. The mean Hoechst intensity of the nuclei was kept constant to be able to compare exposure times.
While the difference in resolution between bin 1 and 2 is not visible for these object structures, the exposure time already increased 4-fold. When switching to the water immersion objective, the acquisition time experiences a dramatic 50-fold reduction. 90 % of all image analysis tasks perform equally well with binning switched on.

2.2.2 Optimizing acquisition conditions for different purposes

This chapter is only relevant for owners of a multi-camera Opera Phenix as only then simultaneous measurements of several channels are possible.

The sample quality, the fluorescence spectra, the focus height and crosstalk – all these parameters influence the final order and conditions in which channels should be measured to a great extent. To understand the impact of each decision, here we guide you through an example step by step explaining the logic behind it. Using the HCS Imaging Application plate, we set up different experiment conditions on a 4-camera Opera Phenix and explain the decisions for or against the selection:

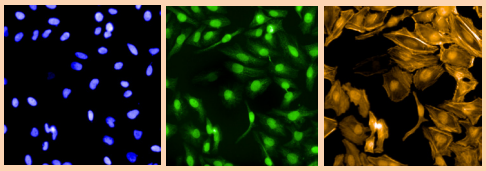
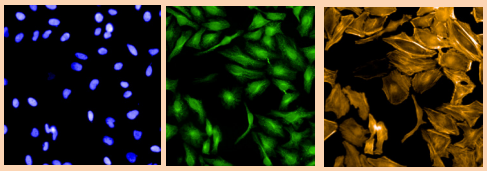
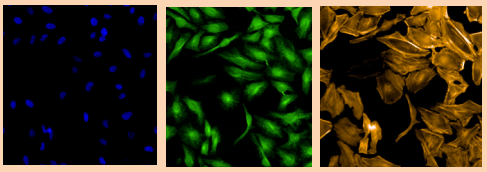
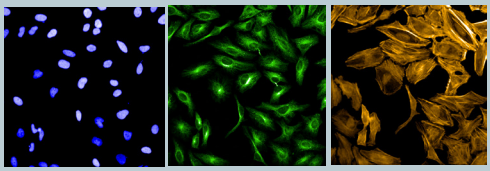
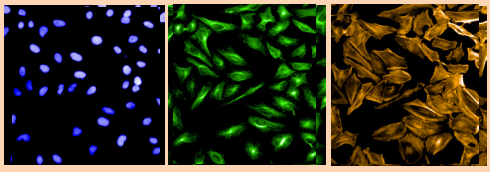
Exposure Settings	Channel Sequence	Consequence / Decision	Resulting Images
Shortest expected experiment time: non confocal/bin 2 measurement, 3 channels acquired simultaneously			
20xW, non confocal, bin 2 Laser power / exposure time Hoechst 20 %, 200 ms Alexa 488 20 %, 200 ms TRITC 30 %, 200 ms	**1 parallel exposure:** 3 channels acquired simultaneously	● Restriction to one focus height in all 3 channels ● Hoechst intensity about 5000 ● No crosstalk suppression in non confocal measurement mode: nuclei are visible in Alexa 488 and TRITC channel ► **Decide at this point if crosstalk is affecting the analysis quality**	
Reduce crosstalk from Hoechst into Alexa 488 and TRITC channel by reducing the exposure time in 2 channels			
20xW, non confocal, bin 2 Laser power / exposure time Hoechst 20 %, 200 ms Alexa 488 100 %, 40 ms TRITC 100 %, 60 ms	**1 parallel exposure** 3 channels are acquired simultaneously	● Hoechst intensity still about 5000 ● However, less crosstalk captured in Alexa 488 and TRITC channels as only a fraction of the total exposure length of Hoechst can reach the other two cameras (see ***Table 2-4***) ► **Analyse if remaining crosstalk is affecting the analysis quality**	
Further reduce crosstalk from Hoechst into Alexa 488 and TRITC channel by reducing overall Hoechst intensity			
20xW, non confocal, bin 2 Laser power / exposure time Hoechst 5 %, 200 ms Alexa 488 100 %, 40 ms TRITC 100 %, 60 ms	**1 parallel exposure** 3 channels are acquired simultaneously	● Hoechst intensity is brought to a minimum of about 1000 so that segmentation remains functional ● Resulting in less crosstalk into Alexa 488 and TRITC channel ► **Again, please analyse whether the remaining crosstalk is affecting the analysis in the other two channels**	
Now you have two options how to further reduce crosstalk: either switch to confocal mode and use Synchrony Optics or separate the channels and measure them sequentially			
Suppress crosstalk from Hoechst into Alexa 488 channel to a great extent by employing Synchrony Optics technology			
20xW, confocal, bin 2 Laser power / exposure time Hoechst 100 %, 160 ms Alexa 488 100 %, 200 ms TRITC 100 %, 600 ms	**1 parallel exposure** 3 channels in one sequence	● Confocality enables Synchrony Optics to suppress crosstalk into Alexa 488 channel up to 98 % ● Despite a Hoechst intensity of about 5000 there is only a very minor residual crosstalk in the Alexa 488 channel's nuclear area ● However, crosstalk into TRITC channel (overnext emission band) can not suppressed that way ● Increase in sharpness and resolution might improve certain readouts additionally ● Confocal mode results in longer exposure times	
Avoid crosstalk from Hoechst into Alexa 488 and TRITC channel by measuring Hoechst separately			
20xW, non confocal, bin 2 Laser power / exposure time Hoechst 20 %, 200 ms Alexa 488 100 %, 40 ms TRITC 100 %, 60 ms	**2 sequential exposures** exposure 1: Hoechst exposure 2: Alexa 488 and TRITC	● Separating the channels (using the channel sequence editors function "Keep these channels separated") will completely avoid crosstalk if your application requires this ● At the expense of a then elongated measurement time	

Table 2-3: Influence of different exposure conditions on the quality and duration of an experiment as well as different remedies to reduce crosstalk issues. Example images were recorded on a 4-camera Opera Phenix using a 20x water immersion objective and the HCS Imaging Application plate. HeLa cells were stained with Hoechst 33342 (nuclei, blue), Alexa 488-anti-tubulin-antibody (green) and actin fibers stained with TRITC-phalloidin (orange).

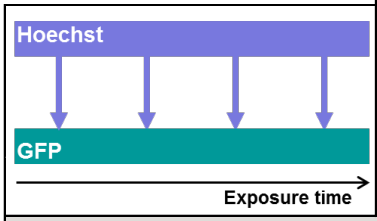
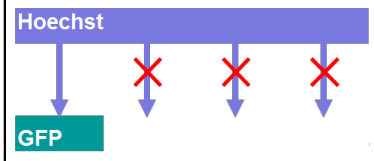
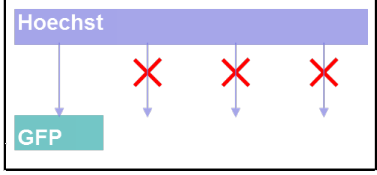
	Exposure Settings Laser power % Exposure time ms	Resulting Intensities	Intensity Crosstalk 20% overlap of emission into GFP channel
	Hoechst 20 %, 200 ms	5000 counts	-
	GFP 20 %, 200 ms	5000 counts	**1000 counts** (20% of 5000) full crosstalk capture with identical exposure times
Shorten exposure time in GFP channel (and increase laser power)			
	Hoechst 20 %, 200 ms	5000 counts	-
	GFP 100 %, 40 ms	5000 counts	**200 counts** reduction of exposure time in GFP channel by factor 5 reduces crosstalk to the same extent
Reduce laser power in Hoechst channel			
	Hoechst 5 %, 200 ms	1000 counts	-
	GFP 100 %, 40 ms	5000 counts	**40 counts** reduction of Hoechst intensity by factor 5 reduces crosstalk to this extent

Table 2-4: Graphical illustration of how different exposure conditions have a great influence on crosstalk (using theoretical intensity values). Here, the most common emission overlap of Hoechst into GFP channel is shown. Both, reducing the exposure time in the crosstalk-affected channel and bringing the intensity in the crosstalk-causing channel to a minimum are very effective measures to reduce crosstalk.

2.2.3 Global Image

For image analysis it is possible to create a montage of all fields in a well called Global Image. This is useful if the object of interest spans more than one field of view, e.g. a wound in a scratch wound assay, stem cell colonies, microtissues or tissue sections (***Figure 2-7***). The montage is based on absolute coordinates and not on features within the image. The resulting image is downscaled to a maximum size of 5 mega pixel using binning. This is done to reduce memory consumption and to speed up image analysis. Global image can be used for segmentation and analysis using most of the Harmony building blocks. Based on the segmentation of the global image, identified populations or regions can subsequently be analyzed on the original full resolution images.

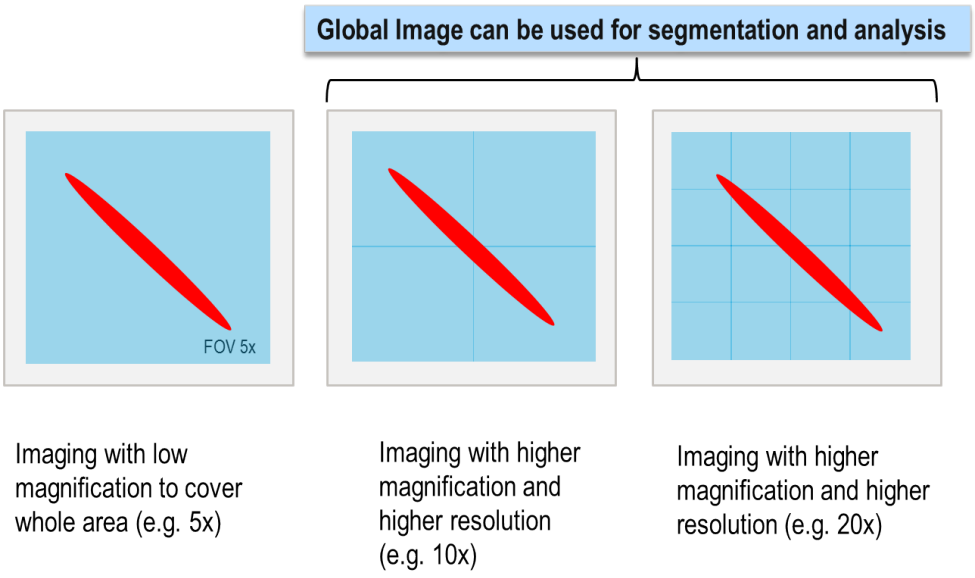


Figure 2-7: A large object or area of interest can either be imaged with a low magnification objective to capture it in one field of view or with high magnification objectives with higher resolution. In that case, the object or area of interest will be spread over multiple fields of views. Global Image will create a montage that can be used for segmentation and analysis.

To achieve an optimal montage result it is recommended to acquire the fields with an overlap of about 5%. The percentage required for a sufficient overlap can be tested by recording a test measurement of multiple fields with overlap. The resulting global image can then be visually evaluated.

Progress of global image creation is indicated by a progress bar and can be stopped by removing the tick from the check box.

2.3 Label-free Imaging: Brightfield and Digital Phase Contrast

2.3.1 Brightfield Imaging – When and How to Use It

The brightfield (or transmission) option (see ***Figure 2-8***) is useful for the following reasons:

- As a cell culture quality control tool before you start your assay protocol. Put the plate on the instrument and perform a small experiment in selected wells to analyze the confluency and status of cells prior to starting the experimental procedures.
- For additional visualization during the experimental setup. If, for example, you cannot find any stained objects when focusing, you can use the brightfield option to verify that cells are present in the well (and have not been lost during washing).
- As an assay readout to detect regions covered with cells without the need to stain them, for example in colony formation, proliferation or migration assays.

However, if you are planning to use unstained cells for whole cell segmentation we recommend digital phase imaging (see section 2.3.2).

In ***Figure 2-8*** examples of brightfield images are shown. It can be seen here, that cells in the focal plane show the least contrast in the brightfield image (B), while slightly defocusing improves the general visibility of the cells (C). Whenever an objective lens has a very large field of view (mostly low magnification objective lenses) an intensity drop at the image's periphery compared to its center can be observed (also called vignetting, A). Brightfield correction can be used to remove this background profile (B). It greatly improves visualization in montages or global images (***Figure 2-9***). The correction algorithm requires only one single brightfield image.

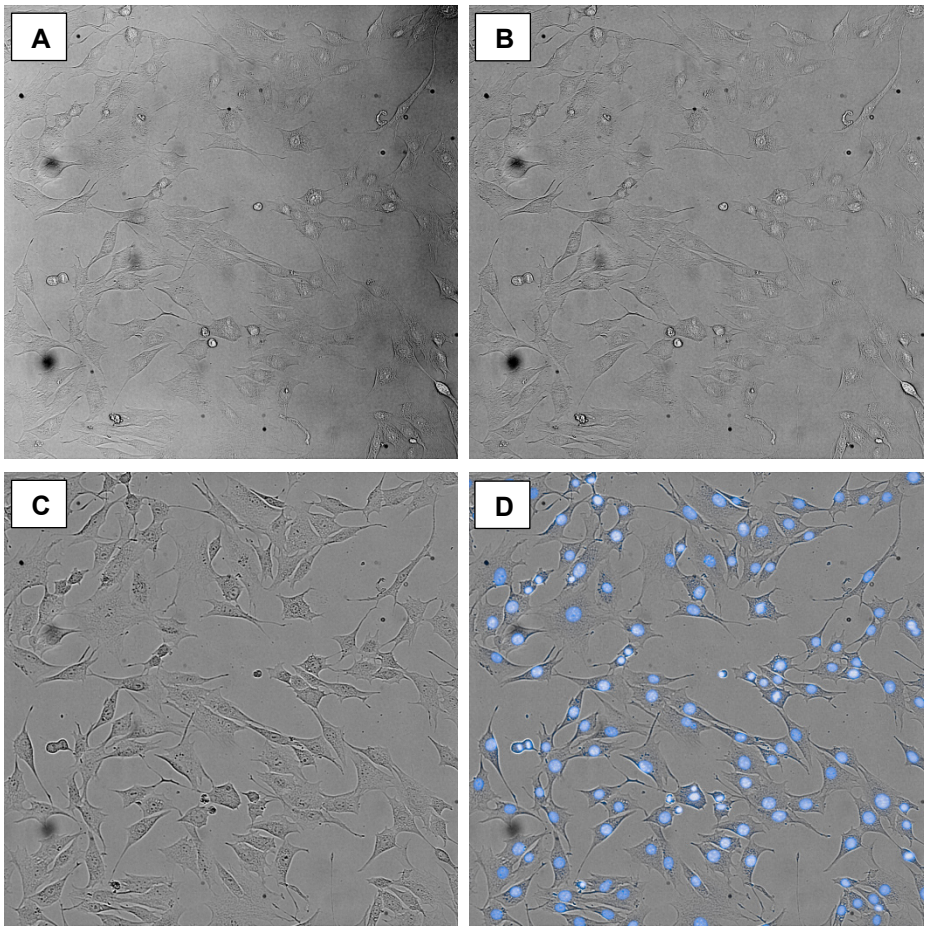


Figure 2-8: Applying brightfield correction removes vignetting effects often seen in brightfield images. To improve contrast in brightfield images the acquisition can be done slightly out of focus. Brightfield and fluorescence images (blue nuclei stained with Hoechst) of NiH3T3 cells recorded on Operetta CLS, widefield mode, 20xhNA objective lens:

A: Original brightfield image in focal plane without brightfield correction

B: Brightfield image in focal plane with brightfield correction

C: Brightfield image slightly defocussed with brightfield correction

D: Overlay of brightfield image (slightly defocussed) and fluorescence channel

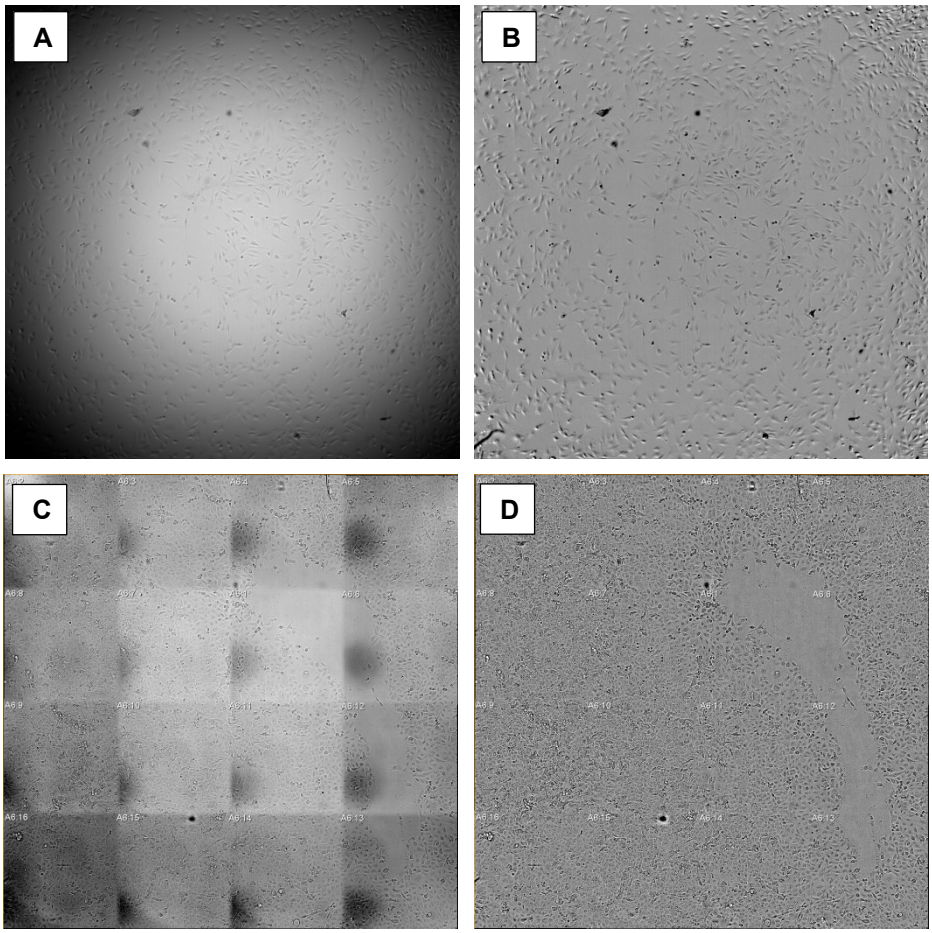


Figure 2-9: Two examples for optimized background flatness after brightfield correction:

A/B: A monolayer of cells acquired with a low magnification objective lens (5x air, widefield, Operetta CLS). Low magnification lenses have a large field of view which typically shows the strongest vignetting effect. Applying brightfield correction removes the background profile thereby improving visibility of all cells and especially the cells at the well border.

C/D: Not only background profiles but additionally other background effects like “dark spots” are removed by the brightfield correction (Operetta CLS, 20x water immersion, widefield).

With the brightfield LED being positioned above the plate please make sure that the transmitted light is not blocked by intransparent sealing, labels, lids or mounting media.

If low magnification objectives are used in combination with the brightfield option, the illumination may experience a strong curvature due to the big field of view. Make sure to always activate brightfield correction in these cases to improve visualization and analysis. In addition, try to attenuate these effects by filling the wells with as much fluid as possible to lift the meniscus.

Brightfield images can be analyzed using the PhenoLOGIC™ machine learning building block Find Texture Regions, see Image Analysis Guide, section

1.14 “PhenoLOGIC™ – Texture Based Segmentation” for more information. Examples for useful applications of the brightfield channel can also be found in the Image Analysis Guide, see sections 2.28 “Texture Based Segmentation – Stem Cell Colonies”, 2.26.5 “RMS Migration – Confluency” and 2.31.2 “RMS Microtissue – Brightfield”.

2.3.2 Digital Phase Imaging – Background, Acquisition and Analysis

Digital phase imaging describes a computational approach for the generation of phase images based on two defocused brightfield images. In contrast to traditional phase contrast microscopy no dedicated objective lenses are required. Digital phase images show an excellent signal to noise ratio without any staining, and therefore allow cell detection without physiological disturbance of the cells by fluorescent labels.

Long-term live-cell imaging benefits from this approach in particular, as the brightfield image acquisition uses a red LED which is at the lowest phototoxic level possible. Typical assays where digital phase imaging is recommended are migration, cell tracking and cell division assays, proliferation, colony formation and kinetic live-cell imaging applications. Moreover, optimal assay end point and quality parameters such as confluency and morphology are easily obtained.

Digital phase imaging replaces cytoplasmic staining for cell segmentation, freeing one fluorescence channel for other markers of interest. As it reduces both experimental complexity and costs while enabling robust cell segmentation, it can be considered a substantial advancement in the field of High Content Analysis.

Background

When performing image analysis tasks on brightfield images, the limitations of this approach become obvious. The overall intensity of the background is approximately the same as the intensity of the cell, leaving only texture-based segmentation methods for evaluation. For highly confluent monolayers, very thin samples or ultrathin cell regions, brightfield images yield a particularly low signal to noise ratio which makes the structures hard to visualize. Simply defocusing these brightfield images is known to improve the image contrast and is widely used to visualize cells in brightfield. However, using this technique, morphological details, especially in ultra-thin regions of cells, can get lost and the contrast is still not optimal for cell segmentation.

By combining two defocused brightfield images – one recorded below and one above the sharpest plane in a certain magnification dependent distance – a digital phase image can be constructed showing a significantly improved signal to noise ratio (***Figure 2-10***). This mathematical reconstruction is based on an algorithm which considers the rate of change in light intensity distribution introduced by changes in the refraction index of the specimen. The resulting intensity in the digital phase image is approximately proportional to the magnitude of the phase and therefore to the optical length (product of cell thickness and refractive index).

The generation of digital phase images is realized in a channel called **Digital Phase Contrast** which offers

- two automatic modes called **High Contrast** and **High Detail**,
- one manual mode called **Manual**.

If one of the two automatic modes is selected, a brightfield stack starting below the focal plane and ending above, with a distance of half the focal depth of the selected objective, is recorded and two appropriate planes are determined from this stack to compute the digital phase image. Depending on the selected mode, the absolute Z-height of the two planes will differ. In the **Manual** mode, the Z-height of the two brightfield images required for the digital phase contrast (DPC) image are entered by the user manually.

When optimal cell segmentation of the phase image is important, the **High Contrast** mode is recommended over the **High Detail** mode which yields images richer in structural details at the expense of contrast. As a result, high detail images could be a better option for texture-based evaluations or simply an improved visual presentation of results (see ***Figure 2-10***).

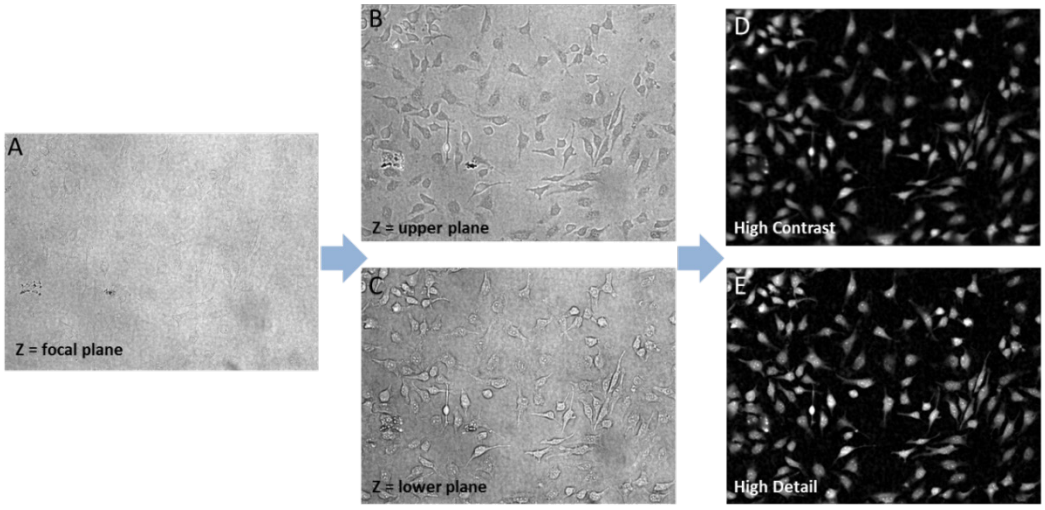


Figure 2-10: Construction of a digital phase contrast (DPC) image from two defocussed brightfield images (20xhNA, non confocal, Operetta). Using the focal plane as a starting point, two planes of a multiplane stack are selected to calculate the DPC image. Depending on the selected mode – **High Detail** or **High Contrast** – these two planes will originate from different focus heights.
Panel A: brightfield image of HeLa cells in the focal plane.
Panel B/C: a pair of defocussed brightfield images from above and below the focal plane.
Panel D/E: resulting DPC images, D contrast optimized and E displaying more details.

The **Manual** mode not only allows users to set the upper and lower brightfield planes, but also to tune two parameters which influence the images: **Filter** and **Speckle Scale**. Generally, we recommend to use this mode:

- if the automatic plane selection of the DPC algorithm fails due to objects in different Z heights or if the objects are too thick.
- if the image quality requires further improvement by fine tuning the parameters.

For the 1.25x objective, DPC is generally disabled for all modes; this lens is causing a strong field curvature which is not compatible with the DPC algorithm.

Cells in the digital phase image have high resemblance to fluorescently labeled cells (***Figure 2-11***) and consequently cell segmentation results in comparable read outs for confluency and cell counting (***Figure 2-12***). While fluorescently labeled cells are optimally segmented by sequentially applying the **Find Nuclei** and **Find Cytoplasm** building blocks, you achieve optimal segmentation on DPC images by simply using the dedicated method “P” in the **Find Cells** building block. Like other images, DPC

images can be used as an input for all other building blocks. Typical examples for digital phase imaging and segmentation are given in the Image Analysis Guide, RMS "Cell Tracking – Migration Analysis" (see section 2.29) and RMS "Cell Tracking – Cell Division" (see section 2.30).

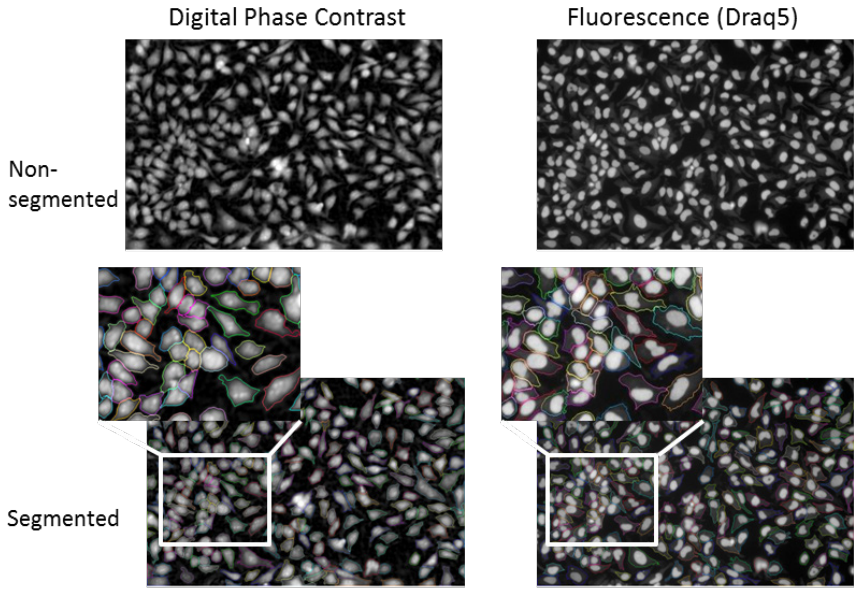


Figure 2-11: Comparison of whole cell segmentation on High Contrast DPC images of HeLa cells using method "P" of **Find Cells** building block (left panels) with whole cell segmentation of the same cells stained with Draq5 by sequentially applying the building blocks **Find Nuclei** and **Find Cytoplasm** (right panels). Original images (20x hNA, non-confocal, Operetta) are displayed in the upper panel, segmentation results below.

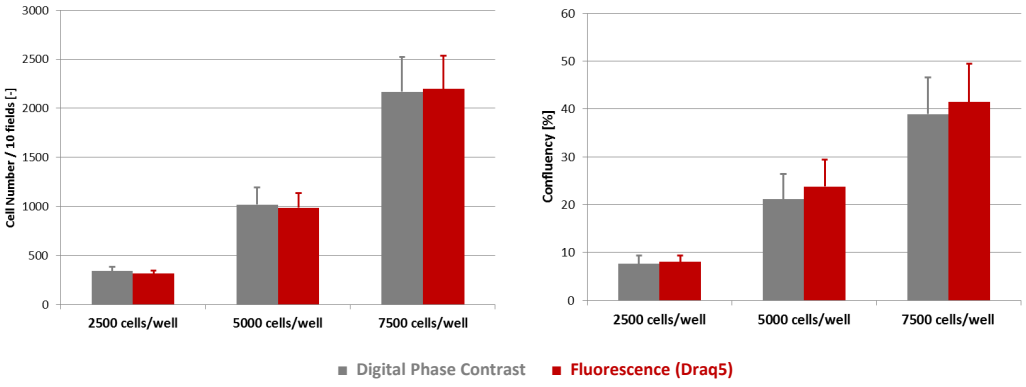


Figure 2-12: Calculation of cell number (left) and confluency (right) based on **Find Cells** whole cell segmentation of non labeled HeLa cells vs. fluorescently labeled (Draq5) HeLa cells segmented by applying the building blocks **Find Nuclei** and **Find Cytoplasm**. Results are mean values of 10 wells for three different cell densities each.

Tips and Hints for Acquisition and Analysis of Digital Phase Images

As the workflow for digital phase imaging differs in some aspects from fluorescence based imaging, here are some hints and tips to support you with your first steps:

Experimental Settings

- The first step in configuring the DPC channel is finding the sharpest plane using BF snapshot. As the contours of cells are hardly visible in the focus plane of a brightfield image, you might find it difficult to select the sharpest plane. However, the DPC algorithm is tuned for robustness and allows you to set the **Height** of the focal plane within a range of about ± 5 x half the focal depth of the objective lens without compromising digital phase image quality.
- The exposure settings should be optimized for a brightfield intensity within the dynamic range of the camera. Measuring in non-confocal mode and with binning 2 is recommended as this will shorten the exposure and also the experiment time considerably.

Overexposure of the brightfield images as well as the absence of transmission light will result in a black DPC image without an error message being displayed. Slight overexposure might lead to a very dark DPC image with bright, round spots.

- Once **DPC Snapshot** is pressed for the first representative well/field, the image will be displayed with a delay: the reason is, that – in the automatic modes **High Contrast** and **High Detail** – the **Snapshot DPC** button combines two functions – the recording of a stack (duration is exposure time dependent) as well as applying an algorithm for plane selection. However, after changing the well or field to confirm the image quality in another representative well, the image display will be faster, because the two required planes are known already and only two exposures have to be performed. In the **Manual** mode, the presentation of the DPC image after selecting **Snapshot DPC** is faster as the two planes are preselected and the stack recording is obsolete.

The quality (contrast) of the DPC images generated with the 5x objective, depends on the sample carrier (due to optical phenomena). In general, we have observed that the contrast is better in 384 well plates, less good in 96 well plates and worst with cells on slides.

DPC images are best displayed using the coloring mode “Enhanced” in Image Control.

- Please carefully select the best possible well/field (i.e. without artifacts such as scratches or dark spots in the brightfield image) for the first **Snapshot DPC** in order to avoid imprecise or faulty automatic plane calculations. It can be helpful to filter your cell culture medium to reduce artifacts in DPC image calculation. By filling your wells in reverse pipetting mode, air bubbles in the wells (which can cause dark areas in the brightfield image) can be avoided.

For reliable phase image reconstruction, please ensure that your images do not contain any microplate well borders. The phase reconstruction algorithm does not compensate for border effects. Borders are more likely to appear in the image field when working with low magnification objectives or when all fields of a well are being acquired (***Figure 2-13***).

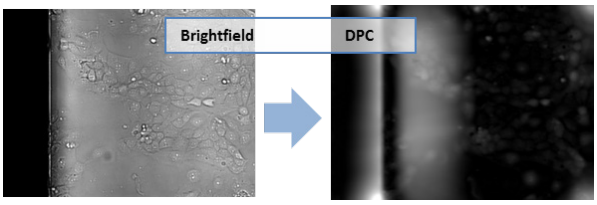
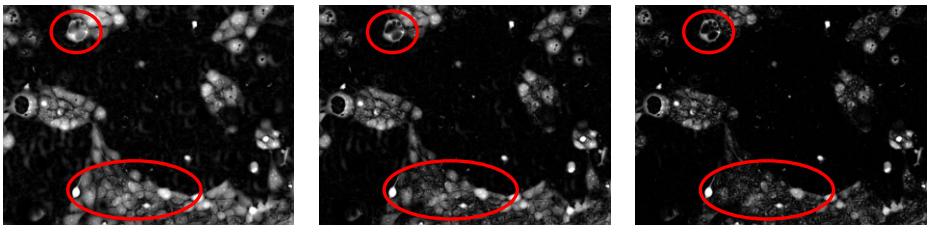


Figure 2-13: Border effect on digital phase images

- If you want to reset the calculated values for upper and lower planes in the **High Detail** or **High Contrast** mode, simply change the height or mode of the DPC channel. After changing the settings, the **Snapshot DPC** function initiates a new stack recording and re-calculates the DPC image.
- With the **Manual** mode, you can tailor DPC image generation to your sample and further improve image quality. There are four tunable parameters: upper and lower plane, **Filter** and **Speckle Scale**.
  - The optimal position for upper and lower plane strongly depends on the sample and the objective lens. For contrast enriched images, good starting values are generally between 3 – 4x the focal depth of the objective lens, above and below the focal plane. For high NA objective lenses, the optimal position of the two planes is asymmetric around the focal plane. The distance of the upper plane from the focal plane often needs to be 1.3 – 2x the distance between the lower plane and the focal plane. The closer the upper and lower planes lie to one another, the more detail will be displayed in the image, but at the expense of higher contrast.
  - **Filter** affects the whole image and is generally used to remove large scale background noise. The default value 1.0 corresponds with a typical background threshold at a given magnification, values between 0.5 and 3 are normally used. E.g. in ***Figure 2-14***, increasing the value to 2 improves the details of the previously blurred cell in the upper left corner. Setting the *Filter* too high can result in black areas within the cells (***Figure 2-14***), while keeping it too low results in background structures being over pronounced or even the appearance of artifacts.



DPC *High Detail*
Filter 1
A431 cells, 20xhNA, non-confocal

DPC *High Detail*
Filter 2

DPC *High Detail*
Filter 4

Figure 2-14: Effect of different **filter** settings on a DPC image (images taken on Operetta)

  - **Speckle Scale** acts locally within an image and can improve artifacts caused by e.g. scratches, detached cells, dust or fuzzes which present themselves as dark spots in the brightfield image and bright patches in phase images. **Speckle Scale** refers to the diameter of these dark spots and is set to 10 µm by default. Typical values have been observed to be in the range of 5 and 30 µm. Examples below show that increasing the *Speckle Scale* from 10 to 20 µm helped to remove a long artifact (***Figure 2-15***) and reversed the unintentional removal of a mitotic cell (***Figure 2-16***).

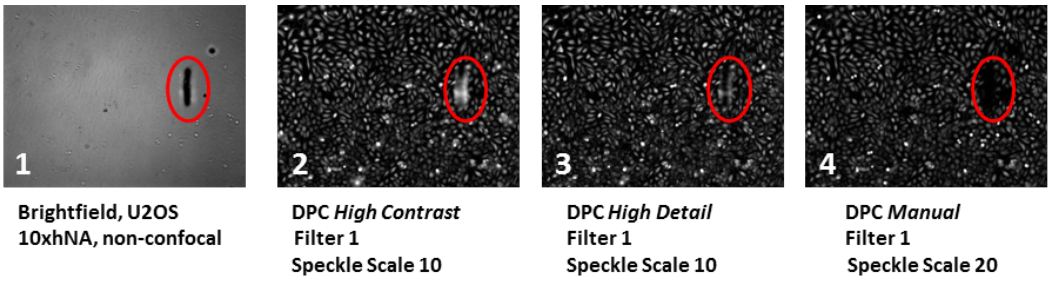


Figure 2-15: Removal of a dust particle by changing the DPC mode and tuning the parameter **Speckle Scale**

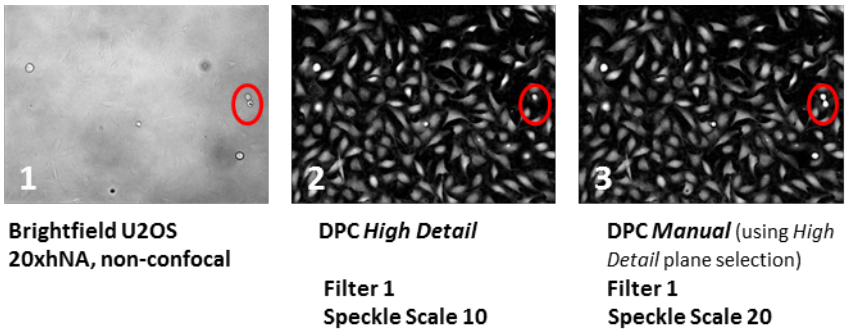


Figure 2-16: **Speckle Scale** adjustment for mitotic U2OS cells (images taken on Operetta)

Special attention should be given to **Speckle Scale** when cell tracking experiments are configured. The variety of cell shapes in a living cell population requires this parameter to be carefully adjusted pre-experiment in order to make sure that the smaller, rounder and brighter dividing cells are still visualized by the DPC algorithm (and not removed as "dust particles"). In ***Figure 2-16***, mitotic U2OS cells could only be seen in the DPC image when the *Speckle Scale* was adjusted from 10 to 20 µm.

- Please keep in mind that the automatic modes of the DPC channel trigger the recording of a stack which reaches above the Z height entered in **Height**. Therefore, if your measurement height is very close to the plate bottom and rim already, restrictions might appear in the selected well once **Snapshot DPC** is clicked. In such cases, it might help to change the well to a more central plate position.
- When setting up the digital phase channel in confocal mode, you may see stripes in your brightfield images (independent from the exposure time) which originate from the pattern of the confocal spinning disc. However, these stripes will disappear in the generated digital phase image.
- Even if a fluorescence or brightfield z-stack has been acquired in combination with a DPC channel in an experiment only one digital phase image will be available since these images cannot be calculated for different Z planes. Consequently, the very same digital phase image will be displayed together with the different z-stack images.

Automatic Measurements

- As the DPC images are calculated from two acquired brightfield images, the duration of the experiment will be longer and the online image representation slightly delayed.
- If more than one camera is present in your instrument, DPC cannot be measured simultaneously with any other channel as always two z-planes per image are acquired.
- For digital phase images, flatfield correction is not applicable: DPC images are inherently optimized for flatness and therefore do not need to undergo flatfield correction procedures. However, if an experiment includes both DPC and fluorescence channels, a flatfield correction profile estimation will launch automatically.

Image Analysis

- Digital phase imaging is primarily optimized for the segmentation of whole cells. Method “P” in the **Find Cells** building block was introduced as a new method optimized and dedicated for digital phase images. In addition, DPC images can be used as an input for other building blocks as well (see RMS “Cell Tracking – Cell Division”, section 2.30).

2.4 FRET

2.4.1 Principles

FRET (Förster resonance energy transfer) can be utilized to study protein-protein interactions or intramolecular conformation changes in living or fixed cells. To this end, the relevant proteins are usually fused to fluorescent proteins. These fusion proteins serve as the donor or acceptor molecule in a FRET assay. In a FRET measurement up to 3 images may be of interest:

- **Donor image:** Excitation Donor (Ex_D) – Emission Donor (Em_D): Shows the level of donor fluorophore
- **FRET image:** Excitation Donor (Ex_D) – Emission Acceptor (Em_A): Shows the level of Förster resonance energy transfer
- **Acceptor image:** Excitation Acceptor (Ex_A) – Emission Acceptor (Em_A): Shows the level of acceptor fluorophore

In ratiometric FRET assays, donor and acceptor fluorophores are encoded on a single protein, which is typically referred to as a FRET reporter. Since the ratio of donor to acceptor is always 1:1 in these assays, the acceptor image is usually omitted. FRET reporters are available for different biological applications such as measuring kinase or protease activity or intracellular Ca^{2+} levels.

2.4.2 FRET measurements on single camera instruments

On a single camera system all images have to be acquired in sequential exposures. For most fluorophores predefined channels can be selected from the Harmony database to acquire the donor and the acceptor image. To acquire the FRET image, a custom channel has to be defined that uses the laser for the donor excitation and the emission filter for the acceptor fluorophore (***Figure 2-17***).

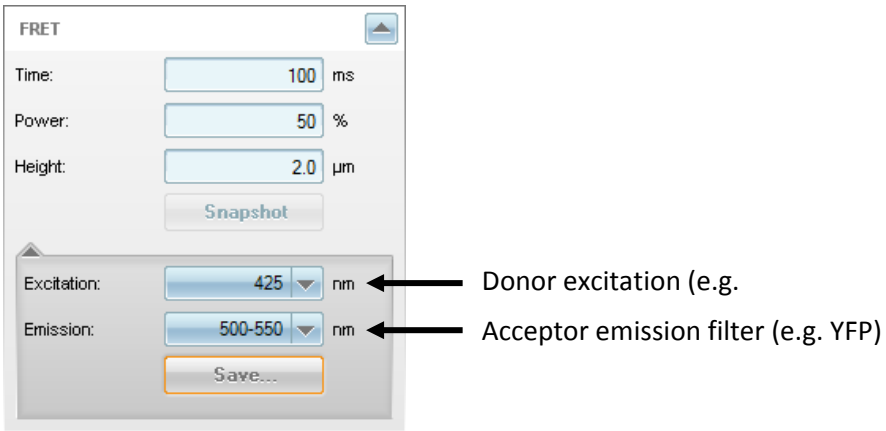


Figure 2-17: Setting up a custom channel to acquire the FRET image. The laser line for the donor excitation and emission filter have to be selected depending on the chosen FRET pair.

FRET measurements in confocal mode are subject to restrictions due to Opera Phenix's Synchrony Optics. Please refer to section 2.4.4 for further details.

2.4.3 FRET measurements on multi camera instruments

On a multi camera instrument, the emission light of the donor and the acceptor fluorophore can be acquired simultaneously after excitation of the donor. This speeds up the image acquisition in FRET assays and reduces phototoxicity as only one excitation is needed. To utilize this option special FRET channels have to be selected from the Harmony database:

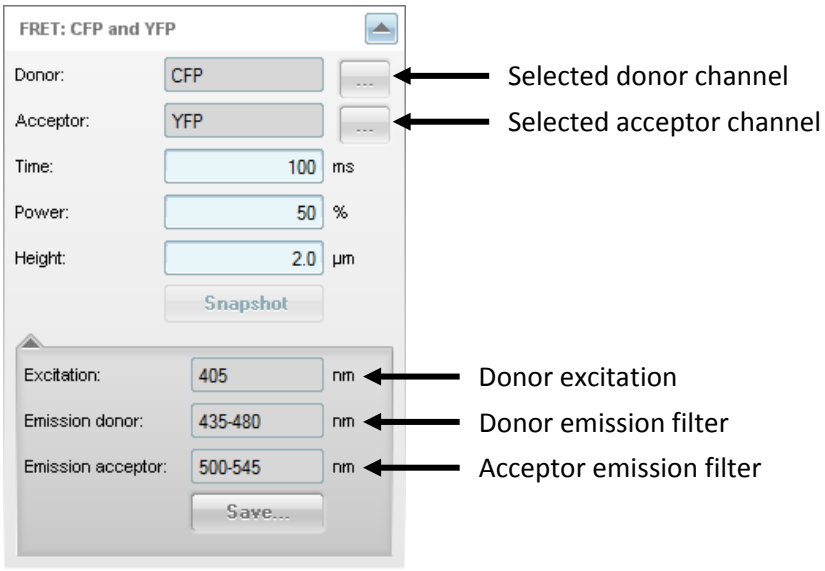


Figure 2-18: Setting up a FRET channel for simultaneous acquisition of donor and acceptor emission. Alternative channels can be created by selecting the respective donor and acceptor channels from the Harmony database.

FRET measurements in confocal mode are subject to restrictions due to Opera Phenix's Synchrony Optics. Please refer to section 2.4.4 for further details.

2.4.4 FRET measurements with the Opera Phenix System

Opera Phenix's Synchrony Optics strongly reduce the emission crosstalk when measuring in confocal mode. However, in a similar way the Synchrony Optics block the acceptor emission in a FRET measurement (see chapter 1.5.3 for details).

There are no such limitations when measuring in **non-confocal** mode. However, further channels acquired in the same measurement (e.g. additional markers) are subject to emission crosstalk.

To enable FRET measurement in confocal mode, Opera Phenix is equipped with an alternative configuration of the dichroic mirrors. This allows the measurement of the CFP::YFP FRET pair even in confocal mode (see ***Figure 2-19***).

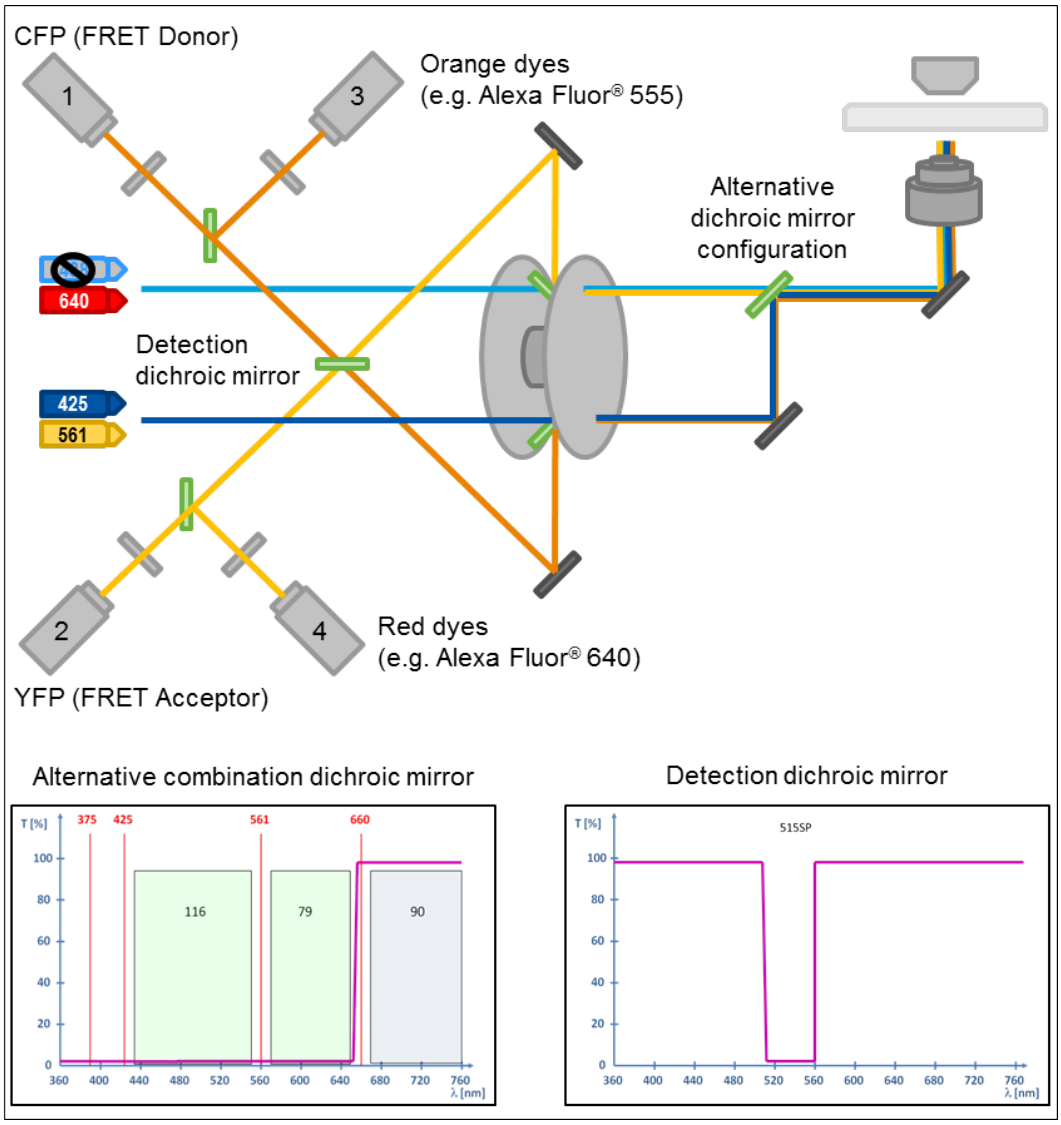


Figure 2-19: Optical path for CFP::YFP FRET in confocal mode. The donor fluorophore is excited by the 425 nm laser (alternatively by the 405 nm laser) via the lower light path. Emission light from the donor and the acceptor fluorophore is reflected by the alternative dichroic mirror configuration as this dichroic mirror is not light-transmissive up to 650 nm. Donor and acceptor emission light passes through the pinhole disk via the lower light path. Then the donor emission light is reflected at the detection dichroic mirror and detected on camera 2. In a 4 camera system, 2 additional dyes can be measured. They are excited by the 561 nm laser and the 640 nm laser, and the emission light is collected on cameras 3 and 4, respectively.

In summary, the following FRET pairs can be measured in **confocal** mode:

- CFP::YFP FRET using the alternative dichroic mirror configuration (Donor excitation up to 425 nm; Acceptor emission 435-550 nm)
- Long stokes shift FRET pairs with donor excitation up to 425 nm and acceptor emission 570-635 nm using the standard dichroic mirror configuration (e.g. CC2-DMPE::Oxonol)
- Long stokes shift FRET pairs with donor excitation at 488 nm and acceptor emission >645 nm using standard dichroic mirror configuration (e.g. mCitrine::mKate)

When measuring FRET assays where the ratio between donor and acceptor is not 1:1, it may be necessary to acquire the acceptor image to measure the acceptor concentration, resulting in a slower image acquisition.

During assay development it may be worth considering whether a purely ratiometric evaluation is a fair approximation of the actual FRET signal. If so, the acceptor image can be omitted in screening runs to increase the acquisition speed.

2.5 Time series measurements

Monitoring kinetic live cell experiments requires the acquisition of time series measurements with previously defined time points using the Harmony® High Content Imaging and Analysis Software.

2.5.1 Measurement intervals

The minimum interval between two time points is dependent on the time it takes for one time point to be measured. This is directly dependent on the exposure time, number of channels, number of measured fields and the number of planes and wells. The shortest possible interval is calculated by Harmony and displayed in the shortest interval box under the time series definition. The shortest interval is the duration of one time point measurement while the estimated duration refers to the whole experiment (all time points). If the interval between measurements selected by the user is too short to be measurable (e.g. the shortest interval is 3:30 min and a fixed interval of 3 min is selected in the definition), a message is displayed that requests the user to either increase the interval time or measure less fields, planes or wells. As the shortest interval is an approximate value that contains a security buffer, you may try shorter intervals if a faster acquisition is desired. Harmony will try to accomplish the defined measurement interval.

The shortest interval calculation is only possible on the instrument PC and not in the Office version of Harmony. To empirically determine if your imaging interval can be shorter than the calculated interval, run a measurement with only 2 time points using the same conditions as in your experiment.

If the endpoint of an assay has not been reached by the end of a time series measurement, additional single measurements can be performed later on. These can be attached using the **Combine Measurements** function.

In general, there are three different time series sequences that allow the use of different measurement intervals (***Figure 2-20***). The easiest case is to use just one sequence with the option to measure a fixed interval, or as fast as possible. If the process monitored slows down over time then a second time series sequence can be used. In this sequence you can choose longer intervals between measurements to find the endpoint of the reaction without increasing the file size inappropriately. For fast kinetic events it can be beneficial to use the break function. The break stops the measurement and allows the user to manually add a compound (a ligand, agonist, drug, etc.). Additionally, a sequence before the break is created to capture the baseline of a reaction.

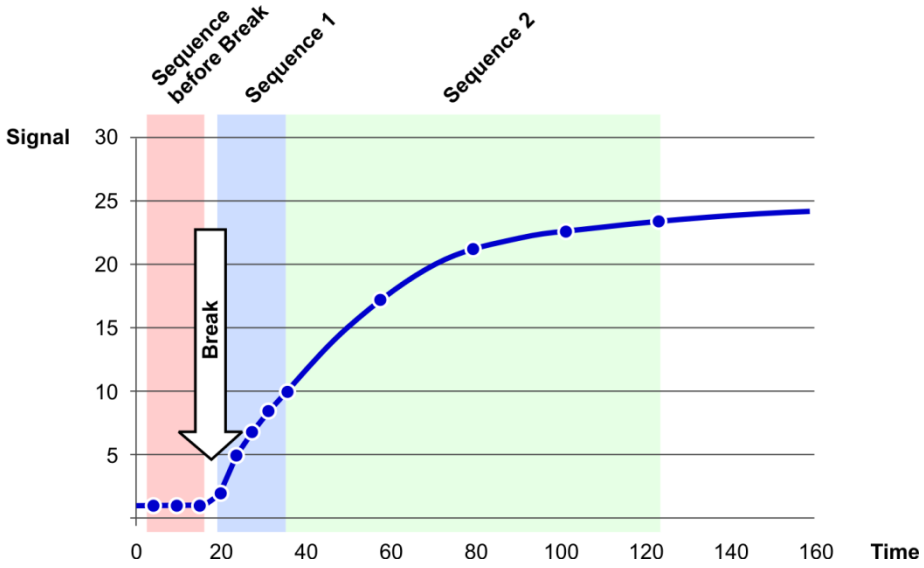


Figure 2-20: Schematic overview of a time series sequences. In principle, three time series sequences with different measurement intervals can be used. If a break is included, the user can manually add compounds into the wells to start a kinetic reaction (represented by the arrow). A time series sequence before the break can be used to monitor the baseline of a reaction. Sequence 1 and 2 allow you to follow a reaction with fast intervals at the beginning and longer intervals for capturing the endpoint.

A time series can be tested on a single well by activating the **Use in Test** function. This function is intended to optimize the exposure settings of a time series, especially if large intensity changes are expected upon compound addition. Intensity changes could be due to an internalization reaction leading to a higher localized fluorescence (e.g. in the cytosol), or a fluorescence enhancement of a biosensor.

Use additional test wells on the measurement plate to optimize the exposure settings of a live cell experiment.

2.5.2 Time stamp

The time stamp of a measurement is always a relative value. The first measurement is, by definition, T0 and has the time stamp 00:00:00 (hh:mm:ss). The time stamps of the following measurements (T1, T2, T3 …) are calculated relative to this starting point. If the break function is enabled, the measurements before the break get a negative signature (T-1, T-2, T-3 …). Additionally, the duration of the break is recorded and the time stamps before the break are corrected for this value.

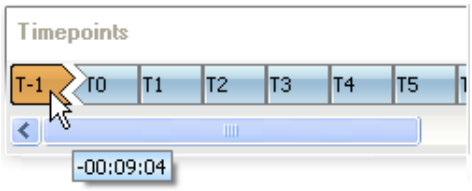


Figure 2-21: Time stamp for the sequence before the break measurement. Measurements before the break get a negative signature (T-1) and a negative value. The duration of the break is tracked and the value of sequences before the break measurements is corrected afterwards. In this case, the T-1 measurement and the break together took 9 min and 4 sec.

All fields of a well get the same time stamp (the time stamp from field one).

2.5.3 Considerations for live cell experiments

To run reliable live cell experiments, it is important to ensure the viability of the cells throughout the measurement. Besides keeping the cellular environment (temperature and CO_2 concentration) constant, reducing the light-induced toxicity is a crucial key factor for cell health. Consider using **digital phase imaging**, as it causes virtually no phototoxicity. If digital phase imaging is used, it is recommended to filter the medium prior to imaging since small particles in the medium may result in artifacts in digital phase images. Illumination of fluorophores leads to the generation of free radicals upon photobleaching (Stephens and Allan, 2003). Therefore, the duration and intensity of illumination should be kept at a minimum.

The following factors can help to reduce phototoxicity:

- Reduce the light source power and exposure times as much as possible.
- Try to reduce the number of exposures (e.g. longer interval times).
- Use high NA objectives to collect as much light from the sample as possible.
- Always use medium without phenol red, as phenol red introduces high background fluorescence.
- If the cells can tolerate it, use medium without serum to reduce unspecific background and improve the signal to noise ratio.
- Use plates with bottoms of high optical quality (e.g. glass).
- Do not use nuclear dyes that are excitated with ultraviolet (UV) light (e.g. Hoechst), as UV light causes DNA damage (Frigault *et al.,* 2009).
- Use the minimum amount of fluorescent probe required.
- The spinning disk confocal system is known to cause significantly less photodamage than other confocal systems (Frigault *et al.,* 2009; Stephens and Allan, 2003). However, if confocality is not essential for your live cell application, we recommend that you measure in the non-confocal mode. Confocal measurements require longer exposure times or higher concentrations of fluorophores than non-confocal measurements. Both might be unfavorable for a live cell assay.

Digital phase imaging is a non-phototoxic alternative to fluorescent imaging. In particular, digital phase imaging can be used to replace fluorescent whole cell stains intended for object segmentation (for more details see section 2.3.2).

Additional control wells with unlabeled cells (at least without nuclear labeling) can help to assess the phototoxicity of the dyes used in the experiment.

2.5.4 Kinetic measurements

Potential application examples for kinetic measurements over several hours could be:

- Translocation assays (e.g. receptor internalization, cytosol-to-nucleus translocation…).
- Apoptosis assays.
- Monitoring drug induced (cytotoxic) effects.
- Determination of the endpoint of an assay during assay development.

As an example of a kinetic measurement, we performed a receptor internalization assay using a fluorescently labeled G protein coupled receptor (GPCR). The Endothelin-A receptor (ET_AR) is present on the plasma membrane until stimulated by its ligand endothelin-1 (ET-1), a peptide hormone. ET-1 induces the internalization of ET_AR into the perinuclear region of the cell (***Figure 2-22***).

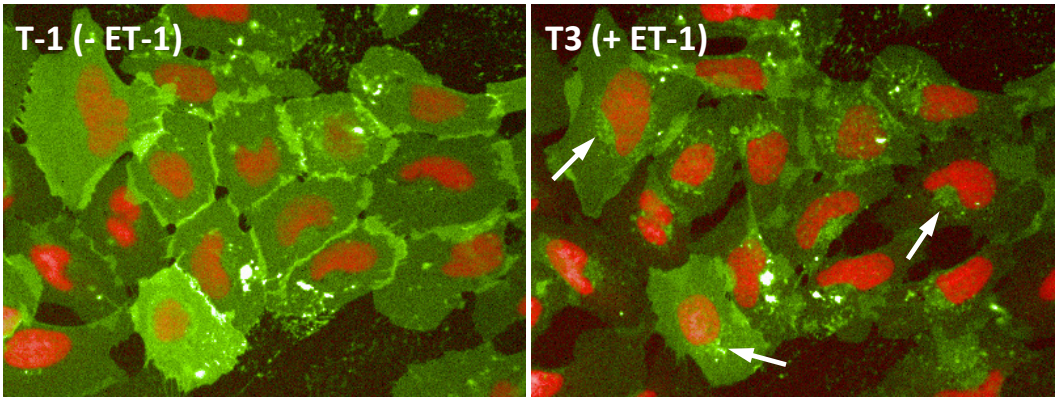


Figure 2-22: Confocal Images of U2OS cells expressing a fluorescently labeled ET_A receptor (green). Nuclei are labeled with Draq5™ (red). Left: At time point -1 (T-1), before Endothelin-1 (ET-1) addition, ET_AR is mainly located on the plasma membrane. Right: After addition of 100 nM ET-1, translocation of the receptor to the perinuclear region is apparent after 60 min (T3).

To run this experiment, a time series was defined using one sequence with an interval of 20 min and 10 time points, resulting in a total duration of 3 h. The break function was included to allow the addition of endothelin-1 into the designated wells. One image was acquired before the break, as a control, prior to the addition of ET-1.

The time series was analyzed using the RMS “Receptor Internalization” (please see Image Analysis Guide, section 2.11). This RMS uses the **Select Cell Region** building block to define a cytoplasmic region of interest around the nucleus. The final readout is the internalized ET_AR intensity (***Figure 2-23***).

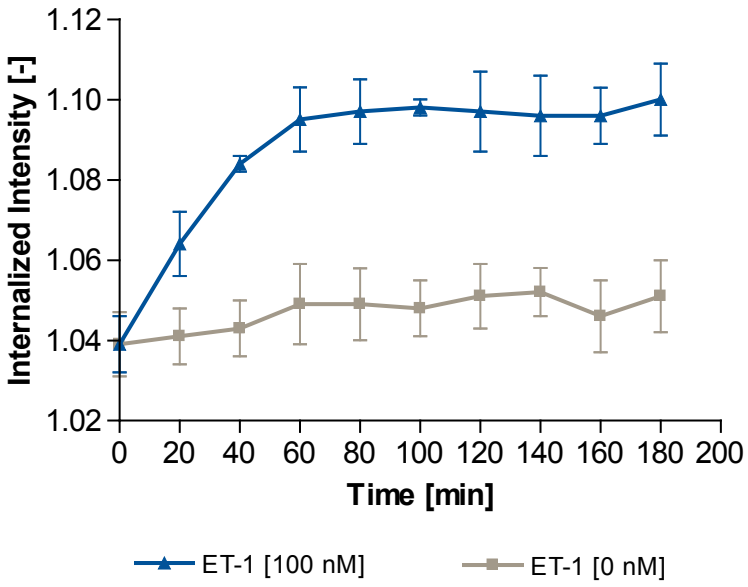


Figure 2-23: Time course of ET_AR internalization up to 180 min. U2OS cells internalize the ET_A receptor upon incubation with 100 nM Endothelin-1 (ET-1). After 60 min, the maximum internalized intensity is reached. No significant increase in the internalized ET_AR intensity can be detected without ET-1 (0 nM). N = 3 wells.

If a fixed interval is defined in the sequence before the break, this interval is used between the time points, but the break will start directly after the last measurement.

The Overview function allows you to assemble time series images and thus facilitates a visual inspection of the biological effect investigated (for an example, see ***Figure 2-24***).

The wells of a plate are always measured with a vertical meander, starting in the lower left corner of the plate (e.g. well 1P). Uneven columns are always measured from P to A, and even columns from A to P. This may be of interest for very fast experiments where the order of pipetting might be important. See ***Figure 1-3***.

2.5.5 Long kinetic measurements

For long term monitoring of cells up to several days, individual measurements can be performed and combined into a time series measurement at a later point. This allows for plates to be moved back into the cell culture incubator where an optimal humidity control is assured throughout the duration of the experiment. The Combine Measurements function is a useful tool for combining measurements that were generated either manually or by automated systems. The combination into one time series enables simultaneous evaluation of all time points, rather than performing individual evaluations and then combining the results in external software (e.g. Microsoft® Excel®).

Potential application examples are:

- Long term monitoring of drug induced (cytotoxic) effects.
- Monitoring cell cycle specific effects.
- Colony formation assays (please also refer to the RMS “Colony Formation Analysis”, Image Analysis Guide, section 2.25).
- Migration and invasion assays.

As an example of a long kinetic measurement, we performed an Oris™ Pro Migration Assay that was monitored for 68 h. The 96 wells of the Oris™ Pro plates contain a dissolving biocompatible gel droplet that creates a cell free zone in the center of the wells. HT-1080 fibrosarcoma cells were incubated in the presence of various concentrations of the actin disrupting drug Cytochalasin D (Cyt D) and were labeled with Cell Tracker™ Green CMFDA. Migration into the cell free zone was monitored with a 2x objective. The measurements were done with variable intervals and combined at a later point of time. By using the combine measurements function a new time series measurement was created.

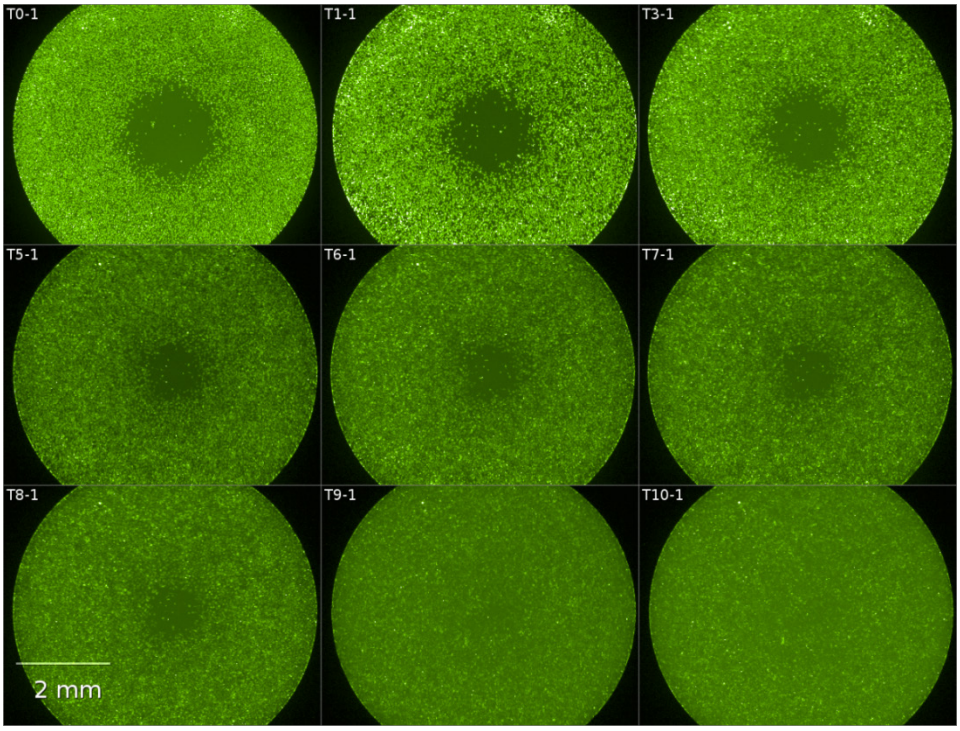


Figure 2-24: Overview of a time series showing the migration of HT-1080 cells into the cell free zone in the center of the well. The cells were labeled with Cell Tracker green CMFDA and were incubated in the presence of 0.1 µM Cytochalasin D. Individual time points were selected and combined to give the overview. The labels in the upper left corner display the selected time point and field number. Using the 2x objective, just one field per well was recorded.

To analyze the time series, the “RMS Migration- Hole in Cell Layer” (see Image Analysis Guide, section 2.26.2) was loaded from the database. This RMS is intended for applications performed with a low magnification objective. Final readouts are the cell layer area [mm^2], open area [mm^2] and the overall well confluence [%].

2.5.6 Measurements for object tracking

The acquisition of images for object tracking needs some special considerations. If the sampling interval is set too large, object tracking will result in lost object tracks. Sampling too frequently will accumulate an excess data volume and may result in unwanted phototoxic effects if fluorescent dyes are used. Therefore, it is highly recommended to determine an optimal imaging interval.

To determine the optimal sampling interval for object tracking prepare a sample where your objects exhibit the highest expected movement speed. Acquire a measurement with a very high sampling rate and prepare your image analysis sequence. Compare the outcome of the object tracking while using only every second, every third, etc. time point for your analysis sequence.

Another point to consider is thermal effects. The corpus of microplates expands by approximately 7 µm per Kelvin over the entire plate dimension, which results in a visible shift of objects towards the upper left corner of the images. Therefore, thermal equilibration of microplates in the environmental chamber is highly recommended.

Using the break function in the time series setting allows compounds to be added to a specimen after acquiring a baseline read. However, removing the plate from the instrument will result in a significant offset in object positions on continuation of the time series. In addition, the thermal equilibration of the microplate will be disturbed. Therefore, carefully check if objects are successfully tracked across the break in the time series. If this is not the case, the time points before and after the break can be analyzed separately.

To reduce the time needed for thermal equilibration, avoid significantly cooling down the plate by shortening the time span for the plate transfer from an incubator to the instrument.

2.5.7 Generation of videos from a time series measurement

Once a time series measurement has been acquired, it is possible to generate a video from a selection of time points or the entire time series. On the Harmony Image Analysis Screen select the Input Image building block and select all the desired time points using the Timepoints selector of the Navigation Area. Using the context menu set the selected time points as a Time Window. After setting a time window Harmony Software will load all the images for the selected time points and automatically generate a video, which can be played within the Harmony Software. Note that it is not possible to select different well, fields, time points and planes when the time window is active. The video can be exported to WMV format by right clicking on the image.

2.6 Temperature and CO_2 control Option

The **T**emperature and **C**CO_2 control **O**ption (TCO) allows you to set a temperature (37-42°C) and a CO_2 concentration (1-10%). These set values are constantly maintained within a range of ± 1 °C and ± 0.5 % respectively.

The use of the TCO and the time series function are independent of each other. On one hand, this allows constant temperature and CO_2 conditions for all wells of a complex live cell experiment (many wells and fields) without doing a time series. On the other hand, it is possible to perform time series measurements without using the temperature and CO_2 control.

2.6.1 Temperature and CO_2

The set temperature of the live cell chamber is reached after a variable heating period. The time needed to stabilize the temperature depends on the set value and the environmental temperature and can range from a couple of minutes to more than an hour. At 22 °C it will take approximately 1 h to heat the instrument to 37 °C. A CO_2 concentration of 5 % is stabilized after 5 min. The status of the TCO is shown by the progress indicators on the device and by symbols in the global control panel of the Harmony software.

If you open the lid (e.g. to adjust the correction collar), it takes a couple of minutes to re-stabilize temperature and CO_2 concentration.

Although the temperature and CO_2 sensors have been placed as close as possible to the plate, they are not measuring the actual temperature and CO_2 concentration in the medium that is surrounding the cells. The medium itself stabilizes the temperature and CO_2 concentration for some time, thus a temporary drop in temperature or CO_2 will not immediately affect the sample. Please note that avoidance of phototoxicity is also crucial for cell viability (please refer also to section 2.5.3).

Any aberration from the set values for the TCO that might have occurred during the experiment will be recorded with the measurement. This information can be accessed on the evaluation screen.

For long experiments, it is recommended that you use the shutdown function to switch off temperature and CO_2 automatically after the experiment has finished. The components for the shutdown have to be selected first in the Settings dialog (“Shutdown/Standby Settings”). Then you can activate the “Shutdown” check box on the Run Experiment screen and start the measurement.

It is possible to start a measurement before reaching the target temperature and CO_2 values. Especially after loading the plate again (e.g. after a break in a time series), the actual values may differ slightly from the target values, but the measurement can nevertheless be continued without delay.

The heating mechanism is carefully optimized to avoid any transient overheating of the sample even during the warm-up cycle. As a result, it is safe to load the plate into the live cell chamber as soon as the green checkmark appears for the very first time.

2.6.2 Evaporation

The Temperature and CO_2 control option does not include a humidity control; therefore evaporation can occur, especially in wells at the plate edges. If evaporation is a critical factor for the success of the experiment, these wells should be excluded and possibly just filled with buffer or medium.

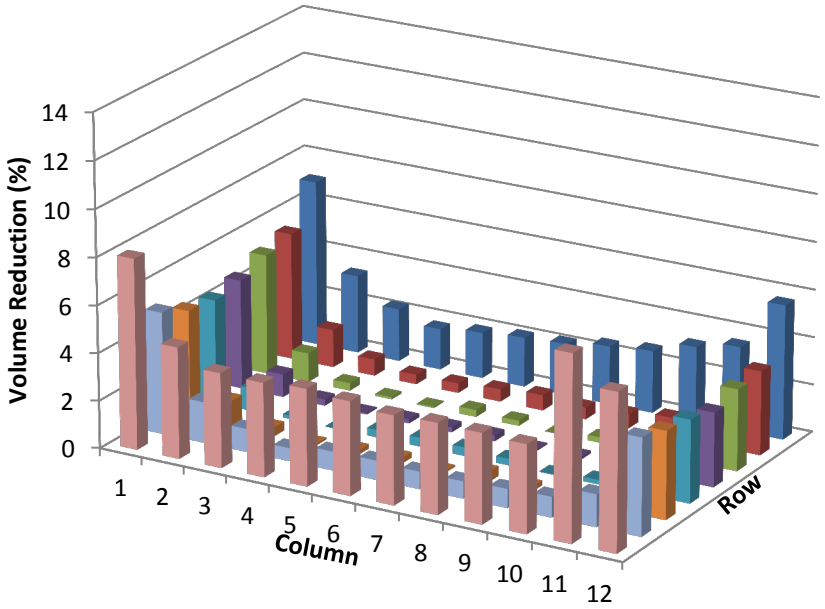


Figure 2-25: Evaporation in a 96 well plate after 4 h of incubation at 37 °C and 5 % CO_2. The volume reduction varies between 2 – 8 % in the wells at the edge, and is below 1 % in the wells at the center of the plate.

If the wells at the edge are necessary for the application, some recommendations are given:

Use special lids that contain a wet filter paper to reduce the evaporation (Labcyte, MicroClime® Environmental Lid, Cat. No. LLS-0310-IP).

Use plates with a gas permeable bottom (e.g. from ibidi) and either seal them with a non-permeable foil, or overlay the samples with silicone oil.

For long term experiments (more than 24 h), we recommend the use of the automation setup, where plates are stored in an incubator between measurements. Please contact your local sales representative for further information.

2.7 Basic facts about Laser-based Autofocus systems

For low magnification objectives (such as the 1.25x), and the measurement of slides (see sections 1.3 and 2.9), special autofocus routines have been implemented for the instrument. However, to understand the resulting functionality, and especially its limitations, it is essential to understand the basic principles behind a laser-based autofocus system.

A laser-based autofocus system relies on the detection of reflections of the laser beam at surfaces. These reflections are caused by variations in the refractive index of different surfaces. The typical application for a laser based automated measurement is an adherent cell sample in a microplate with buffer in the well. In this case, the differences in refractive index between the components (air, glass and buffer) are large and two peaks can be detected, as illustrated in ***Figure 2-26***. When the autofocus system moves upwards, a peak between the air and plate bottom is detected first, followed by a second peak at the interface between the glass bottom and the buffer.

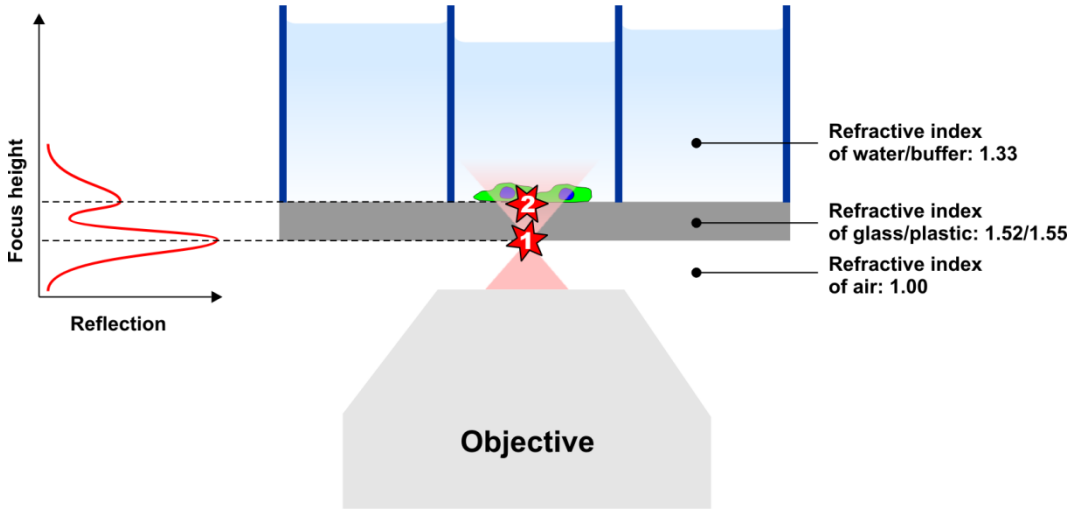


Figure 2-26: Seen from the bottom side of the plate, the first autofocus peak is detected at the interface between air and the lower plate bottom, and the second peak is detected at the interface between the upper plate bottom and the aqueous solution.

The second peak is the essential reference point for focusing of the samples. The user-defined focus height is added to the height of this second peak (see ***Figure 2-27***).

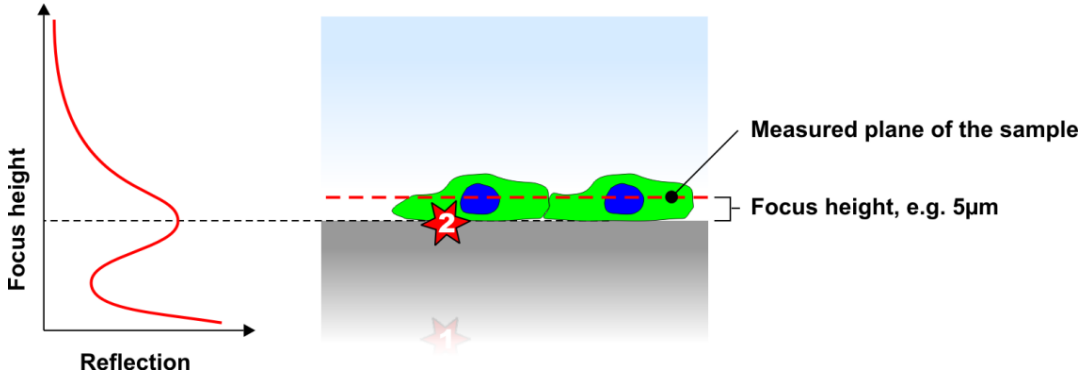


Figure 2-27: The second peak is detected at the interface between glass and buffer. Using this as a starting point, the focus height is then added where each sample will be measured.

The thickness of the plate bottom may vary over tens of microns within a short distance in the well, even with very high quality plates. By using the second peak as reference point and measuring this at every single measurement position (i.e. image field), the system ensures that the measurement is performed at exactly the same focus height (relative to the plate bottom) at every location in the well (see ***Figure 2-28*** for an illustration). This is particularly important for confocal measurements when even slight variations in focus height would become apparent.

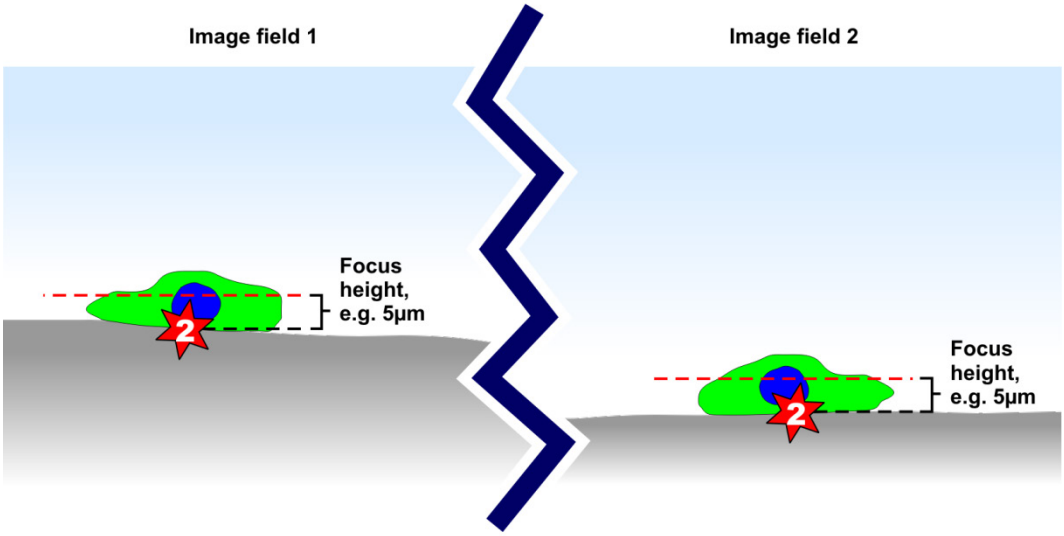


Figure 2-28: As shown in this exaggerated example, the height of the plate bottom may vary between different image fields. As the focus height is added to the second focus peak, which represents the upper plate bottom, each sample will be measured at the same relative height.

However, for low magnification objectives and slides, the use of the second peak is not feasible. Please see the following sections (2.8 and 2.9) for an explanation of the reasons and further recommendations.

In these cases, the autofocus system will take the first peak as a reference and calculate a theoretical second peak by adding the plate bottom thickness. However, this may result in less precise focusing, as illustrated in ***Figure 2-29***, as the thickness of plate bottoms or cover slips has a certain tolerance.

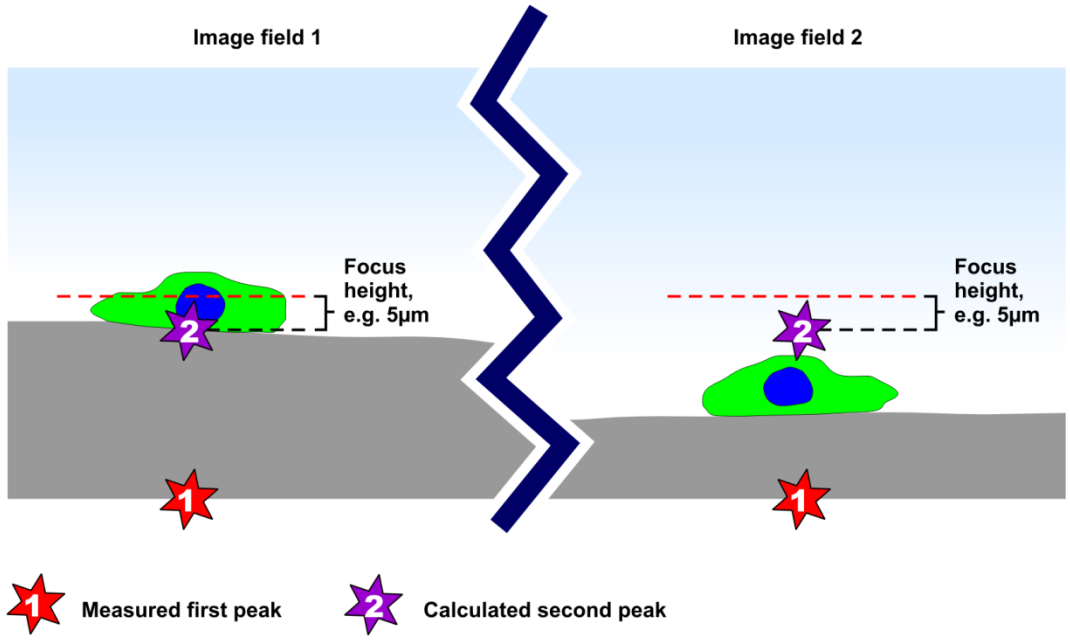


Figure 2-29: If the bottom thickness varies a lot within a well (plate, slide or cover slip), the use of the first peak as a reference for focusing will result in partially unfocussed images.

It is essential to use high quality plates or cover slips with minimal thickness tolerances in order to minimize the risk of partially unfocused images.

2.8 How to measure with low magnification objectives

Low magnification objectives like the1.25x have a very large depth of focus, e.g. the 1501 µm. Therefore, only large changes in the focus height of > 500-1000 µm will result in visible changes in the object sharpness. See ***Table 5-5*** in the Appendix for details.

The depth of focus of 1501 µm also means that this objective has a very low z-resolution. As a consequence, also the autofocus system's resolution is not high enough to detect the upper and lower plate bottom like it is done with higher magnification objectives, see ***Figure 2-30***. Only the lower plate bottom is detected, because the change of the refractive index is very high at this point (air to glass/plastic interface). See section 2.7 for details on how the autofocus system detects the plate bottom.

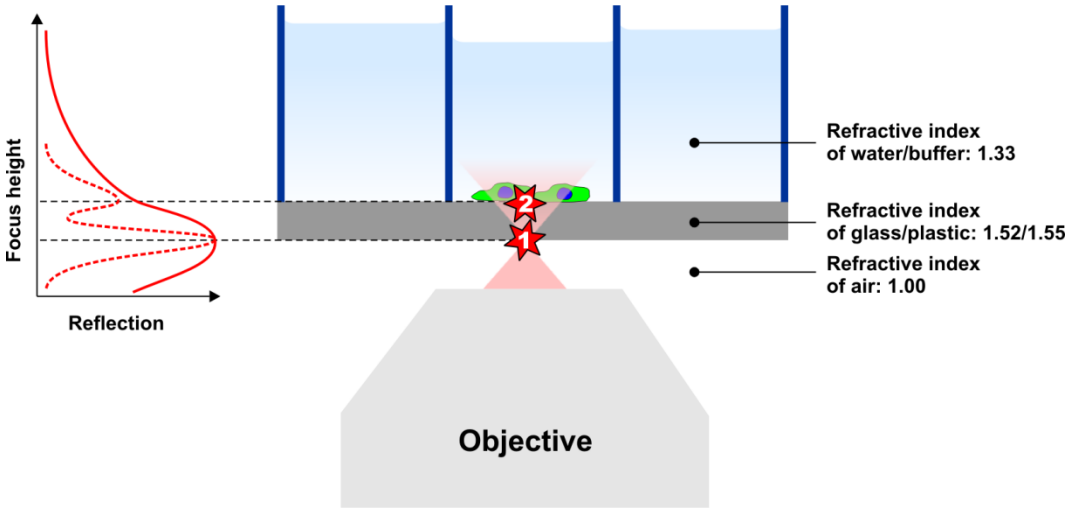


Figure 2-30: With a low magnification objective (1.25x), only the first autofocus peak is detected at the interface between air and the lower plate bottom, whereas the second, weaker peak is not taken into account. Please compare this figure to ***Figure 2-26*** in section 2.7 showing the situation for higher magnification objectives.

Note that when using the 1.25x objective, the way you determine the focus height in a microplate will differ from the description given in section 2.1.2 "Setting the focus height, excitation power and exposure time". Due to the low z-resolution and the single autofocus peak, the optimal focus height of the sample will also include the optical plate bottom thickness:

Assuming that the sample is about 50 µm and the plate bottom is about 200 µm thick (optical thickness!), a good starting point for searching for the focus height would be around 250 µm. For further explanation of the optical thickness, please refer to section 1.2.3 "Plate geometry".

However, there is one exception: if you use the 1.25x objective for measuring a microscope slide then you don't need to add the optical thickness of the slide or cover slip to the focus height – just determine the focus height as normal from e.g. the 20x objective and a microplate. As described in section 2.9, the autofocus system will use the thickness of the slide or cover slip automatically.

Although the sample focusing will be slightly inexact, this will not negatively affect your measurement as the low magnification objective has a high depth of focus.

There are some aspects that need to be considered when working with a low magnification objective:

- The low magnification objective will only work reliably up to a plate bottom thickness of 1 mm. Due to the second focus peak partially re-appearing, thicker plates might result in unstable focusing.
- Also double-check that the used plate's geometry matches with the selected plate type definition. If the well's boundaries are in the middle of the image, this might lead to focus failures.
- Similar to the fact that you might find a negative focus height (as described in section 1.2.3 "Plate geometry"), with the low magnification objective you might have to start at +200 µm when fluorescent dyes are used.
- The very wide depth of focus of a low magnification objective necessitates a long scan range. This could lead to the detection of a second peak at the upper buffer/air interface if the wells are only filled with a minimum amount of buffer (see ***Figure 2-31***). As this would seriously affect focusing, please ensure that an appropriate amount of buffer is added to the well, resulting in a height of the liquid surface of at least 1 mm above the plate bottom.

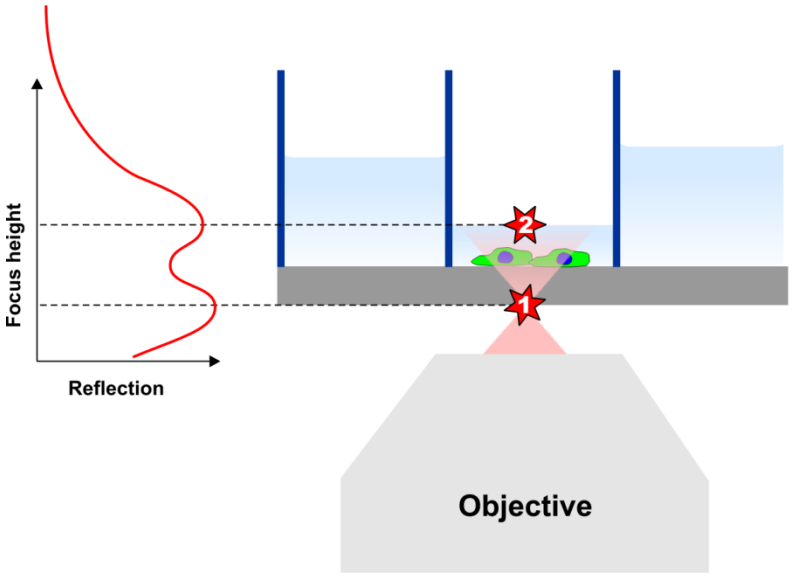


Figure 2-31: As explained in the text, the system will only detect the first peak and perform a very wide scan for focusing. If the fill level in the well is within the scan range then a second peak will be detected which will lead to a focus failure. This is because with a low magnification objective, no second peak is expected.

2.9 An introduction to the measurement of tissue samples on slides

Slides are very different from microplates. There are numerous possible ways to prepare a sample on a slide and unfortunately, most of them are not optimally compatible with the previously described use of the autofocus system since the second reference peak is not usable. This is because the second focus peak either does not exist (see ***Figure 2-32***), or else it is obscured by additional peaks (see ***Figure 2-33***).

Mounting media are optimized to have the same refractive index as glass in order to minimize any reflections. These reflections occur at the surface between glass and buffer and may reduce image quality. However, a laser based autofocus system relies on exactly these reflections. Therefore, in the case of a sample with mounting medium, the second peak is missing and only one peak (at the air-glass interface) is present.

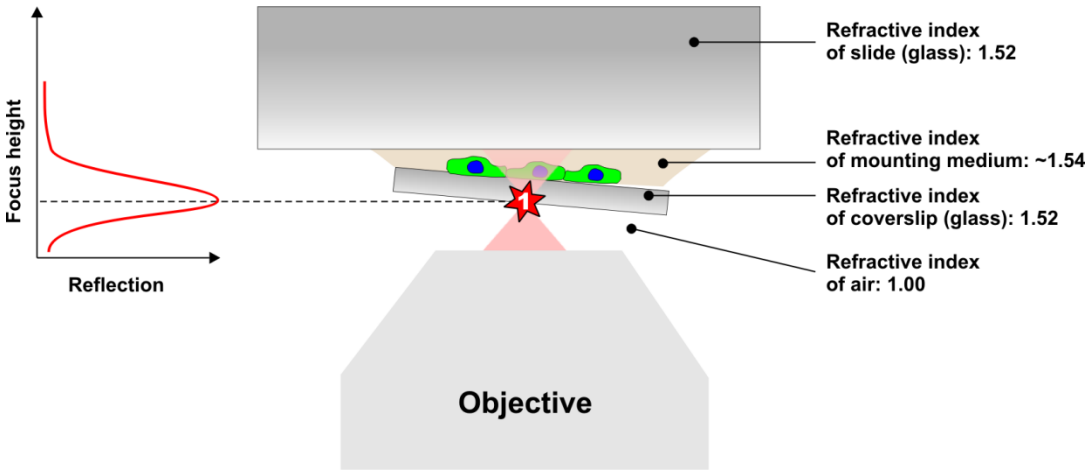


Figure 2-32: Illustration of a slide sample which uses mounting medium. The only focus peak will occur at the interface between air and glass since the refractive indexes of glass and mounting medium are almost identical.

Instead of mounting medium, you could use buffer to cover the sample which will help the cover slip to align in parallel to the slide. In this case, the second peak is present, but due to the complex 'sandwich' of slide/buffer/cover slip an additional peak (very close to the second, usually within only a few µm) is also present that deteriorates the second peak and makes it unsuitable as reliable reference point.

Here, again, the first peak is the only available reliable reference point for focusing.

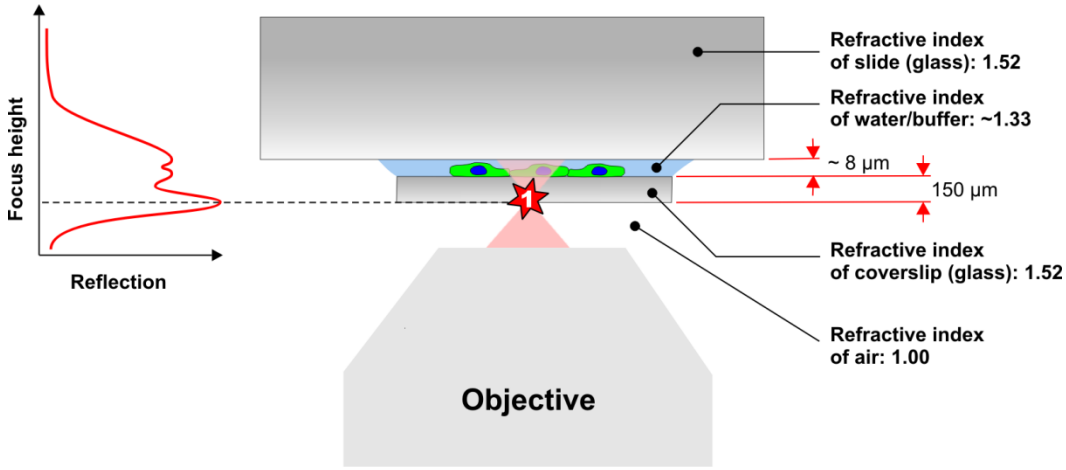


Figure 2-33: Illustration (not-to-scale) of a slide sample which uses buffer to cover the sample. As the distance between the cover slip and slide is only a few µm, the second peak is almost identical to the third peak, meaning that they cannot be separated reliably. The autofocus system takes the first peak as a reference point and will internally calculate a theoretical position for the second peak.

However, the signal ratio between the first and the second peak is changed when using water immersion objectives. The refractive index of water (n=1.33) is closer to the refractive index of the coverslip (n=1.52) than air (n=1). As a result the peak at this interface will be smaller compared to air objectives. Therefore, if the refractive index of the mounting medium is close to that of water, the system could mistake the second peak for the first one and use this as reference point for focusing of the samples. The result will be defocused images. Hence, it is important to use mounting media with high (n>1.46) refractive indices.

Whenever a slide is chosen as plate type, the autofocus system will automatically use the first peak as this is the only available reliable reference point.

The autofocus system will then internally add the thickness of the cover slip (or slide) to the first peak, which calculates the theoretical position of the second peak. As a result, you can select your focus height as normal (even with a low magnification objective – please refer to section 2.8).

The automatic addition of the “bottom” thickness means that the quality of focusing critically depends on the thickness tolerance of the cover slip or slide as illustrated earlier in ***Figure 2-29***.

Consider the following when measuring slide samples:

- The one peak autofocus can be easily thrown off by the use of a mountant with a refractive index similar to water, especially when using a water immersion objective. This leads to defocussed images as an incorrect peak has been used for fitting. No autofocus error messages are seen.
- Cover the sample with a mounting medium that has a similar refractive index to glass.
- Avoid enclosing air bubbles within the region of interest.
- Whenever possible, turn the slide upside down so that the cover slip is below the slide for measurement. Usually, cover slips have a smaller thickness tolerance than slides.
- Locate the sample on the cover slip, not on the slide.
  See ***Figure 2-34*** for explanation.

- Measure only a small area of e.g. 100 fields to avoid any variation in the “bottom” thickness (more likely to occur over a large distance), which would influence the image quality.
- To avoid focus failures, make sure you locate your well layout in a sample region without air bubbles.

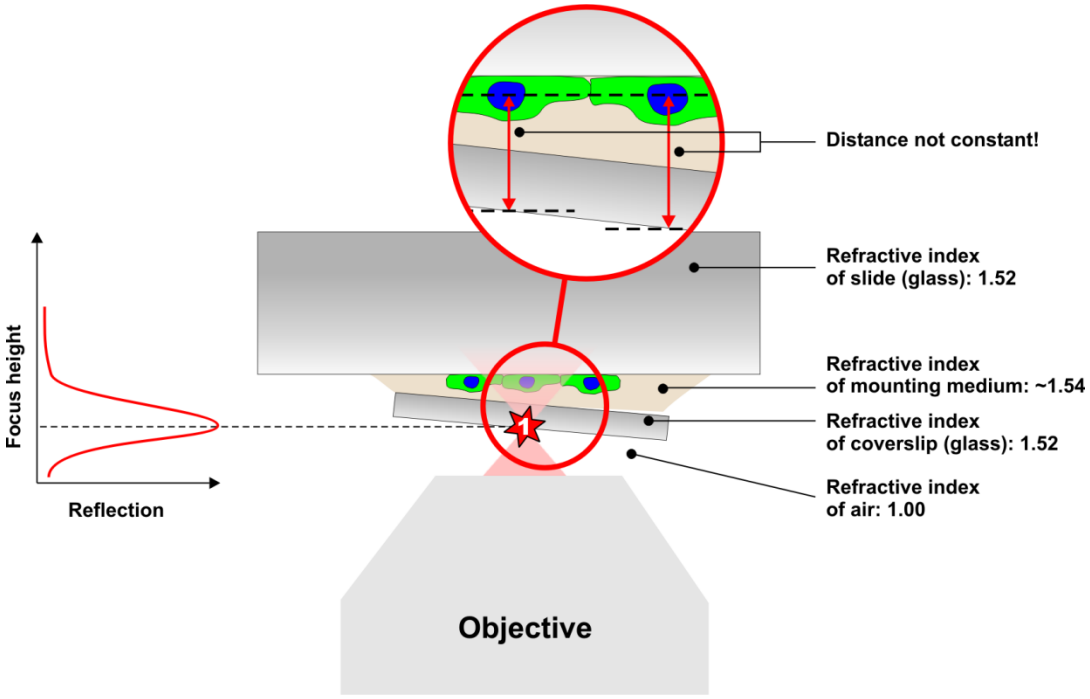


Figure 2-34: If the sample is located on the slide instead of the cover slip, and mounting medium is used, you are likely to get unfocused images because the distance between the focus peak and the given focus height is not constant. Consequently, locating the sample on the cover slip is strongly recommended.

For repositioning the well layout, either the manual PreScan / ReScan or automated PreciScan option can be useful (see section 2.10 and 2.11 for details). Try a low magnification objective to locate air bubbles within the area of interest if you get many focus failures.

If tissue sections are to be imaged and analyzed the Global Image feature might be helpful both for automated PreciScan and image segmentation. Global Image is available in the Image Analysis tab. It creates a montage image based on absolute coordinates out of all fields and downscales the image by binning to a final image size of max. 5 MP.

2.10 Manual PreScan/ReScan option

The PreScan/ReScan option has been introduced to facilitate measurements, particularly of slides or similar large sample holders, but it also works with microplates. You can measure (prescan) the whole well layout with a low magnification objective and then select a region of interest for a rescan using the desired higher magnification. Please see the Harmony Help or User Manual for further explanation. Additionally, an automated routine called PreciScan is available as well (see section 2.11 “Automated PreScan/ReScan – PreciScan™”).

An example is given in ***Figure 2-35*** (selection of fields) and ***Figure 2-36*** (result) below.

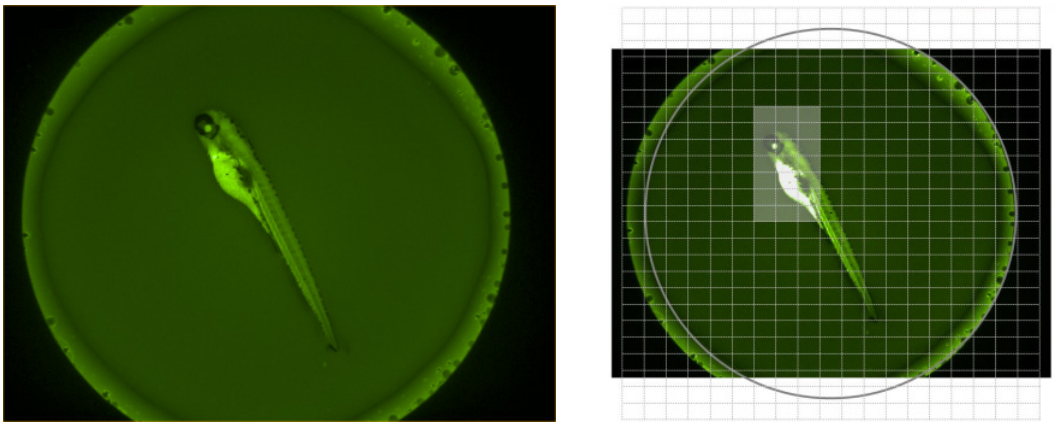


Figure 2-35: Selection of the image fields for a 40x ReScan on a 2x PreScan image (GFP labeled zebra fish in a 96 well plate). Left: 2x magnification PreScan image. Right: Screenshot of the Harmony well layout control for image field selection (selected fields are highlighted). The grid indicates the user-selected field positions for the 40x ReScan objective. The PreScan image was selected by the “Background for Well” option, available from the right mouse click menu on the well layout.

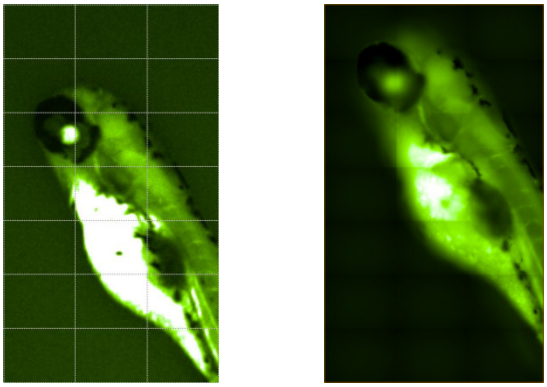


Figure 2-36: PreScan/ReScan of a GFP labeled zebra fish in a 96 well plate. Left: The 2x PreScan image, with gridlines, shows the positions of the selected 40x objective image fields (shown in the right part of ***Figure 2-35***). Right: Montage of the resulting 40x images from the ReScan. Note that the ReScan image has shifted compared to the PreScan image due to unavoidable mechanical tolerances when switching the objective. Please be sure to select a slightly larger area for ReScan to account for this (this will not occur when using the automated PreciScan option, see section 2.11).

The PreScan and selection of the well layout for the ReScan is a manual process that has to be repeated for each sample (e.g. slide or well) to be measured.

When selecting the well layout for the rescan you may need to surround the object that you are interested in by a border of one or more fields in order to fully capture it with the higher magnification objective.

This is due to the unavoidable shift of the image when switching objectives. This effect is caused by mechanical tolerances, which you may be familiar with from research microscopes. In the example shown in ***Figure 2-36***, if the top row of fields was not selected in the prescan image, the rescan will not cover the entire head of the zebra fish.

Consider the following when using the PreScan/ReScan option:

- Carefully check the restrictions of the measurable plate area prior to using the PreScan/ReScan option with microplates. A well at the edge of the plate may be measurable with the low magnification PreScan objective, but may not be accessible for the high magnification objective in the ReScan. For further information on objective restrictions, see section 1.2.4 “Measurement restrictions”, section 1.4 “Objectives” and the Harmony Help or User Manual.
- If you can see air bubbles in the prescanned images, omit the respective image fields in the rescan measurement wherever possible. The air bubbles may cause autofocus errors in the ReScan.
- For analysis the feature Global Image, available in the Image Analysis tab, will create a montage image of ReScan images based on absolute coordinates and downscale it to max. 5 MP.

2.11 Automated PreScan/ReScan – PreciScan™

Typically, objects of interests are rather small in relation to the well size and sometimes users might be interested in only a small sub-population of cells (rare events). An example could be the search for single cells positive for a specific marker among a majority of other cells, like phospho histone 3 (pHH3) as a marker for mitotic cells. These marker positive cells could be located anywhere in the well and of course the distribution and number of these objects varies from well to well. To capture all cells of interest the user would need to scan the whole well including all fields. This can result in very large number of fields being imaged, especially when using a high magnification objective that provides sufficient resolution for a detailed subcellular analysis. Hence, this leads to very time consuming measurements and additionally huge measurement files in which the majority of images will not contain any valuable information.

The PreciScan procedure allows the user to image only the objects of interest in an automated manner. PreciScan basically consists of 3 subsequent steps (***Figure 2-37***). The first step is a PreScan measurement of the sample using a low magnification objective. In a second step images of the PreScan measurement are automatically analyzed using an online analysis to determine x and y positions of desired objects. In a third and final step, these absolute positions will be used to set image fields capturing these objects in a ReScan measurement with a higher magnification objective.

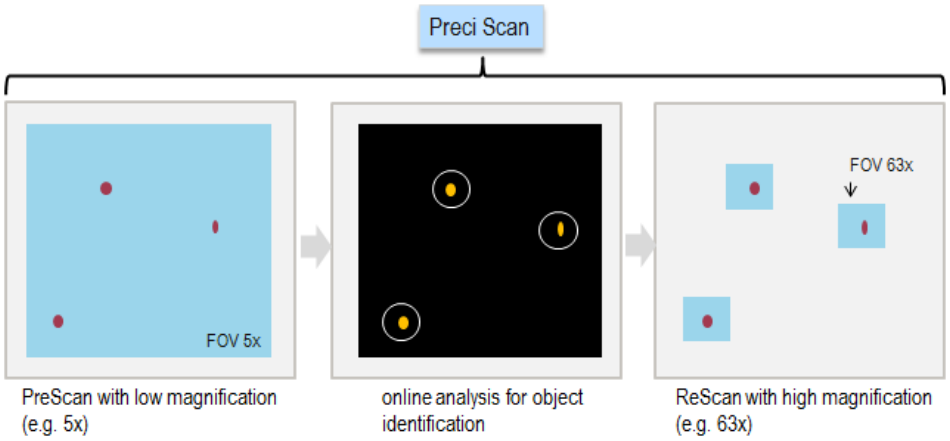
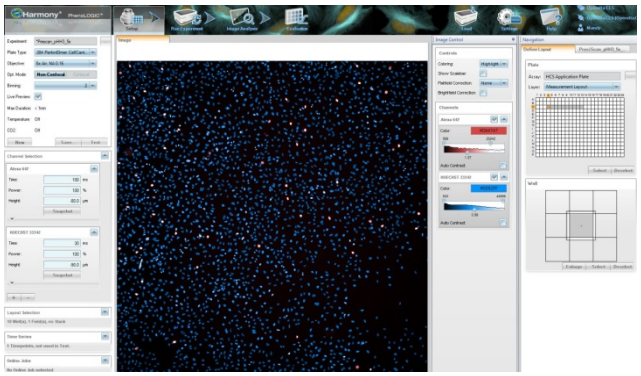


Figure 2-37: The three subsequent steps of a PreciScan procedure: The first step (left panel) is a PreScan measurement of the sample using a low magnification objective, e.g. 5x. In a second step an online analysis of the PreScan measurement identifies x and y positions of desired objects (middle panel, orange spots). These absolute positions will be used to set image fields capturing these objects in a ReScan measurement with a higher magnification objective as a third step (right panel).

PreciScan Example 1 – Imaging of pHH3 positive cells (HCS Application Plate)

HeLa cells were seeded in a 384-well microtiter plate (CellCarrier™, PerkinElmer, Inc.) and treated with Demecolcine to study cytoskeletal rearrangements and the mitotic index. Cells were labelled with an anti-α-tubulin and an anti-phospho-histone H3 primary antibody and visualized by binding of Alexa-488 and Alexa-647 labelled secondary antibodies. The nuclei were stained with Hoechst 33342. PHH3 positive cells are to be scanned with 63x magnification to study the morphology of the mitotic cells. The following PreciScan setup could be used to identify pHH3 positive cells using a 5x objective and to re-image these cells with a 63x water immersion objective:

Setup **PreScan** experiment



Setup tab:

- Choose 5x lens, load Hoechst and Alexa647 channel.
- Optimize exposure settings and z heights for each channel.
- Select wells and run a test measurement that will be used to setup the online analysis sequence.

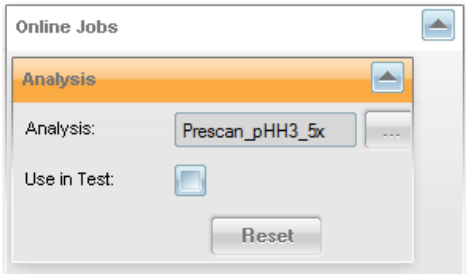
Setup image analysis sequence

Analysis Sequence "Prescan_pHH3_5x"			
Input Image	**Flatfield Correction :** None Brightfield Correction **Stack Processing :** Individual Planes **Min. Global Binning :** Dynamic		
Find Nuclei	**Channel :** HOECHST 33342 **ROI :** None	**Method :** B Common Threshold : 0.4 Area : > 30 µm² Split Factor : 7.0 Individual Threshold : 0.4 Contrast : > 0.1	Output Population : Nuclei
Select Population	**Population :** Nuclei	**Method :** Common Filters Remove Border Objects Region : Nucleus	Output Population : Nuclei w/o border
Calculate Morphology Properties	**Population :** Nuclei w/o border **Region :** Nucleus	**Method :** Standard Area	Property Prefix : Nucleus
Select Population (2)	**Population :** Nuclei w/o border	**Method :** Filter by Property Nucleus Area [µm²] : < 900	Output Population : Nuclei final
Calculate Intensity Properties	**Channel :** Alexa 647 **Population :** Nuclei final **Region :** Nucleus	**Method :** Standard Mean	Property Prefix : Intensity Nucleus Alexa 647
Select Population (3)	**Population :** Nuclei final	**Method :** Filter by Property Intensity Nucleus Alexa 647 Mean : > 2000	Output Population : pHH3 positive nuclei
Determine Well Layout	**Population :** pHH3 positive nuclei **Region :** Nucleus	**Method :** Standard Rescan Magnification : 63x Max. No Fields : 1 Object Margin : 10 µm Field Overlap : 0 %	Output Population : Well Layout
Define Results	**Method :** List of Outputs **Population : Nuclei final** Number of Objects **Method :** Formula Output Formula : a/b*100 Population Type : Objects Variable A : pHH3 positive nuclei - Number of Objects Variable B : Nuclei final - Number of Objects Output Name : Percent pHH3 positive cells **Object Results** Population : Nuclei final : None Population : Nuclei w/o border : None Population : Nuclei : None Population : pHH3 positive nuclei : None		

Image Analysis tab:

- Use images from test measurement to generate an analysis sequence to find pHH3 positive cells.
- Generate a population of all nuclei using the Hoechst channel (**Find Nuclei**).
- Create new population of pHH3 positive cells (**Calc Intensity Properties** / **Select Population**).
- Configure well layout for ReScan, select pHH3 pos cell population, select 63x magnification (**Determine Well Layout**)
- Expand **Define Results** building block and calculate ratio of pHH3 positive cells using a formula output: pHH3 positive cells divided by all cells from Hoechst nuclei population.
- Save analysis sequence “PreScan pHH3 5x”.

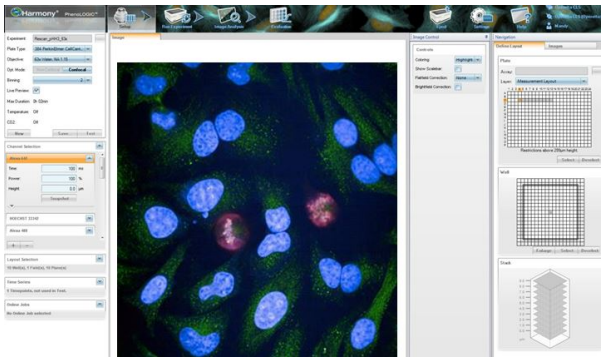
Save final **ReScan** experiment



Setup tab:

- Add analysis sequence “PreScan pHH3 5x” as online job to the PreScan experiment and save.

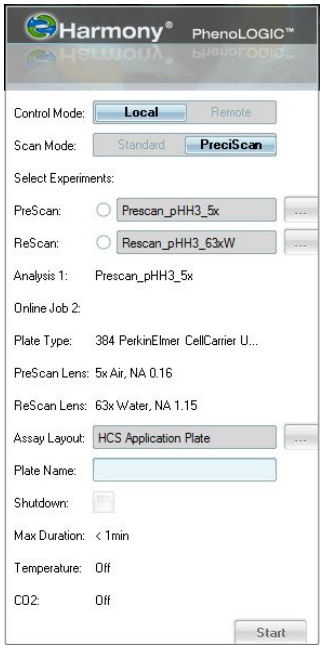
Setup **ReScan** experiment



Setup tab:

- Snapshot one well with PreScan lens and use **Background For Well** function (context menu of “Well” pane) in test images to find pHH3 positive cells in well.
- Select ReScan lens (63xW). Select channels.
- Define exposure settings and stack with 10 planes.
- Select at least one well.
- Check measurement restrictions by selecting all subfields.
- Save the ReScan experiment.

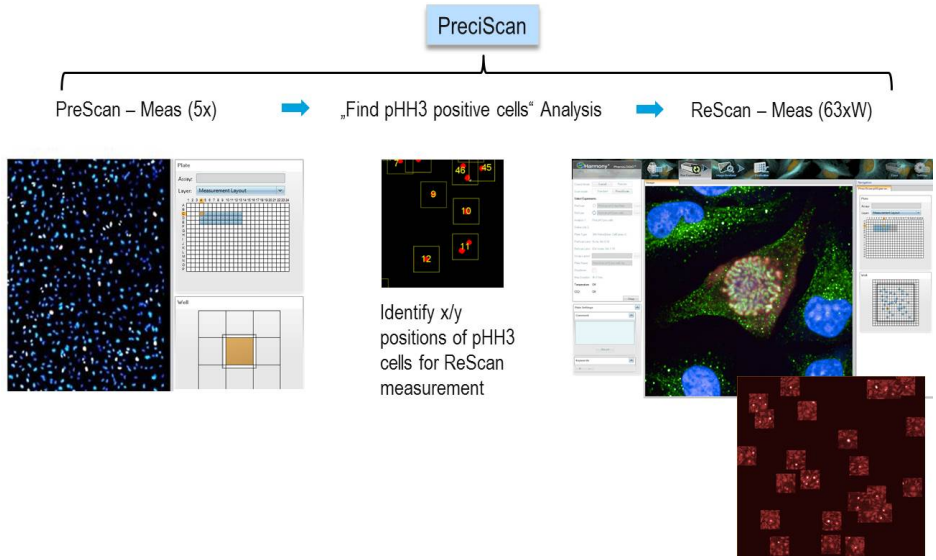
Run **PreciScan**



Run Experiment tab:

- Select scan mode **PreciScan**
- Select PreScan experiment from database.
- Select ReScan experiment from database.
- Define plate name and click **Start**.

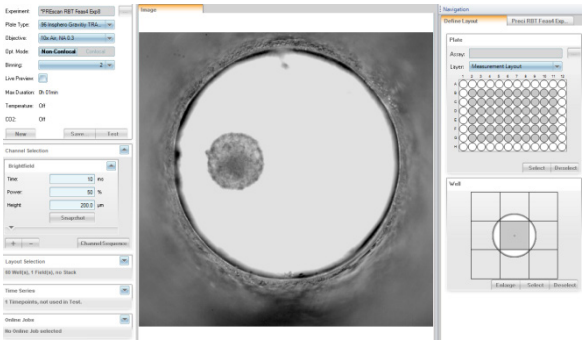
Result of the PreciScan – Example 1



PreciScan Example 2 – Imaging of Microtissues

Microtissues were cultured and stained in an InSphero GravityTRAP™ ULA Plate 96-well. As microtissues do not attach to the well bottom of this plate due to the ultra-low attachment coating (ULA), they can be positioned anywhere in the well. The following PreciScan setup can be used to image the microtissues with a 20x air objective.

Setup **PreScan** experiment



Setup tab:

- Choose 5x lens and Brightfield channel.
- Set z height to half the microtissue diameter and slightly overexpose to get rid of dust particles present on well bottom.
- Select all wells and run a test measurement that will be used to setup the online analysis sequence.

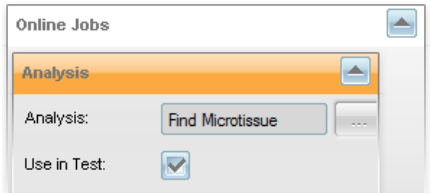
Setup image analysis sequence

Analysis Sequence "Find Microtissue"			
Input Image	**Flatfield Correction :** None **Brightfield Correction :** 0 **Stack Processing :** Individual Planes **Create Global Image :** 0 **Min. Global Binning :** Dynamic		
Find Image Region	**Channel :** Brightfield **ROI :** None	**Method :** Common Threshold Threshold : 0.69 Split into Objects Area : > 300000 px² Fill Holes	Output Population : Well Output Region : Well
Select Region	**Population :** Well **Region :** Well	**Method :** Standard Border Geometrical Center	Region Prefix : Well
Select Region (2)	**Population :** Well **Region :** Well Geometrical Center	**Method :** Resize Region [µm/px] Outer Border : -370 px Inner Border : INF px Restrictive Population : None Restrictive Region :	Output Region : Image Region Geometrical Center Resized
Filter Image	**Channel :** Brightfield	**Method :** Invert Image Cut-off Quantile : 100	Output Image : Inverted
Filter Image (2)	**Channel :** Inverted	**Method :** Smoothing Filter : Gaussian Width : 20 px	Output Image : Gaussian Smoothed
Find Image Region (2)	**Channel :** Gaussian Smoothed **ROI :** Well **ROI Region :** Image Region Geometrical Center Resized	**Method :** Common Threshold Threshold : 0.92 Split into Objects Area : > 20000 px² Fill Holes	Output Population : RBT Tissue Output Region : RBT Tissue
Calculate Intensity Properties	**Channel :** Gaussian Smoothed **Population :** RBT Tissue **Region :** RBT Tissue	**Method :** Standard Mean	Property Prefix : Intensity RBT Tissue Gaussian Smoothed
Select Population	**Population :** RBT Tissue	**Method :** Filter by Property Intensity RBT Tissue Gaussian Smoothed Mean : > 10000	Output Population : RBT Tissue Selected
Determine Well Layout	**Population :** RBT Tissue Selected **Region :** RBT Tissue	**Method :** Standard Rescan Magnification : 20x Max. No Fields : Object Margin : 10 µm Field Overlap : 10 %	Output Population : Well Layout
Define Results	**Method :** List of Outputs **Population : RBT Tissue Selected** Number of Objects **Population : RBT Tissue :** None Population : Well : None Population : RBT Tissue Selected : None		

Image Analysis tab:

- Use images from test measurement to generate analysis sequence to find microtissue region.
- Find well area in Brightfield channel (**Find Image Region**).
- Use geometrical center region (**Select Region**).
- Resize geometrical center region (**Select Region**).
- Invert Brightfield image (**Filter Image**).
- Find tissue in well region (**Find Image Region**).
- Configure well layout for ReScan with 20x (**Determine Well Layout**).
- Click **Define Results** and save sequence “Find Microtissues”.

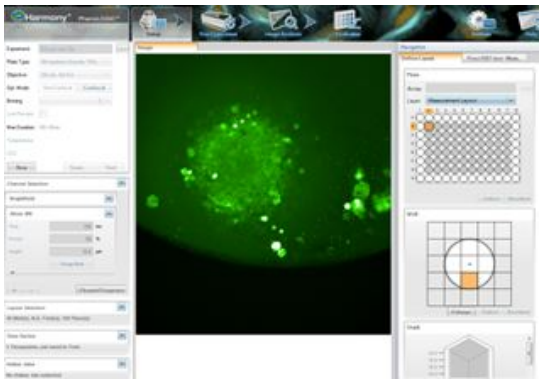
Save final **PreScan** experiment



Setup tab:

- Add analysis sequence “Find Microtissues” as online job to PreScan experiment and save.

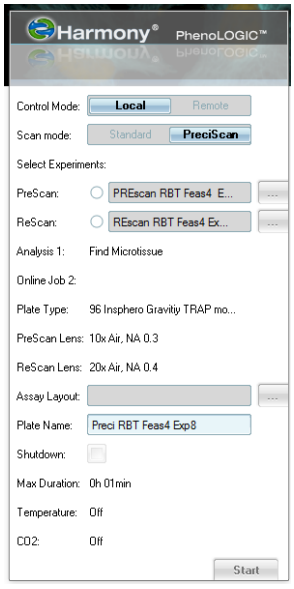
Setup ReScan experiment



Setup tab:

- Snapshot one well with PreScan lens and use **Background for Well function** in test images to find microtissues in well.
- Select ReScan lens, 20xA and Alexa488 channel, define exposure settings and stack with several planes.
- Select at least one well to save the ReScan experiment.
- Check measurement restrictions by selecting all subfields.

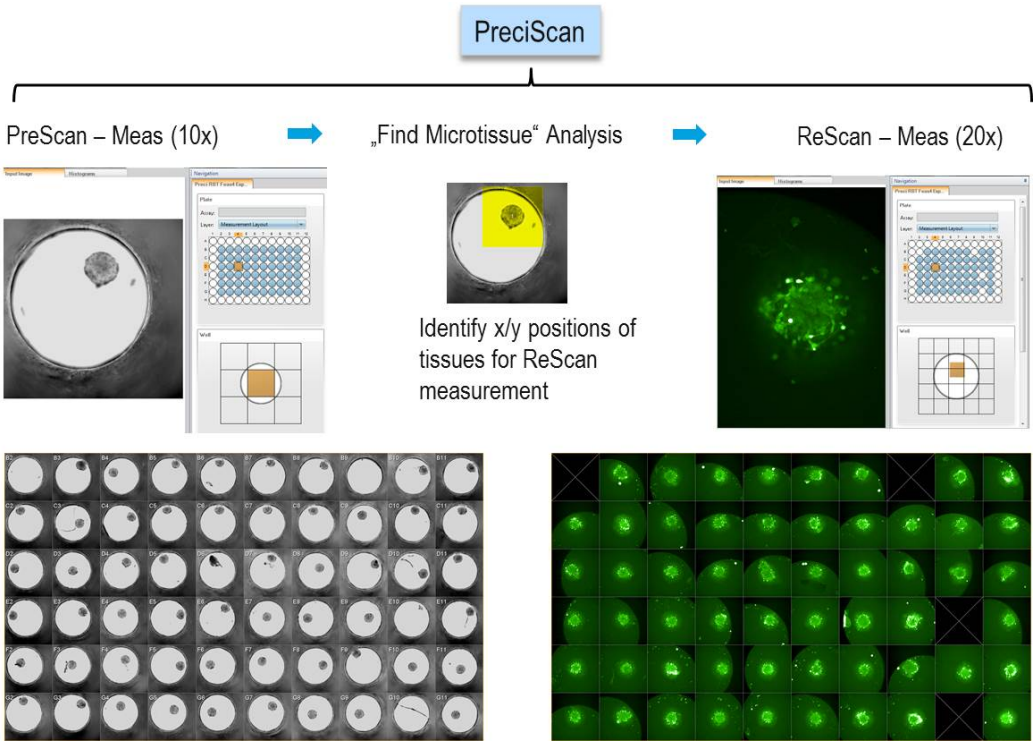
Run **PreciScan**



Run Experiment tab:

- Select scan mode **PreciScan**.
- Select PreScan experiment from database.
- Select ReScan experiment from database.
- Define plate name and click **Start**.

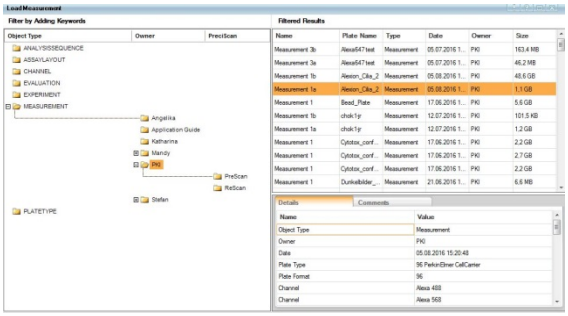
Result of the PreciScan – Example 2



Hints for management of PreciScan data

Image Analysis tab:

- The PreScan and a ReScan measurement are connected by the PreScan evaluation. Once PreScan or a ReScan measurement is loaded you can easily switch back and forth between both measurement using the **Load PreScan/ReScan** button.



Database Browser:

- PreScan measurements are tagged with an “a” in the database.
- ReScans measurements are tagged with a “b” in the database.
- An additional “PreciScan” keyword can be added in the browser to group and find PreScans and ReScans in the database.

Evaluation1	Preci RBT Feas6 Exp1
Measurement 1a	Preci RBT Feas6 Exp1
Measurement 1b	Preci RBT Feas6 Exp1

Data transfer:

- A complete PreciScan consists of 3 database items: The PreScan measurement “a”, the ReScan measurement “b” and the appropriate PreScan evaluation. The PreScan analysis sequence is part of the evaluation.
- For a data transfer of a PreciScan into another ODA all three items must be archived to get the analysis sequence and the functionality of switching between measurements.

Tips and tricks

Running an experiment in PreciScan mode requires appropriate PreScan and ReScan experiments. Please use the Harmony Help function, section “PreciScan” to learn more.

Use the lowest magnification possible for the PreScan to limit scan time.

Use several wells across the plate including compound treated and control cells to test the functionality of your PreScan analysis sequence.

You can use “snapshot” or “test” to generate images and test the analysis sequence by switching between the Setup- and Image Analysis tabs. Note that “snapshot” or “test” images are not saved in the database.

Alternatively, a measurement of several wells across the plate using the PreScan lens could be done and the analysis sequence tested on this measurement. This data is saved on the database and no switching between setup and image analysis is needed to generate the analysis sequence.

When setting up the ReScan experiment to optimize exposure parameters, use the “Background for Well” function to do a manual PreScan-ReScan for better object location.

The PreScan and its following analysis are mainly used to identify objects to be rescanned. However, it can also be used to generate additional readouts in the define results building block, e.g. object number ratios of different cell populations which cannot be necessarily determined from the ReScan images.

When setting up the ReScan experiment select all subfields of the well to check the measurement restrictions for the ReScan lens across the plate (see section 1.2.4 “Measurement restrictions”).

A ReScan experiment cannot be used separately. It always needs a PreScan and subsequent analysis to be executed.

Setup parameters of an existing ReScan experiment associated with a measurement cannot be used for setting up a new experiment. Please load the “original” ReScan experiment instead.

Due to the nature of the application with PreciScan, the number of fields per well in the ReScan run might not be known before executing the PreScan. Hence, the size of the resulting file cannot be calculated before the PreScan run has finished. Subsequently, if the hard disk space is insufficient the PreciScan experiment will stop at this point.

The total number of fields per well is limited to 4200. When using for example a slide-based sample in combination with a high magnification objective like the 40x or 63x objectives, 4200 fields might not be enough to cover the whole object region identified in the PreScan run.

Chapter 3 – Multiparametric Applications with Assay Protocol

This chapter describes our assay protocols, for specific applications, and how they can be used with our multiparametric ready-made image analysis solutions.

You will find protocols for:

3.1 Ready-Made Application: Apoptosis-1

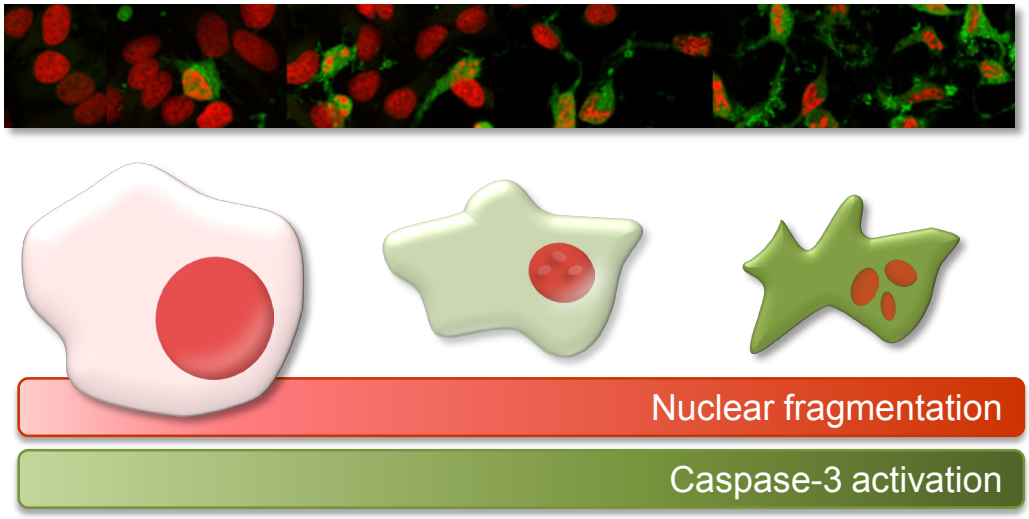


Figure 3-1: Schematic representation of the two main read-outs of the RMA "Apoptosis-1": nuclear fragmentation and caspase-3 activation.

3.1.1 Introduction

There are two main pathways for the induction of apoptosis, the death receptor-mediated pathway, and the mitochondrial pathway. Induction of either pathway will ultimately result in the activation of caspases, a class of intracellular cysteine proteases which are considered to be the central components of the apoptotic response [Hengartner, 2000; Riedl and Shi, 2004].

Here, we describe a high-content imaging apoptosis assay performed on HeLa cells. To experimentally trigger apoptosis in a cell population, we used the cell toxin staurosporine[1] (see the glossary in section 3.1.5). By immunofluorescently labeling the activated caspase-3, a strong increase in fluorescence intensity is observed. In addition, we evaluate nuclear morphological changes connected to apoptosis: the size and fragmentation of the nucleus.

3.1.2 Materials and Methods

CellCarrier 384-well (PerkinElmer #6007920); SilverSeal aluminium foil (greiner-bioone #676090); HeLa cells (DSMZ #ACC-57); RPMI-1640 (Sigma #72400[6]) + 10 % FBS (Sigma #F9665); Trypsin / EDTA (Sigma #T3924); PBS (PAA #H15-002[6]); Staurosporin (Sigma #S5921); Goat Serum (Sigma #9023); 5 mM DRAQ5™ (Biostatus, #DR51000); Cleaved Caspase-3 (Asp175) Antibody, Alexa Fluor® 488 conjugate (Cell Signaling, #9669); 37 % Formaldehyde (Sigma #25,254-9); Triton X-100 (Promega #H5142); Tween® 20 (Sigma #P7949); BSA (Sigma #05473).

Experimental Procedure

- Grow HeLa cells in RPMI-1640 media.
- Plate 6000 cells (in 50 µl growth medium) per well into a 384-well CellCarrier microplate (see ***Figure 3-2***).
- Incubate for 16 h at 37 °C, 5 % CO_2 before adding the apoptosis inducer (staurosporine).
- Remove the growth medium and expose the cells to staurosporine, diluted in growth medium, 25 µl total volume per well (the staurosporine stock solution (5 mM) was prepared in $DMSO_2$, see section 3.1.5)
- Incubate for 4 h at 37 °C, 5 % CO_2
- Caspase-3 labeling:
  - Fix with formaldehyde (3.7 % (v / v), 15 min, room temperature (RT).
  - Permeabilize with Triton-X-100 (0.5 % (v / v)), 5 min, RT.
  - Block for 30 min with 3 % BSA[3] in PBS[4] (section 3.1.5).
  - Add Alexa Fluor® 488 conjugated anti-caspase-3 antibody (diluted to 0.75 µg / ml in 3 % (w / v) BSA in TBS[5]) and incubate overnight at 4 °C.
  - Wash with 0.05 % Tween® 20.
- Stain the nuclei with 10 µM DRAQ5™ for 15 min at RT.

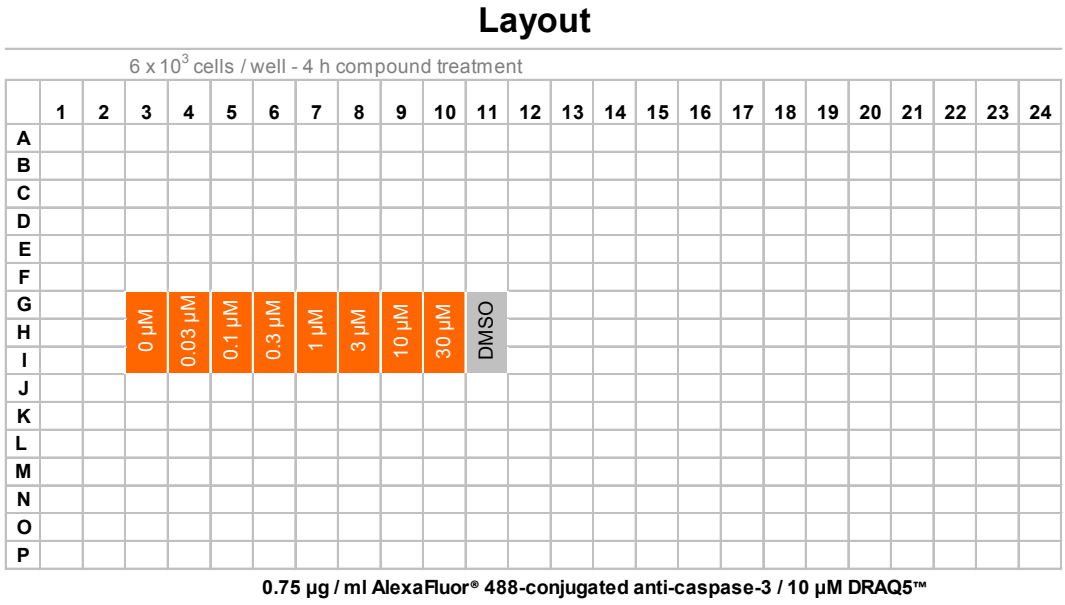


Figure 3-2: Layout for compound treatment and staining.

Image Acquisition

- Use the 20x Air objective
- Open the Harmony **Setup** screen
- Add the DRAQ™ channel and the AlexaFluor® 488 channel to your experiment

- Select the wells that are to be measured (see ***Figure 3-2***) and 4-5 image fields per well to obtain good assay statistics
- Adapt the settings (focus height, exposure time and excitation power) to your samples (see section 2.1.2).
- Switch to the **Run Experiment** screen.
- Optional: Load the pre-defined assay layout “RMA Apoptosis 1”.
- Start the automated plate measurement.

Both channels can be acquired simultaneously if your instrument has more than one camera. Due do to the large spectral separation of the used dyes crosstalk is precluded.

If you are not using the same plate, or plate layout, please refer to chapter 2 for details on how to set-up your experiment.

Dye	Ex./ Em. Max. [nm]	Channel name in Harmony	Readouts
DRAQ5™ (nucleus/ cytoplasm, specific stain for nucleic acids)	647 / 670	DRAQ5™	Loss of cells Nuclear shrinking Nuclear fragmentation
Cleaved-caspase-3 antibody (AlexaFluor® 488 conjugate)	494 / 520	AlexaFluor® 488	Apoptosis activation

Table 3-1: Fluorescence characteristics of each dye used, and the corresponding readouts.

Image Analysis

- Load the analysis sequence “RMS Apoptosis 1” from the Harmony database.
- Select the **Find Nuclei** building block and check the illustration for correct detection of the nuclei. Adjust the tuning parameters if needed. See Image Analysis Guide, section 1.4 “Optimizing Image Analysis (on Single Images)” for a more general description of parameter optimization. Also refer to the RMS “Nuclear Fragmentation” (Image Analysis Guide, section 2.13) for specific hints on detecting fragmented nuclei. Make sure that clusters of fragments are detected as one nucleus.
- Adjust the threshold for caspase-3 positive cell classification in the **Select Population** building block. See the RMS “Quantification of Cytoplasmic Marker ” (Image Analysis Guide, section 2.6) for an example and specific hints.

The ready-made analysis sequence “RMS Apoptosis 1”contains the following building blocks:

Image Analysis: RMS Apoptosis 1		
Input Images	DRAQ5™ nuclear stain channel	AlexaFluor® 488 Caspase-3 channel

Image Segmentation	
Find Nuclei	Detects Nuclei in the DRAQ5™ channel

Definitions of Regions of Interest	
Select Cell Region	Define an enlarged region of interest (ROI) in the DRAQ5™ channel by setting the outer border of the nuclear region to -80 % and the inner border to 100 % (see Image Analysis Guide, section 2.11.3). This region (**MarkerROI**) will later be used to quantify the intensity of the AlexaFluor® 488 detection channel
Select Region	Define a shrunken region of the nuclei (**NucFragROI**) by scaling down the outer border by 2 pixels (px)

Quantifying Properties in Regions	
Calculate Intensity Properties	Use the **MarkerROI** to quantify properties of the marker channel (caspase-3): – Mean Intensity of the marker – Standard Deviation of the marker – Median Intensity of the marker
Calculate Intensity Properties (2)	Quantify the properties of the nucleus, using **NucFragROI** as a region in the nuclear channel: – Coefficient of Variance (CV) Intensity – Sum Intensity
Calculate Morphology Properties	Quantify the properties of the nucleus using the standard Nuclear region: – Area of a nucleus

Select Subpopulation	
Select Population	Apply a threshold to find a population which has a high mean marker intensity (to be adjusted). This population is defined as 'apoptotic cells'.

Calculate Readout Values	
Define Results	Select and calculate values to report back as the results for each well

Readout Values	
Number of Cells	Equivalent to the number of nuclei in the DRAQ5™ channel
Fragmentation Index	Corresponds to the **NucFragROI** Intensity coefficient of variation which measures the degree of fragmented nuclei. The higher this index, the more apoptotic cells are present
Mean Area of nuclei	Average detected nuclear area, in μm^2
Mean DNA concentration	Corresponds to the integrated intensity (sum intensity) of the nuclear channel, and correlates to the DNA content
Caspase-3: Mean Intensity	Average intensity of objects found in the marker channel
Percentage of apoptotic cells	Corresponds to the ratio of apoptotic cells and the number of detected nuclei $= \frac{Caspase\ positive\ cells}{Total\ number\ of\ cells} \cdot 100$

3.1.3 Example Results

This section outlines the results of an example measurement to analyze the influence of three different compounds on HeLa cells.

Six parameters were analyzed simultaneously:

- Cell count
- Fragmentation index
- Nuclear size and intensity
- Caspase-3 intensity
- Percentage of apoptotic cells

Caspase-3 Detection in Apoptotic Cells

A strong increase in cytoplasmic fluorescence intensity, connected to the activation of caspase-3, can be observed and translated into numerical data as shown in the dose-response curve in ***Figure 3-4***.

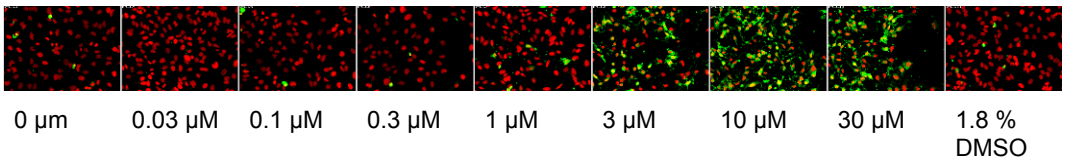


Figure 3-3: Assembly of images taken from one selected subfield per well. The two channels were merged and artificially colored red (nuclei) and green (caspase-3 emission). HeLa cells were exposed to different concentrations of staurosporine for 4 h, and exhibited a dose-dependent response to this inducer. DMSO did not have any effects at the highest concentration used in this experiment. Images were captured with a 20x long WD objective on the Operetta system.

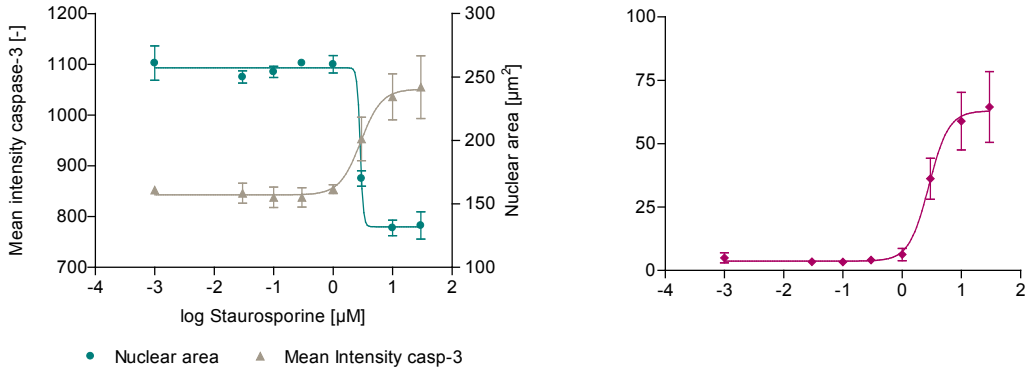


Figure 3-4: Left: The graph shows the staurosporine- dose-response curve. With increasing staurosporine concentration the mean caspase-3 intensity also increases. The typical nuclear morphological changes (pyknosis and karyorrhexis) are accompanied by a decrease in nuclear size starting at a staurosporine concentration of ~ 1 µM. Right: In order to quantify cells according to their apoptotic state, a threshold with respect to caspase-3-related fluorescence intensity was applied to the data (the threshold was variable and was adapted according to the intensity results of individual experiments, here 600). In this way the subpopulation of apoptotic cells was classified, and the resulting numerical readout was called the "percentage of apoptosis positive cells". Based on these percentages, an EC_{50} of 2.82 µM for staurosporine was calculated, using the Prism software to fit the curve.

Analysis of Nuclear Fragmentation upon Apoptosis Induction

The apoptotic state of the cell population was validated using another important and recognized morphology-based apoptotic marker – the degree of nuclear fragmentation. This is an indirect measurement deduced from the intensity fluctuation of the nuclear stain. The nuclear fragmentation index will be evaluated in terms of the CV value (coefficient of variation, standard deviation divided by mean value). The index increases with increasing nuclear fragmentation and therefore serves as an indirect indicator (***Figure 3-6***).

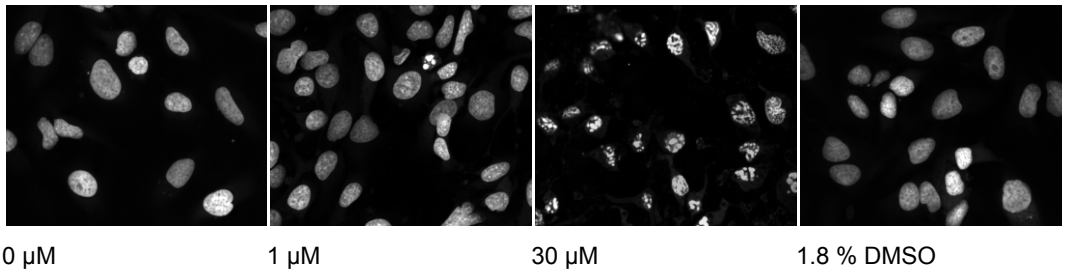


Figure 3-5: Nuclei of HeLa cells after treatment with different concentrations of staurosporine for 4 h. Significant levels of karyorrhetic nuclei become increasingly visible as the concentration of staurosporine is increased. The degree of fragmentation is measured as intensity variance due to the high fluctuation of the nuclear stain.

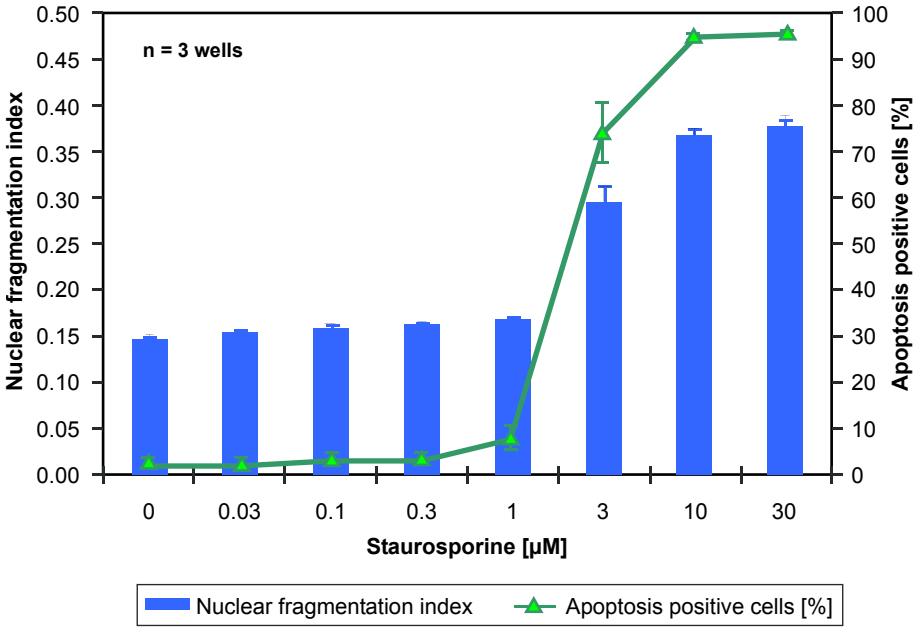


Figure 3-6: Validation of apoptosis in staurosporine treated HeLa cells using a combination of the two apoptotic markers. The switch of the cell population to a highly apoptotic state is confirmed by matching the curves generated by the morphological readout (the nuclear fragmentation) to a biochemical one (the level of cytoplasmic active caspase-3).

3.1.4 Hints and Tips

- The use of a direct fluorophore conjugated antibody for caspase-3 makes the assay more automation-friendly (less incubation and wash steps) and can increase signal specificity.
- It is easy to multiplex this with additional apoptotic markers, such as Bax or Bak, or MitoTracker®.

3.1.5 Glossary

[1] Staurosporine is a broad spectrum protein kinase inhibitor [Rueggs and Burgess, 1989].

[2] DMSO: Dimethyl Sulfoxide

[3] BSA: Bovine Serum Albumin

[4] PBS: Phosphate Buffered Saline

[5] TBS: Tris Buffered Saline, pH 7.5

[6] These products are no longer commercially available from the designated suppliers. They might still be obtained from alternative suppliers. Please note that the protocol might have to be adjusted accordingly.

3.1.6 References

Hengartner, M.O. (2000) "The biochemistry of apoptosis". Nature 407, pp 770-776.

Riedl, S. and Shi, Y. (2004) "Molecular Mechanisms of Caspase Regulation During Apoptosis". Nature Cell Biology 5, pp 897-907.

Rueggs, U.T. and Burgess, G.M. (1989) **"**Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases". Trends Pharmacol. Sci. 10, pp 218-220.

Zhang, J.H., Chung, T.D. and 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.

3.2 Ready-Made Application: Cytotoxicity-1

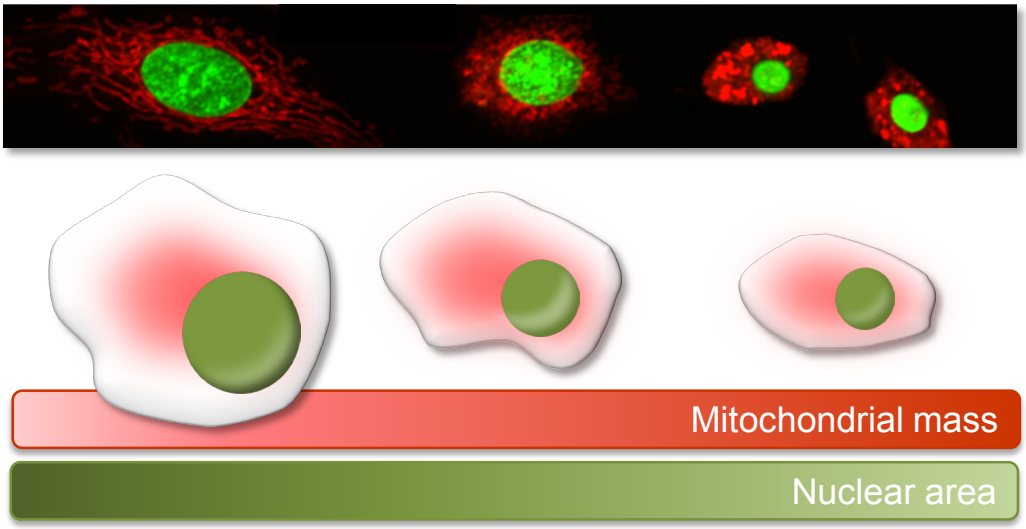


***Figure 3-7*:** Schematic representation of the two main read-outs of the RMA "Cytotoxicity-1": mitochondrial mass and nuclear area.

3.2.1 Introduction

The ability to measure early indicators of toxicity is an essential part of drug discovery. *In vitro* cytotoxicity assays involving tissue specific cell cultures are considered valuable predictors of human drug toxicity. The liver, as a primary organ for drug metabolism, is a target organ for many toxic effects therefore *in vitro* cellular cytotoxicity studies focus on human hepatocytes [O'Brien *et al., 2006*].

Here, we describe a rapid and flexible live dye-based high content cytotoxicity assay performed using HepG2 (human hepatocellular carcinoma) cells. In order to study the *in vitro* cytotoxicity, cells were treated with three different **compounds:** Tacrine[1], FCCP[2] and Acetaminophen[3] (see the glossary in section 3.2.5), and were measured and analyzed for characteristic morphological changes: nuclear shrinkage, cell membrane disruption, mitochondrial dysfunction and cell-loss.

A dye cocktail containing three fluorescent **organelle dyes** was used:

- **Hoechst 33342** is a popular cell-permeant nuclear dye suitable for live-cell staining. Using the Hoechst stain, we analyzed loss of cells, nuclear intensity and nuclear shrinkage.
- The carbocyanine dimer **BOBO™-3** is a cell-impermeant nuclear dye which binds specifically to double-stranded DNA. It is used to assess cell membrane disruption since it only enters cells with disrupted cell membranes.
- **MitoTracker® Deep Red** is used to determine changes in mitochondrial mass. This cell-permeant organelle dye accumulates in the matrix of metabolically active mitochondria.

3.2.2 Materials and Methods

CellCarrier 384-well Collagen I coated (PerkinElmer #6007920); SilverSeal aluminium foil (greiner-bioone #676090); HepG2 cells (DSMZ #ACC-180); DMEM-F12 (Sigma #D6421)+ 10 % FBS (Sigma #F9665) + 2 mM L-glutamine (Sigma #G7513); Trypsin / EDTA (Sigma #T3924); PBS (PAA #H15-002)[4]; Tacrine (Sigma #A3773); FCCP (Tocris #0453); Acetaminophen (Sigma #A5000); Hoechst 33342 (Invitrogen #H1399); BOBO™-3 (Invitrogen #B3586); MitoTracker® Deep Red (Invitrogen #M22426).

Experimental Procedure

- Grow HepG2 cells in DMEM-F12 media supplemented with 10 % FBS and 2 mM L-Glutamine.
- Plate low-passage (5-20) HepG2 cells in the Collagen I coated 384-well CellCarrier microplates at a density of 4000 cells per well.
- Incubate for 16 – 24 h at 37 °C, 5 % CO_2.
- Remove the growth medium and expose the cells to the appropriate compound, diluted in growth medium (50 µl total volume per well).
- Incubate for 24 h at 37 °C, 5 % CO_2.
- Add 25 µl of a pre-mixed dye-cocktail to each well. The dye cocktail contains:
  - 3 µM Hoechst 33342 (final concentration 1 µM).
  - 2.25 µM Bobo™-3 (final concentration 0.75 µM).
  - 0.9 µM MitoTracker® Deep Red (final concentration 0.3 µM).
- Incubate for a further 45 min at 37 °C, 5 % CO_2.
- Perform the measurement immediately.

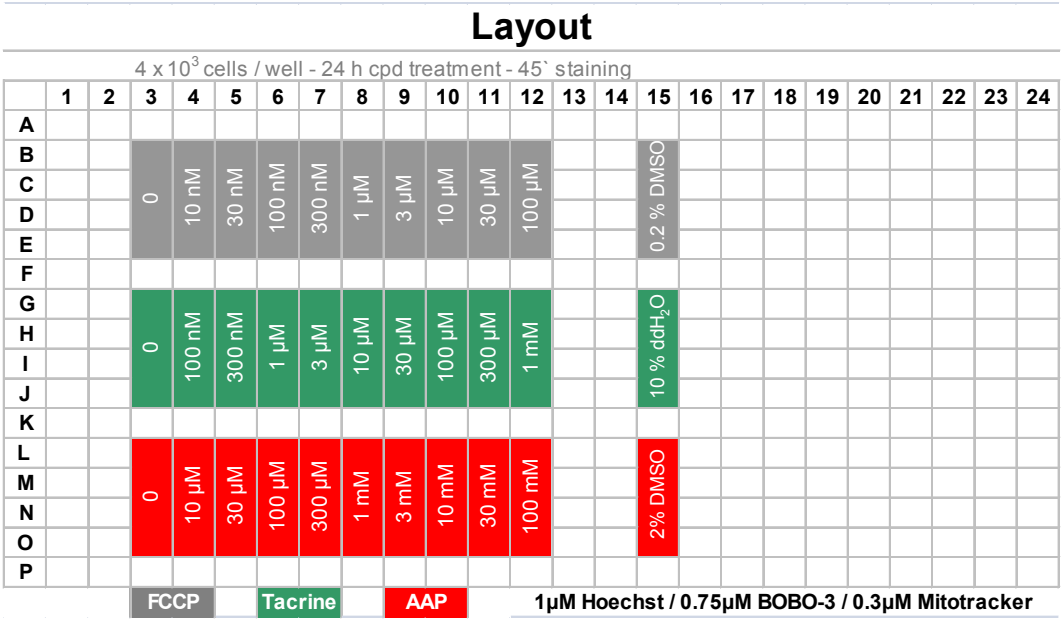


Figure 3-8: Layout for compound treatment and staining.

Image Acquisition

- Use the 20x Air objective
- Open the Harmony **Setup** screen
- Add the HOECHST33342 channel, the BOBO™-3 channel and the MitoTracker® Deep Red channel to your experiment
- Select the wells that are to be measured (see ***Figure 3-8***) and one image field per well to obtain good assay statistics
- Adapt the settings (focus height, exposure time and excitation power) to your samples (see section 2.1.2).
- Switch to the **Run Experiment** screen.
- Optional: Load the pre-defined assay layout “RMA Cytotoxicity 1”.
- Start the automated plate measurement.

Due to the large spectral overlap there can be a significant crosstalk from the BOBO-3™ channel into the MitoTracker® Deep Red FM channel during simultaneous acquisition. This crosstalk can be minimized by acquiring images in confocal mode (see section 1.5 for details). Alternatively, sequential image acquisition will always preclude any crosstalk but will result in longer measurement times overall.

If you are not using the same plate, or plate layout, please refer to chapter 2 for details on how to set-up your experiment.

Dye	Ex./ Em. Max. [nm]	Channel name in Harmony	Readouts
Hoechst 33342 (nucleus / DNA)	350 / 461	HOECHST 33342	Loss of cells Nuclear shrinkage
BOBO™-3 iodide (nucleus / DNA)	570 / 602	BOBO-3™	Cell membrane disruption
MitoTracker® Deep Red FM (mitochondria)	635 / 690	MitoTracker® Deep Red	Changes in mitochondrial mass

Table 3-2: Fluorescence characteristics of each dye used, and the corresponding readouts.

Image Analysis

- Load the analysis sequence “RMS Cytotoxicity 1” from the Harmony database.
- Select the **Find Nuclei** building block and check the illustration for correct detection of nuclei. Adjust the tuning parameters if needed. See Image Analysis Guide, section 2.4 “Optimizing Image Analysis (on Single Images)” for a more general description of parameter optimization.
- Select the **Find Cytoplasm** building block and check the illustration for correct cytoplasmic detection results. Adjust if needed (see Image Analysis Guide, section 2.4).
- Adjust the threshold for detection of BOBO™-3 stained cells in the **Select Population** building block. Use the illustration and the table of properties provided by the building block (see also Image Analysis Guide, section 2.3.3).

The ready-made analysis sequence "RMS Cytotoxicity 1" contains the following building blocks:

Image Analysis			
Input Images	MitoTracker® Deep Red channel	BOBO™-3 nuclear channel (= Dead)	HOECHST 33342 nuclear channel

Image Segmentation	
Find Nuclei	Detects nuclei in the HOECHST 33342 channel
Find Cytoplasm	Detects cytoplasm / mitochondria in the MitoTracker® Deep Red channel

Quantify Properties in Regions	
Calculate Intensity Properties	Calculates the fluorescence intensity of the mitochondria, based on the cytoplasmic outlines in the MitoTracker® Deep Red channel
Calculate Intensity Properties (2)	Calculates the mean fluorescence intensity of the HOECHST 33342 stained nuclei
Calculate Intensity Properties (3)	Calculates the mean fluorescence intensity of the BOBO-3™ stain within the detected nuclei
Calculate Morphology Properties	Calculates the area of the detected nuclei

Identify Subpopulations	
Select Population	Removes nuclei which are not completely visible in the image
Select Population (2)	Identifies nuclei with a specified fluorescence intensity for the BOBO™-3 stain

Calculate Readout Values	
Define Results Select and calculate values to report back as the result for each well	List of Outputs: Number of Nuclei Mitochondria Intensity Nuclear Area Formula Output: Calculates "Membrane permeability [%]" $= \frac{Number\ of\ Dead\ Cells}{Sum\ of\ Live\ \&\ Dead\ Cells} \cdot 100$

Readout Values	
Cell Count	Corresponds to the number of detected nuclei in the Hoechst 33342 stain channel (border cells excluded)
Nuclei Area (Mean + StdDev)	Corresponds to the size of the detected nuclei in the Hoechst 33342 stain channel (border cells excluded)
Mitochondrial mass (Mean + StdDev)	Corresponds to the mean fluorescence intensity of the mitochondria (border cells excluded)
Membrane permeability	Corresponds to the ratio of the number of detected dead nuclei to all nuclei

3.2.3 Example Results

This section outlines the results of an example measurement in which the effect of three different compounds on HepG2 cells was investigated.

Four parameters were analyzed simultaneously:

- Cell count
- Nuclear size
- Mitochondrial mass
- Cell permeability

Effects of the compounds on mitochondrial mass and cell count

The way that mitochondria respond to toxic impact depends on the type of compound being investigated, its concentration and the specific mitochondrial function that is affected. Mitochondrial dysfunctions have differing effects. We observed an enhanced biogenesis of mitochondria, caused by an increase in mitochondrial respiration, after treatment with FCCP, Tacrine and AAP. One of the most sensitive indicators of cell stress is the determination of the live cell count [O'Brien *et al., 2006*]. We observed a significant loss in the number of live cells with increasing concentrations of FCCP, Tacrine and Acetaminophen.

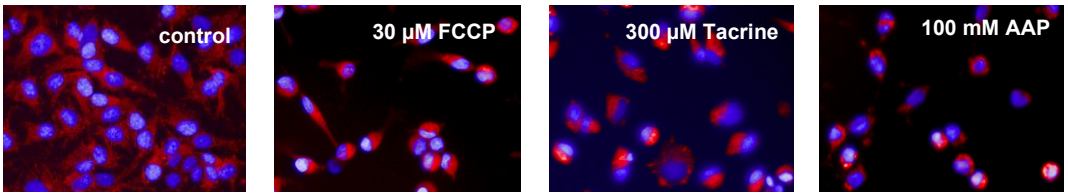


Figure 3-9: False color overlay (blue-nuclei, red-mitochondria) of untreated and FCCP, Tacrine and AAP treated cells. The images show compound-induced changes in phenotype and mitochondrial signal as well as a loss of cells. The 24 h compound treatment of HepG2 cells resulted in an enhanced biogenesis of mitochondria and an increase in mitochondrial mass. A reduction in nuclear size and a loss of cells due to the toxic impact was also observed. The images were acquired on the Operetta using the 20x LWD objectives.

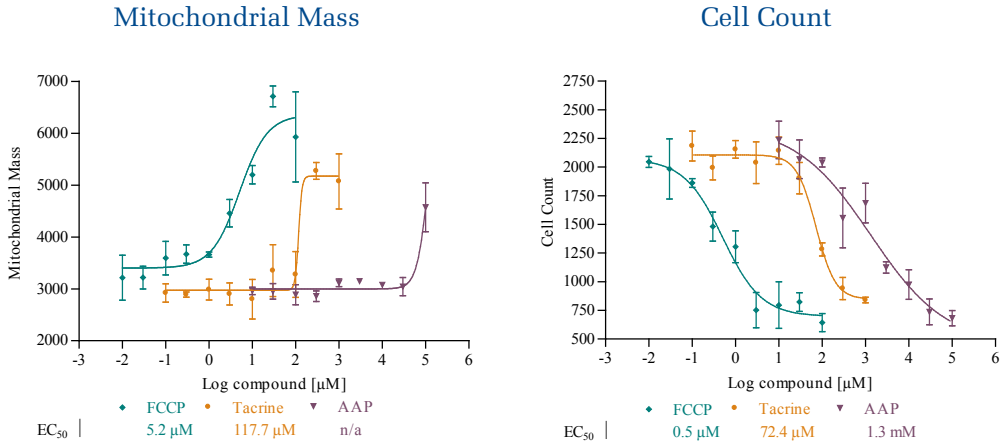


***Figure 3-10*:** FCCP, Tacrine and Acetaminophen dose-response curves, determined from the mitochondrial mass (left) and live cell count (right). N = 4 wells.

Effects of the compounds on cell permeability and nuclear size

Cell membrane disruption usually occurs in the late stages of cytotoxicity and is a common indicator of cell death. FCCP and Tacrine treated HepG2 cells show a significant increase in cell membrane permeability as the concentration of each compound is increased, whereas the Acetaminophen treated cells show marginal cell membrane disruption in response to increasing concentrations.

Nuclear shrinkage due to toxic impact typically occurs with cell injury. The typical cytotoxic effect is a reduction in nuclear size with increasing concentrations of the compound, as the results of the dose-response curves with FCCP and Tacrine demonstrate (***Figure 3-12***). When HepG2 cells were treated with Acetaminophen, an initial swelling of the nuclei was observed, due to compound induced necrosis, prior to nuclear shrinkage [Mirochnitchenko *et al.*, 1999].

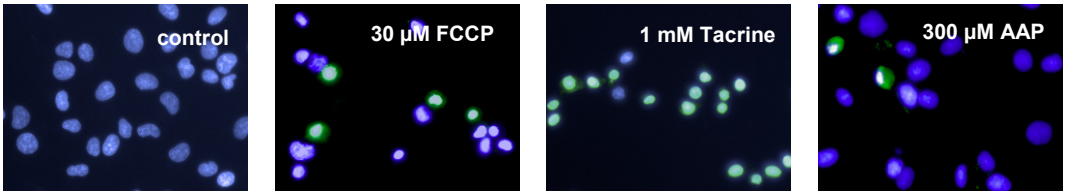


Figure 3-11: False color overlay of untreated and compound-treated cells showing Hoechst (blue) and BOBO™-3 (green) stained nuclei. Cells with intact membranes show Hoechst staining only (control). Loss of membrane integrity allows the cell-impermeant dye BOBO™-3 to enter the cells and bind to the DNA (compound treated cells).

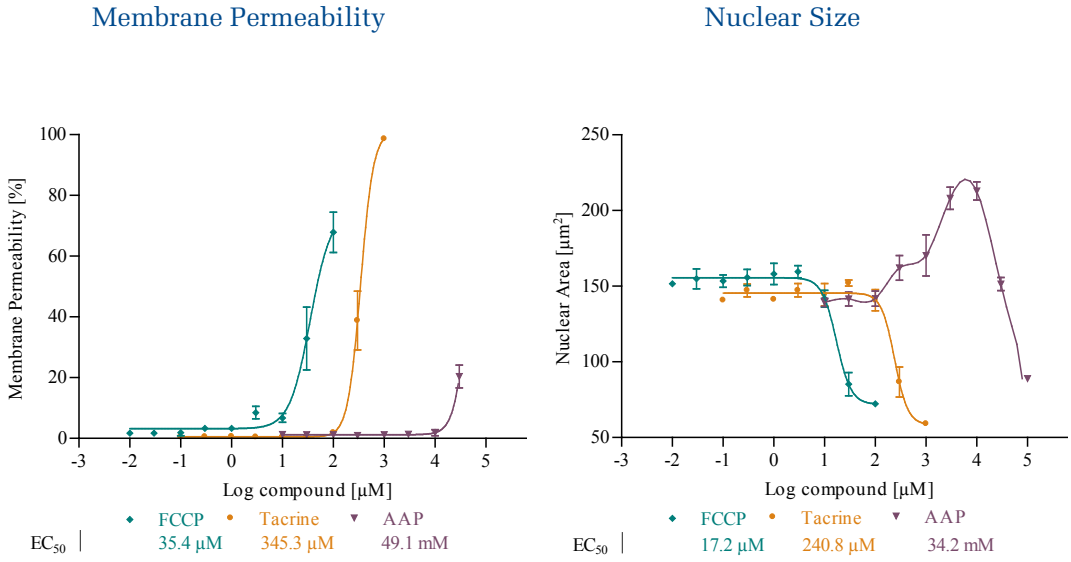


Figure 3-12: FCCP, Tacrine and Acetaminophen-generated dose-response curves deduced from membrane permeability (left) and nuclear size (right). N = 3 wells.

3.2.4 Hints and Tips

- HepG2 cells grow more evenly and more homogenously on coated plates. We used the PerkinElmer CellCarrier™ microplate precoated with Collagen I.
- By applying a pre-mixed dye-cocktail we reduced the number of steps in the protocol to the minimum.
- The organelle dye MitoTracker® Deep Red can be replaced by any organelle dye that is compatible with the fluorescent characteristics of the remaining dyes, e.g. LysoTracker® Probes.

3.2.5 Glossary

[1] Tacrine is a parasympathomimetic and centrally acting cholinesterase inhibitor (anticholinesterase) used in the treatment of Alzheimer`s disease. The cytochrome P450 is the principle isozyme involved in the metabolism of Tacrine in the liver, and results in an active metabolite which is associated with a high frequency of hepatotoxicity [Galisteo *et al., 2000*].

[2] P-fluoromethoxyphenylhydrazone, better known as FCCP, is a very potent uncoupler of oxidative phosphorylation in mitochondria. This compound acts by degrading the linkage between respiratory chain and the phosphorylation system used to generate ATP [Niethammer *et al., 2008*].

[3] Acetaminophen (or Paracetamol) is a widely-used analgesic (painkiller) and antipyretic and is well-known to cause potentially fatal liver damage and hepatic necrosis if overdosed. The toxic effect of Acetaminophen is primarily due to a highly-reactive intermediary metabolite, NAPQI. NAPQI is metabolized via the hepatic cytochrome P450 enzyme system and at normal doses is quickly detoxified by conjugation with gluthathione [James *et al., 2003*].

[4] These products are no longer commercially available from the designated suppliers. They might still be obtained from alternative suppliers. Please note that the protocol might have to be adjusted accordingly.

3.2.6 References

Galisteo, M. et al. (2000) “Hepatotoxicity of Tacrine: Occurrence of Membrane Fluidity Alterations without Involvement of Lipid Peroxidation”. The Journal of Pharmacology and Experimental Therapeutics 294, pp 160-167.

James, L.P., Mayeux, P.R., Hinson, J.A. (2003) “Acetaminophen-Induced Hepatotoxicity”. Drug Metabolism and Disposition 31, pp 1499-1506.

Mirochnitchenko, O. *et al.*, (1999) “Acetaminophen Toxicity”. The Journal of Biological Chemistry 274 (15), pp 10349-10355.

Niethammer, P., Kueh, H.Y. and Mitchison, T.J. (2008) “Spatial Patterning of Metabolism by Mitochondria, Oxygen, and Energy Sinks in a Model Cytoplasm”. Current Biology 18, pp 586-591.

O’Brien ,P. and Haskins, J.R. (2006) “In Vitro Cytotoxicity Assessment”. Book Title: High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery. Methods in Molecular Biology 356, pp 415-425.

3.3 Ready-Made Application: Cell Cycle-1

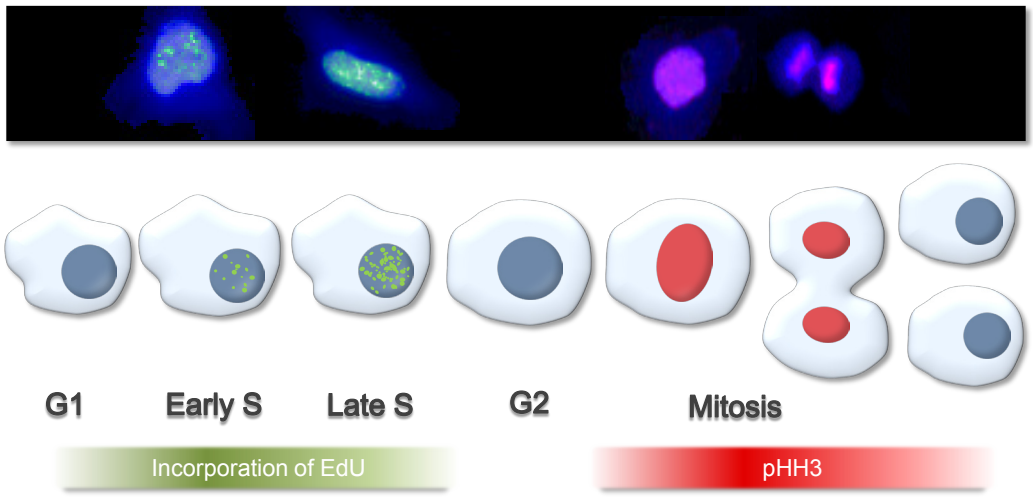


Figure 3-13: Schematic representation of the two main read-outs of the RMA "Cell Cycle-1": incorporation of EdU and pHH3 detection.

3.3.1 Introduction

One of the most important aspects of anti-cancer treatments is the inhibition of cell proliferation and cell division. Both events can be analyzed using HCS approaches by multiplexing cell cycle-specific cellular targets (Gasparri *et al.,* 2006). One novel and powerful technique is EdU staining, which detects the S-phase of the cell cycle through incorporation of the nucleoside analog Uridine into newly synthesized DNA strands (Invitrogen™, Click-iT™ EdU kit). Furthermore, there are well validated protein markers available that are associated with certain cell-cycle phases. One example is the phosphorylated histone H3 (pHH3), which is a common M-phase marker.

In this application, we describe an image-based high content analysis which combines these two specific cell cycle markers, and supplies a user-friendly, novel solution for the precise classification and quantification of cells in S- and M-phase.

3.3.2 Material and Methods

CellCarrier-384 (PerkinElmer #6007550); SilverSeal aluminium foil (greiner-bioone #676090); CASY Model TT Cell Counter (Innovatis #40006); HeLa cells (Sigma #93021013); RPMI1640 (Gibco #72400) + 10 % FBS heat inactivated (Sigma #F9665); Trypsine (Sigma #T3924); Nocodazole[2] (Sigma #M1404); Thymidine[1] (Sigma #T1895); 37 % Formaldehyde (Sigma #25,254-9); PBS (Gibco #14040-091); NH_4Cl (Merck #11450500)[3]; Triton X-100 (Promega #H5142); Tween® 20 (Sigma #P7949); BSA (Sigma #A3059); Monoclonal Anti-phospho-Histone H3 (pSer28) antibody produced in rat (Sigma #H9908); Alexa Fluor® 647 goat anti-rat IgG (Invitrogen #A21247); Click-iT™ EdU Alexa Fluor® 488 Assay kit (Invitrogen #A10027)[3]; Hoechst 33342 (Invitrogen #H1399).

Experimental Procedure

- Culture HeLa cells in RPMI medium + 10 % FBS.
- Seed cells (50 µl final volume) in the CellCarrier-384 tissue culture plates at a density of 5000 cells per well (for the 6 h compound incubation) and 2500 cells per well (for the 18 – 30 h compound incubation) and incubate overnight at 37 °C and 5 % CO_2 (see the plate layout in ***Figure 3-14***).
- Remove the growth medium and expose the cells to various concentrations of the compound (diluted in growth medium), for 6 – 30 h at 37 °C and 5 % CO_2 (50 µl total volume in each well).
- Add an equivalent volume (50 µl) of EdU (25 µM final concentration) in complete culture medium 30 min prior to fixation, and incubate at 37°C and 5 % CO_2.
- Fix the cells by incubating for 20 min with 4 % formaldehyde at room temperature (RT) and permeabilize the cells with 0.5 % Triton X-100 for 5 min at RT as described in the Standard Protocols section in the Appendix (page 169).
- Remove the supernatant and incubate with 50 µl Click-iT™ reaction cocktail for 30 min at RT in the absence of light. Then block with 3 % BSA for 20 min at RT or overnight at 4 °C).
- For histone staining, incubate with 2 µg / ml of anti-pHH3 primary antibody for 1 h at RT in the absence of light. Afterwards, incubate with 10 µg / ml of secondary AlexaFluor® 647 antibody for 1 h at RT in the absence of light. Wash the cells three times with 50 µl 0.05 % Tween® 20 in PBS prior to and after the secondary antibody incubation.
- For nuclei staining, incubate the cells with 16.2 µM Hoechst 33342 for 30 min at RT in the dark.

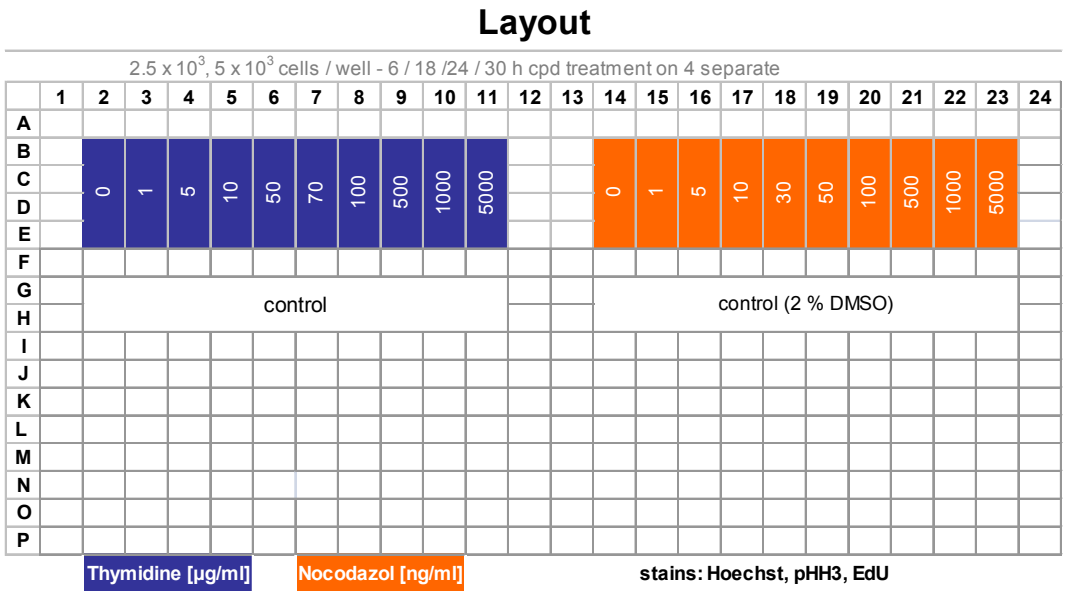


Figure 3-14: Layout for compound treatment and staining.

Image Acquisition

- Use the 20x Air objective
- Open the Harmony **Setup** screen
- Add the HOECHST33342 channel, the AlexaFluor® 488 channel and the AlexaFluor® 633 channel to your experiment
- Select the wells that are to be measured (see ***Figure 3-14***) and 4-5 image fields per well to obtain good assay statistics
- Adapt the settings (focus height, exposure time and excitation power) to your samples (see section 2.1.2).
- Switch to the **Run Experiment** screen.
- Optional: Load the pre-defined assay layout “RMA Cell Cycle 1”.
- Start the automated plate measurement.

Due to the broad emission spectrum of HOECHST33342 there can be significant crosstalk from the HOECHST33342 channel into the AlexaFluor® 488 channel during simultaneous acquisition. This crosstalk can be minimized by acquiring images in confocal mode (see section 1.5 for details). Alternatively, sequential image acquisition will always preclude any crosstalk but will result in longer measurement times overall.

If you are not using the same plate, or plate layout, please refer to chapter 2 for details on how to set-up your experiment.

Dye	Ex./ Em. Max. [nm]	Channel name in Harmony	Readouts
Hoechst 33342 (nucleus / DNA)	350 / 461	HOECHST 33342	Loss of cells Nuclear shrinkage
Alexa Fluor® 488 (EdU)	490 / 519	AlexaFluor® 488	S-phase cells
Alexa Fluor® 647 (pHH3)	653 / 669	AlexaFluor® 633	M-phase cells

Table 3-3: Fluorescence characteristics of each dye used, and the corresponding readouts.

Image analysis

- Load the analysis sequence “RMS Cell Cycle 1” from the Harmony database.
- Select the **Find Nuclei** building block and check the illustration for correct detection of the nuclei. Adjust the tuning parameters if needed. See Image Analysis Guide, section 1.4 “Optimizing Image Analysis (on Single Images)” for a more general description of parameter optimization.
- Interactively set the thresholds for “pHH3-positive” and “EdU-positive” cell classification in the respective **Select Population** building blocks using the illustration and the single cell results table that is provided by the building block.

- Set the threshold for "High DNA content" classification in the **Select Population** building block: Run the analysis for one well, export the single cell results and create a scatter plot to determine the optimal threshold value. See the RMS "Cell Cycle Classification" (Image Analysis Guide, section 2.14.3).

The ready-made analysis sequence "RMS Cell Cycle 1" contains the following building blocks:

Image Analysis: RMS Cell Cycle 1			
Input Images	HOECHST 33342 nuclear channel	AlexaFluor® 488 EdU channel	AlexaFluor® 633 pHH3 channel
Image Segmentation			
Find Nuclei	Nuclear detection on the nuclear stain image		
Quantify Properties in Regions			
Calculate Intensity Properties	Calculates the mean and sum fluorescence intensity of the DNA stain in the detected nuclei		
Calculate Intensity Properties (2)	Calculates the mean fluorescence intensity of the EdU marker stain in the detected nuclei		
Calculate Intensity Properties (3)	Calculates the mean fluorescence intensity of the pHH3 marker stain in the detected nuclei		
Calculate Morphology Properties	Calculates the area of the individual detected nuclei		
Identify Subpopulations			
Select Population	Removes nuclei which are not completely visible in the image		
Select Population (2)	Identifies nuclei with a specified fluorescence intensity for the pHH3 Marker stain		
Select Population (3)	Identifies nuclei with a specified fluorescence intensity for the EdU Marker stain		
Select Population (4)	Identifies nuclei with a high DNA content (greater than the specified threshold)		
Calculate Readout Values			
Define Results	Selects and calculates values to report back as the analysis results for each well		
Readout Values			
Number of Cells	Corresponds to the number of nuclei in the well which are completely visible in the image		
Fraction of Cells with High DNA content	Corresponds to the fraction of G2- / S-phase and early M-phase cells $= \frac{\text{number of nuclei with high DNA content}}{\text{total number of nuclei}} \cdot 100$		
Mean Nuclear Area	Average size of the nuclei		
Fraction of cells in S-phase	Corresponds to EdU positive cells $= \frac{\text{number of EdU positive nuclei}}{\text{total number of nuclei}} \cdot 100$		

Mean intensity of EdU marker	Average fluorescence intensity of the EdU marker in nuclei (EdU positive cells only)
Fraction of cells in M-phase	Corresponds to the pHH3 positive cells $= \frac{\text{number of pHH3 positive nuclei}}{\text{total number of nuclei}} \cdot 100$
Mean intensity of pHH3 marker	Average fluorescence intensity of the pHH3 marker stain in the nuclei (pHH3 positive cells only)

3.3.3 Example Results

Three parameters were analyzed simultaneously:

- pHH3 detection (= M-phase).
- EdU incorporation (= S-phase).
- Cell count.

To study inhibition of the cell cycle, cells were treated with two compounds, Thymidine[1] and Nocodazole[2] (see the glossary in section 3.3.5), for 6 h, 18 h, 24 h and 30 h. Upon incubation of cells with these compounds, the cell cycle will arrest at the G1/S and G2/M checkpoints respectively. The effects of this inhibition were analyzed using the following three fluorescent stains:

- EdU, a nucleoside analog which incorporates into DNA during S-phase. Labeling of the incorporated EdU was performed with an Alexa Fluor® 488 dye from the Click-iT™ EdU Alexa Fluor® HCS Assay Kit supplied by Invitrogen™.
- pHH3 was used as an M-phase marker and is detected via immunofluorescence, using the Alexa Fluor® 647 antibody.
- **Hoechst 33342** is a nuclear stain that was used to calculate the mean and sum fluorescence intensity of the nuclei, as well as the nuclear area as a control.

Typical images of non-treated HeLa cells that are positive for EdU and pHH3 (marking specific phases of the cell cycle), are shown in ***Figure 3-15***.

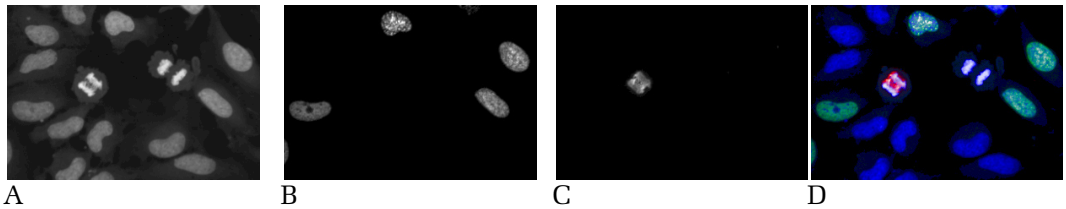


Figure 3-15: Operetta Images (20x magnification) acquired from A – Hoechst channel, B – EdU channel and C – pHH3 channel. Panel D is a color overlay showing cells that are positive for EdU (green), positive for pHH3 (red) or negative for both signals (blue).

Image-based quantification of cell cycle inhibitory compounds

We used a dose-response series of Thymidine and Nocodazole to examine their specific effects in S-phase (Thymidine) or mitosis (Nocodazole), through their influence on the number of EdU / pHH3 positive cells.

Figure 3-16 shows the results of the dose-response series after 18 h of incubation with each compound. The results are based on the image analysis.

Thymidine was shown to decrease the number of HeLa cells that are positive for EdU in S-phase from 32 % to 0 %. It did not affect the mitotic index which is approximately 2 %. Nocodazole is a mitosis inhibiting compound, and increased the number of pHH3 positive cells, increasing the mitotic index from ~ 2 % to ~ 28 %.

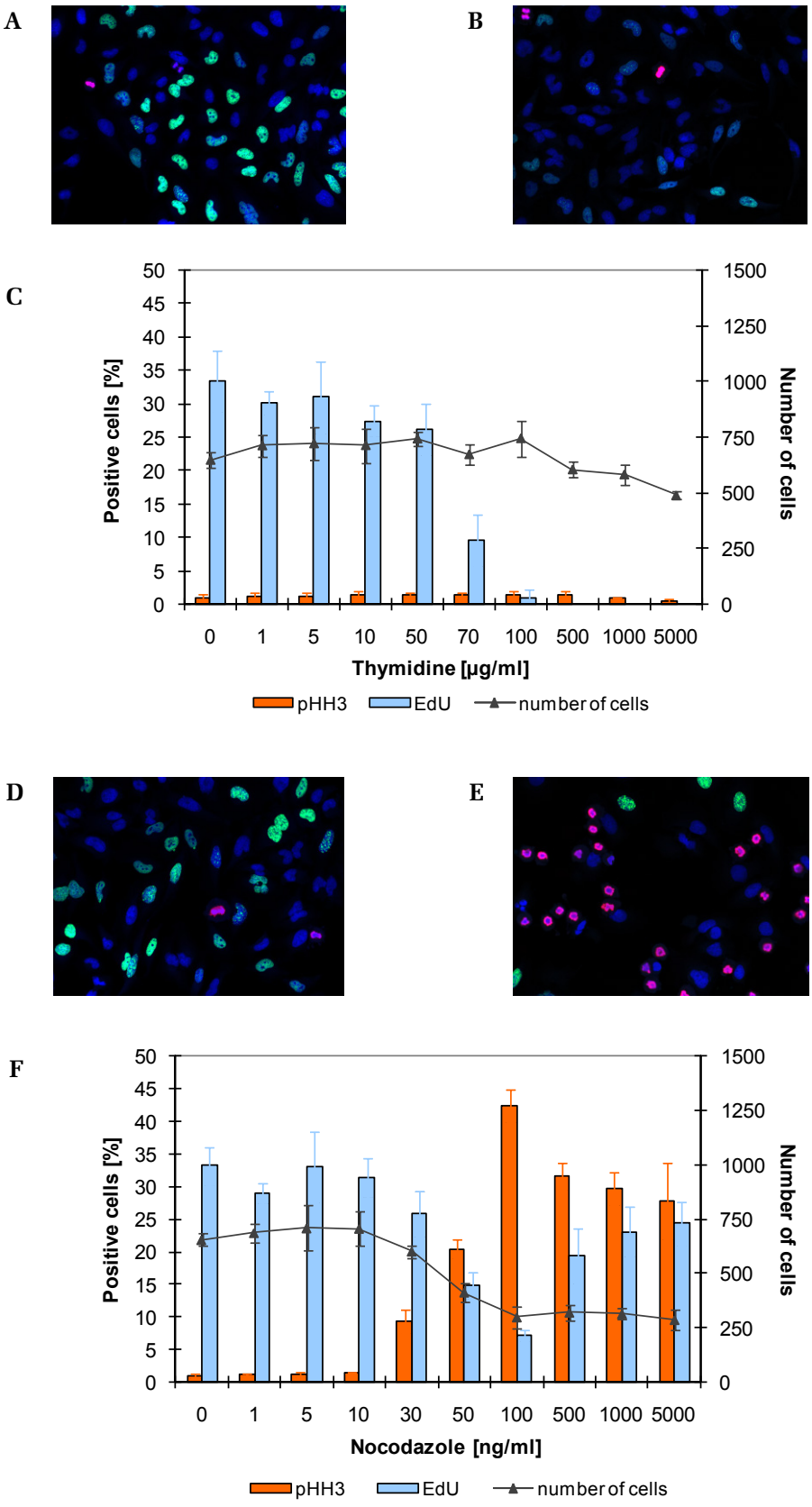


Figure 3-16: False color overlay (green – EdU, red – pHH3 and blue – DNA) of all channels after an 18 h incubation with either Thymidine (A – 0 µg/ml, B – 500 µg/ml) and Nocodazole (D – 0 ng/ml, E – 500 ng/ml). The images show a significant dose-dependent decrease of the EdU signal in response to Thymidine treatment (panels A and B), and a dose-dependent increase of the pHH3 signal in response to Nocodazole. The corresponding Harmony data analysis (panel C – for Thymidine and panel F – for Nocodazole) shows the percentage of cells positive for EdU (representing cells in S-phase) in blue, and the percentage of cells positive for pHH3 (= the mitotic index) in orange. Additionally, a decrease in cell number upon treatment with each compound is shown (black lines in C and F). N=4.

Influence of Thymidine concentrations on the EdU and pHH3 intensities

Figure 3-17 shows the influence of Thymidine concentration on the EdU / pHH3 signals. Thymidine not only suppresses the incorporation of Uridine, which means a depletion of the number of EdU positive cells (as previously shown), it also decreases its signal intensity. We found that the EdU signal intensity is dose-dependent, whereas the pHH3 signal is not. This was expected since Thymidine does not cause cells to arrest in G2- / M-phase.

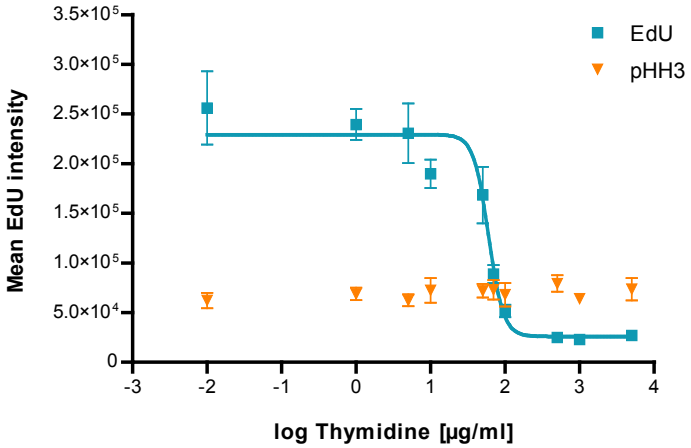


Figure 3-17: EdU intensity (S-phase index) after Thymidine treatment. HeLa cells were exposed to different concentrations of Thymidine for 18 h, and were stained for incorporated EdU. An EC_{50} value of 59.81 µg/ml (derived from the EdU signal) is similar to the calculation shown above. As expected, the pHH3 signal intensity is not affected by incubation with Thymidine. N=4.

Time series analysis

To further study the influence of the compound incubation-time on the percentage of EdU / pHH3 positive cells, cells were incubated with Thymidine and Nocodazole for 6 h 18 h, 24 h and 30 h. The dose-response curves for both compounds are shown in ***Figure 3-18***.

Both compounds show the greatest effect on the number of positive cells after an incubation of 18 h. The half maximal effective concentration (EC_{50}) was determined to be 63.3 µg/ml for Thymidine (from the EdU signal), and 41.7 ng/ml for Nocodazole (from the pHH3 signal).

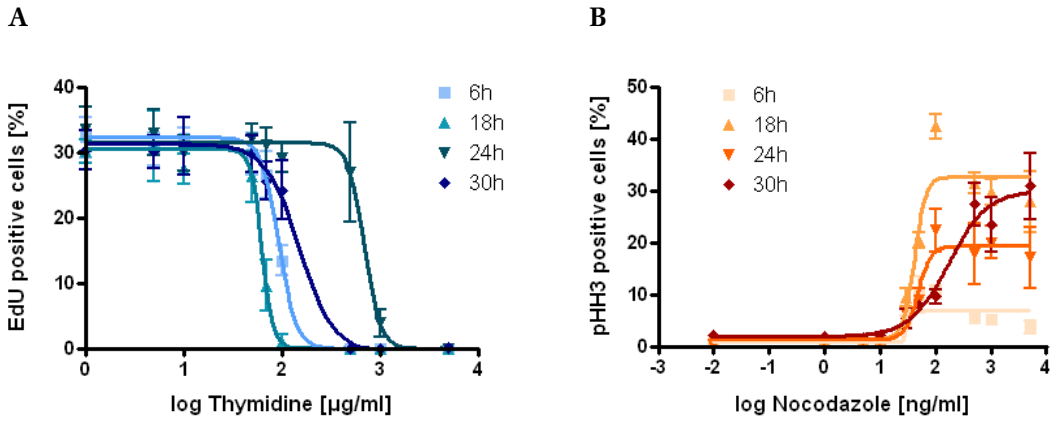


Figure 3-18: Dose-response curves after incubation with each compound for different time periods. HeLa cells were exposed for 6 h, 18 h, 24 h and 30 h to various concentrations of Thymidine (A) and Nocodazole (B) and were stained for DNA, EdU incorporation and pHH3. N=4.

Conclusions

High Content Screening is a powerful tool for detailed investigation of the cellular responses to anti-proliferative drugs. We have shown that our ready-made application "Cell Cycle-1", by multiplexing an S-phase and M-phase marker, is well suited to studying multiple events in a single assay. Furthermore, we have validated the use of Invitrogen's Click-iT™ EdU Assay kit with our imaging platforms and recommend it as a time-saving and reproducible method (compared to the classical BrdU incorporation, Casparri *et al.,* 2006).

3.3.4 Hints and Tips

- The population of cells in certain cell cycle phases can also be calculated by plotting the DNA sum intensity over the DNA mean intensity (or area) of the nuclei (see Image Analysis Guide, section 2.12 "Nuclear Classification – DNA content" for details).
- See the RMS "Cell Cycle Classification" (Image Analysis Guide, section 2.14) for details on how to multiplex measurement of the DNA content distribution with cell cycle markers.
- It is recommended that you use a control of untreated cells, in parallel to the compound titration, to monitor fluctuations in the cell number across the plate (see ***Figure 3-14*** "control" +/- DMSO).

3.3.5 Glossary

[1] Thymidine (deoxythymidine) is part of the DNA. More precisely, it comprises deoxyribose (a pentose sugar), joined to the base thymine. An excess of Thymidine leads to allosteric inhibition of the ribonucleotide reductase, which consequently prevents DNA synthesis (G1- /S-phase blocker).

[2] Nocodazole is an anti-mitotic agent that blocks microtubule formation by binding to ß-tubulin, therefore disabling nuclear and cell division (G2- /M-phase blocker).

[3] These products are no longer commercially available from the designated suppliers. They might still be obtained from alternative suppliers. Please note that the protocol might have to be adjusted accordingly.

3.3.6 References

Gasparri, F., Capella, P. and Galvani, A. (2006) "Multiparametric Cell Cycle Analysis by Automated Microscopy". J. Biomol. Screen 11, pp 586-598.

Chapter 4 – Data Management

This chapter describes how to handle data created with the Operetta, Operetta CLS or Opera Phenix system and Harmony software, and gives recommendations for data safety.

4.1 Data Management

To cope with the amount of data that can be created with the Operetta, Operetta CLS or Opera Phenix system and Harmony software, it is important to understand Harmony's data types, data handling and data management tools. This chapter will cover data safety basics and different data management tasks and tools.

- Data safety
- Data organization and retrieval
- Image relocation to free up local hard disk space
- Data archiving to store completed projects for the long-term
- Data backup to create a copy of your current database and images

4.1.1 Data Safety

Data can be lost for various reasons: accidental deletion, virus damage, hardware malfunction, software crashes, data corruption or even natural disaster. Critical data, therefore, should always be protected. The prevention of data loss can rarely be guaranteed, but the impact of a data loss event can be mitigated with proper precautions.

The nature of the data loss determines the action required, e.g. to prevent data damage by a computer virus, an antivirus software should be used.

A key concept for securing data is storing multiple copies at multiple sites on several different media. As an example, think of the data as the key to a house. It's wise to give one of the keys to parents, friends or neighbors. If the primary key is lost, a copy is in another location.

Next, we will describe the fundamental differences between the commonly used terms: RAID (Redundant Array of Independent Disks) archive and backup.

RAID (Redundant Array of Independent Disks)

The Harmony PC is configured as RAID system with hardware redundancy. RAID stands for Redundant Array of Independent Disks. RAID is a storage technology, and a RAID system is a collection of hard disks which is presented as one logical unit and accessed as a single hard drive.

With this technology, the data are protected from one type of data loss event: damage to a *single* hard drive within the RAID system. Therefore, a RAID system provides data protection to some extent, but is not a substitute for a backup. Please note that a backup storage should always be physically separated from the primary source.

Archive

Data archiving in general is the process of moving data that is no longer being actively used to long-term retention storage. Therefore, an archive is a collection of historical records.

If a project is finished, the corresponding experimental settings, measurements, analysis sequences and evaluations are not needed in the primary storage (here: Harmony PC) anymore. To archive the data, the user should copy it to a secondary location as a first step and delete the original data from the primary source afterwards.

Backup

Data backup is a copy of data files on a storage device which stores the data in a secondary location. A backup is always a short-term insurance to facilitate disaster recovery. A backup application takes periodic snapshots of active data. The backup data are retained only for a few days or weeks on a second storage device such as a portable hard disk or network associated storage (NAS).

The data will be copied and, if the worst case scenario occurs, it can be recovered from the secondary device and restored to the primary location. The backup device must be physically separated from the primary device, e.g. the backup NAS should be in another part of the building than the Harmony PC.

Table 4-1: Comparison of Backup and Archive

Backup	Archive
Copy of information	Primary information
Operational backup and disaster recovery	Regulatory compliance and legal records retention
Short retention	Long retention
Data overwritten on periodic basis	Data retained for compliance

If the Harmony database suffers serious damage and there are no archives or no backup, there is no way to restore your data.

4.1.2 Harmony Data Management – Basics

Logging into Harmony requires a user account and corresponding password. The initial user account is “PKI” with password “PKI”. This account has administrative rights. We recommend just using it for the first login and then creating individual user accounts for each Harmony user. If the data have been created using one user account, they belong to this user account or “Owner”. It is not possible to change this “Owner” afterwards. Therefore data management starts when users log into Harmony. Additionally only the “Owner” of the data or a user account with administrative rights has the authorization to e.g. delete the data.

The data within Harmony are created as objects in the Harmony database. All database objects belong to one of the default object types – related to the experimental workflow. In ***Figure 4-1*** the database object types available are listed.

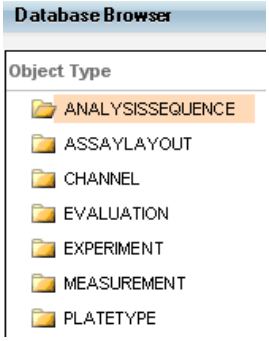


Figure 4-1: Detail of the **Database Browser** interface showing all the distinct object types available in the Harmony database in alphabetical order.

The object type “Experiment”, for example, is created in the **Setup** tab of Harmony by saving the experimental setup data. The defined settings are then used to measure plates in the **Run Experiment** tab. By clicking the **Start** button in the Run Experiment tab a new object type “Measurement” is created. The object “Measurement” includes the images and experimental settings that were used to acquire the images.

To analyze images, an image analysis needs to be defined on the **Image Analysis** tab. Saving an analysis sequence will create the object type “Analysis Sequence” in the Harmony database.

The **Evaluation** tab uses the analysis sequence generated to analyze measurements and to visualize the evaluation results. By running the analysis sequences in the Evaluation tab an “Evaluation” is created and becomes visible in the **Database Browser**. The evaluation inherits experiment, measurement and analysis sequence information (see also chapter 4.1.5 “Inheritance”).

4.1.3 Keywords

Keywords describe the properties of a database object. Each object has the same four default keywords (Object Type, Owner, Date and Signature). Keywords can have different values, e.g. the value of the keyword “Owner” is the name of the user account that was used to perform the experiment.

In addition to default keywords, database objects are further described by a huge number of specific object type (or individual) keywords. Keywords are automatically created by Harmony (please refer to section **About Keywords** in the Harmony Help or User Manual).

Example: Keywords for object type “Channel”:

Default keywords:

- SIGNATURE (unique identifier)
- OWNER (user account used to create the database object)
- DATE (creation date)
- OBJECT TYPE (Harmony recognizes seven different object types)

Individual keywords:

- INSTRUMENT TYPE (suitable instrument type, Opera Phenix, Operetta CLS or Operetta)
- CHANNEL (name of the channel)
- CHANNEL TYPE (Fluorescence, Brightfield, FRET or Phase)
- EMFILTER (emission filter name)
- EXFILTER (excitation filter name)

Another option to describe database objects are user-defined keywords (please refer to the next chapter or to sections **Define Keywords** and **User-defined Keywords** in the Harmony Help or User Manual). These enable the user to assign additional keywords such as “Project” or “Cost Center” to a measurement. Their value is determined by the individual user (e.g. user-defined Keyword “Project” – value “Project A”).

4.1.4 User-defined keywords

The **Run Experiment** tab enables the user to attach **user-defined keywords** to a measurement. Like other keywords, user-defined keywords can be used in the navigation area of the **Database Browser** to find database objects. User-defined keywords are inherited by the following database objects in the hierarchy. It is also possible to edit user-defined keywords later on, by opening the context menu of a measurement in the object list of the Database Browser.

For example creating a user-defined keyword “Project” under **Define Keywords** in the **Data Management** interface can be very helpful. By sorting the Database Browser with the project keyword in the first column, all database objects that belong to a project are displayed in the object list.

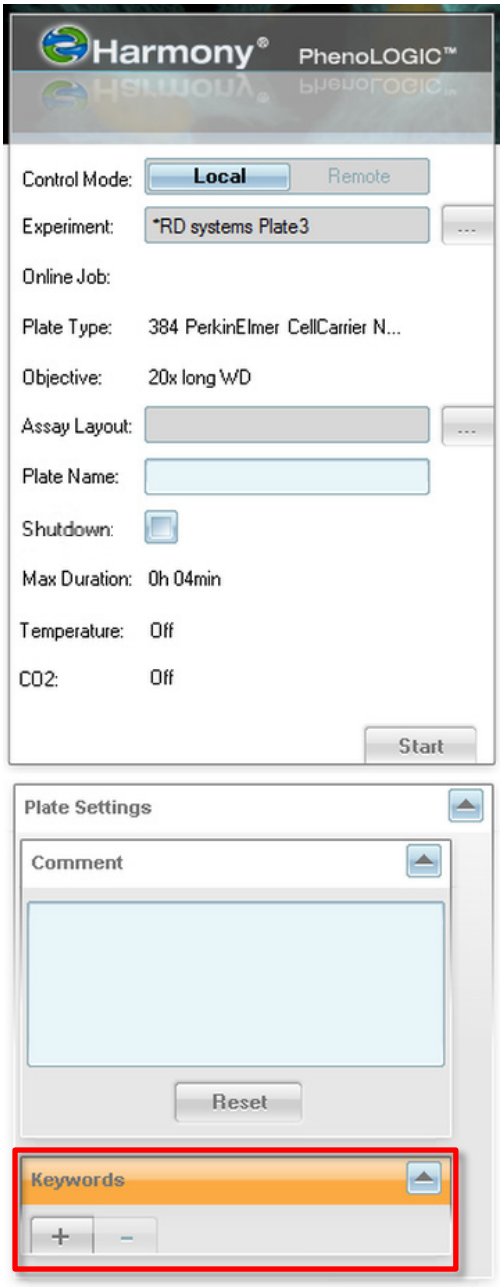


Figure 4-2: Interface to add user-defined keywords in the Run Experiment tab. At the top: the control area to define the experiment. At the bottom you will find the field to attach user-defined keywords. A user-defined keyword may be a given project. It is possible to add several different user-defined keywords.

4.1.5 Inheritance

Due to the multi-tiered object hierarchy, database objects in one tier inherit the information that was used to generate them from the tiers before. Measurements contain not only the images, but also the information about the experiment, like the channels and the plate type. An evaluation contains not only the results but also the image analysis sequence, the images and all experimental settings. If an evaluation is loaded into the **Evaluation** tab, it is possible to go back to the **Image Analysis** or **Setup** tabs to access the corresponding analysis sequence or the experimental settings. Due to this inheritance it is possible to extract all information stored from an evaluation or a measurement or their archives (see also section 4.2.2).

Objects that are used for an experiment, measurement or evaluation pass on their individual keywords and user-defined keywords to the following objects in the hierarchy.

A measurement for example can have an assay layout attached and additional user-defined keywords. Evaluations inherit the current assay layout of the measurement and use its information to display the data in the evaluation results table. If an assay layout is changed (e.g. detach and attach a new one) the assay layout of existing evaluations will be updated accordingly.

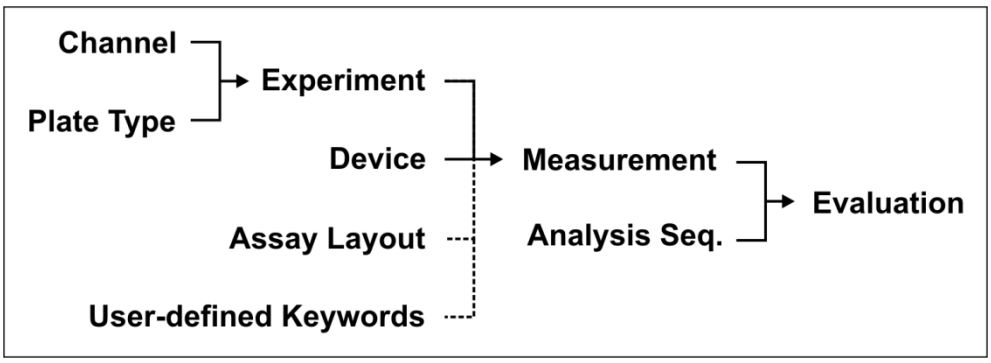


Figure 4-3: Inheritance of object information and keywords in Harmony. It is possible to restore all information from an evaluation or its archive. Assay layouts and user-defined keywords are not created by default. They have to be manually attached to be passed on (punctuated line).

4.1.6 How to find objects in the database

The **Database Browser** is the interface for all database-related tasks. Keywords help to filter the database and find the desired database objects quickly. In the Database Browser multiple keywords can be combined to a navigation “tree”, narrowing down the number of objects from left to right until the desired object has been found.

In addition, the **Search…** option (search in comments) can be used. It can be accessed by clicking the Add/Remove Keyword-Buttons (***Figure 4-4***) in the Database Browser. The entered term will be searched for in all the comments attached to database objects. By tagging high quality measurements or successful analysis sequences with meaningful comments these items can be easily retrieved from the database later.

The Database Browser can be opened in many different ways. Depending on the context, it will open with slightly different presetting functionality (please refer to section **Database Browser** in the Harmony Help or User Manual). Navigating to the **Settings** dialog and opening Database Browser leads to the Database Browser without presets.

Figure 4-4 gives an overview of the general structure of the Database Browser.

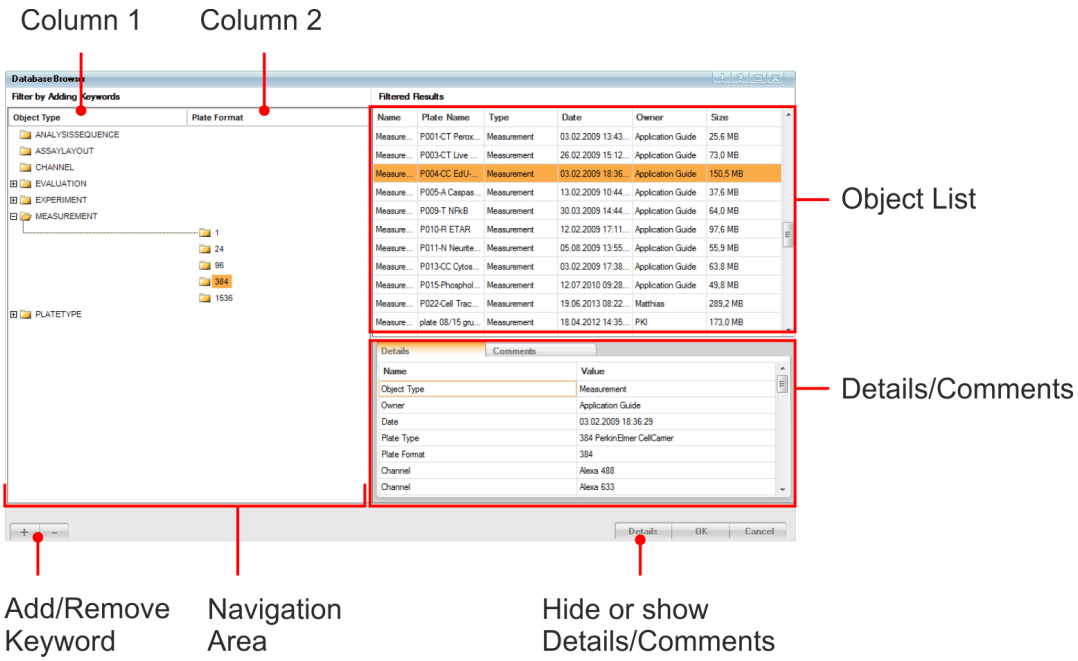


Figure 4-4: Example of a Database Browser interface. Left side: Columns displaying the sorting path, Navigation Area and Add/Remove Keyword-Buttons to change the sorting path. Right side: Object List of the data found, Details/Comments are displayed for the highlighted object.

To go directly to an object listed in the navigation area, select the respective column and start typing the first characters of the object name. After pressing the Enter key, the first object in the column that matches the query will be highlighted.

4.1.7 How to temporarily free disk space

Over time, the Operetta, Operetta CLS or Opera Phenix can produce a lot of data, especially when the biological application requires kinetic live cell measurements or stack measurements. As on other storage devices, the space on the Harmony PC hard drive is limited. To ensure that the instrument is able to acquire new images and perform new measurements it is necessary to have enough free disk space on the Harmony PC.

If all images in the database need to remain accessible to the user, the **Relocate Images** function can be used. Relocate Images moves selected images to a secondary hard disk or computer. Only the images are moved from the Harmony database, the metadata (keywords, numerical data) remain in the Harmony database.

As a result, Relocate Images extends the hard drive capabilities of the Harmony PC temporarily. Relocate Images can be done as a **Scheduled Task**. By creating a Scheduled Task, images will be regularly relocated and the Harmony PC will have enough free space to perform new measurements. We recommend setting up a Scheduled Task with a time period suitable for the amount of data created (please refer to section **Scheduled Tasks** in the Harmony Help or User Manual).

4.1.8 How to store and remove finished projects

If a project is finished and no immediate access to the images is needed, the project should be archived using the **Write Archive** function. Write Archive generates a Harmony compliant data copy on a secondary hard disk or computer. For instance using the project keyword in the navigation tree, all database objects that belong to the project are displayed and can be selected easily for archiving (***Figure 4-5***). Due to the inheritance of information in Harmony it is sufficient to archive all evaluations and unevaluated measurements. All other database objects used for a project can be restored from evaluation or measurement archives (see also chapter 4.2.2).

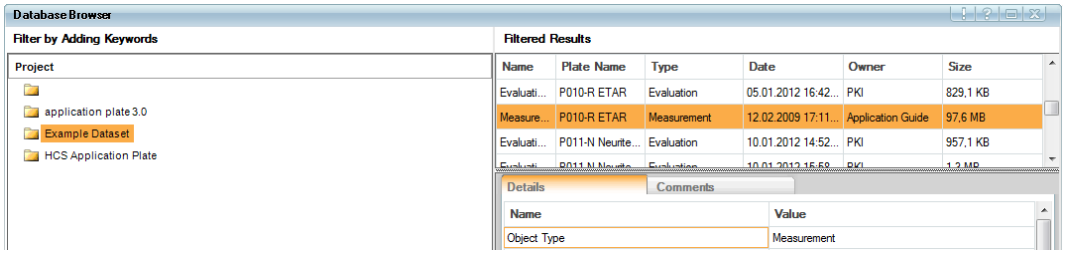


Figure 4-5: Example for archiving a finished project: By selecting the keyword "Project" to list the different projects in the navigation area, all data belonging to a project (evaluations and measurements) can easily be selected for archiving.

Write Archive just writes a data copy and the originals remain in the Harmony database. To free up more disk space, it is necessary to remove the original data from the database by using the **Delete Data** function. To delete data from the database that has already been archived, open the Delete Data dialog directly after successful archiving. The archived database objects will be selected as a preset and can be deleted easily without making a new selection.

To delete data in the **Database Browser** you have to be logged in as the owner of the data or with an account that has administrative rights.

Write Archive also works for images on a secondary image location (if relocated by the **Relocate Images** function). It is not necessary to relocate these images back to the Harmony database to run the Write Archive task.

4.1.9 How to share data

To share data with colleagues owning an Operetta, Operetta CLS or Opera Phenix system, a Harmony compliant archive can be written (with **Write Archive**) and shared.

Harmony archives provide the option to share Harmony datasets. They contain all information and can be loaded into another Harmony database by **Read Archive**.

To share data with colleagues who do not have an Operetta, Operetta CLS or Opera Phenix or do not use Harmony, selected images and results can be exported by **Export Data**. Exported data can be used by third-party applications (e.g. for visualizing images in other software or post-processing of results in Microsoft® Excel®).

4.1.10 Automated data transfer to Columbus

For transferring images directly into the image database in the Columbus™ system, the automated **Columbus Transfer** offers a fast and easy solution.

A copy of all image files in TIFF format will be transferred, as well as all files associated with the selected measurements such as assay layouts, flatfield correction

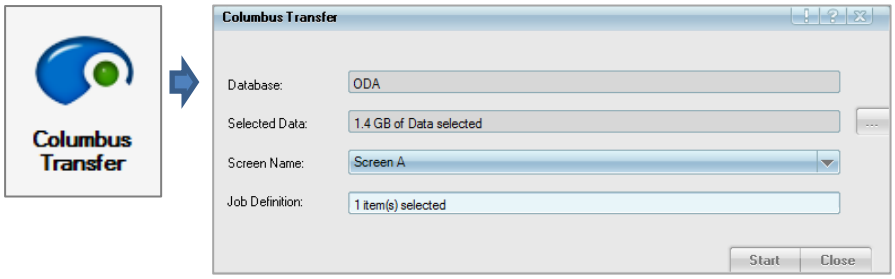
parameters, analysis sequences and evaluations. The transfer will be processed as a background job, so that users can continue to use Harmony.

To use this function, the URL of the Columbus server and a Columbus user account must have been configured in the **User Accounts** dialog.

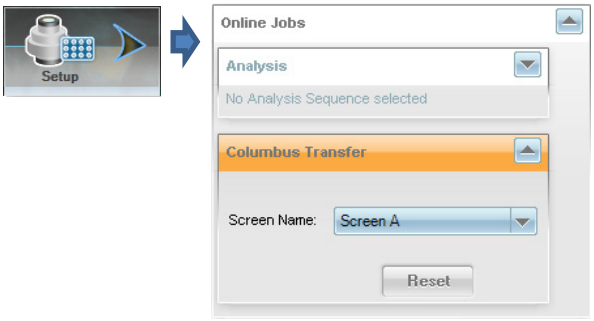
Each Harmony user can enter their own Columbus user account. Each Columbus user account has its own list of screens in Columbus. The list of available screen names in Harmony (to be selected before transfer) depends on the Columbus account selected and on the logged in Harmony user.

Once a Columbus user account has been configured, data can be transferred using the **Columbus Transfer** dialog by double clicking the Columbus Transfer icon in **Settings – Data Management**. Data can also be transferred automatically to a Columbus system within the network as soon as a measurement has been finished. For this purpose, an **Online Job** can be defined when setting up an experiment (***Figure 4-6***).

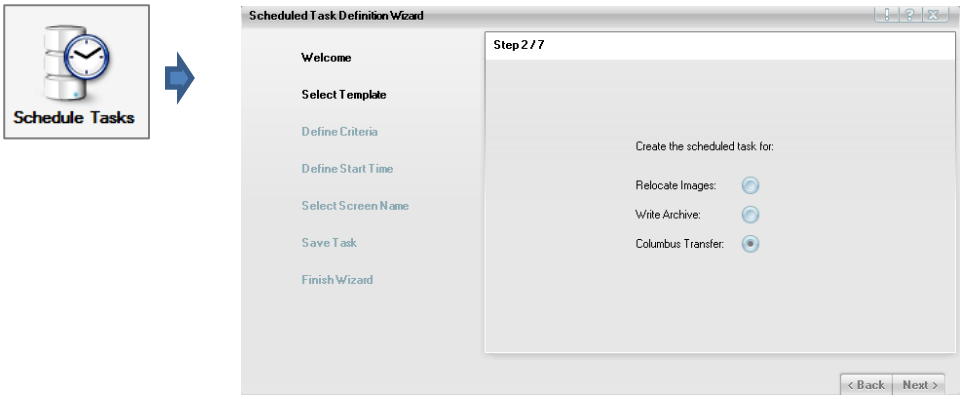
A) Columbus Transfer – manually



B) Columbus Transfer – automated as an online job



C) Columbus Transfer – as a scheduled task



D) Export Data – manually import to Columbus

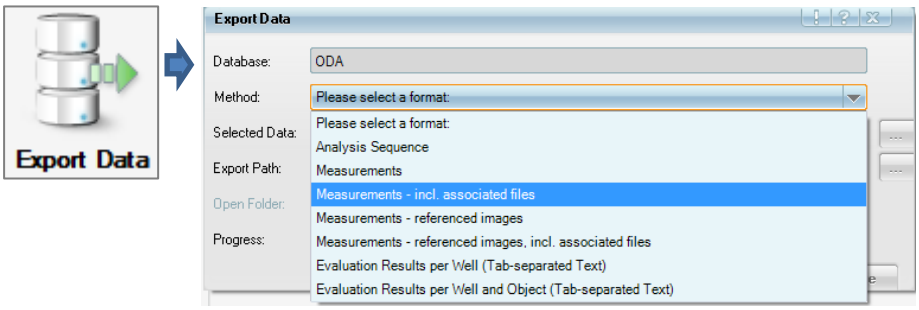


Figure 4-6: **A:** Data transfer to a Columbus system. If the Columbus system is within a network, data can be transferred manually using the Columbus Tranfer dialog in Settings – Data Management. **B:** If the Columbus Tranfer has been defined as an online job in the experiment setup, the data will be transferred automatically after the measurement has been finished. **C:** Also a defined scheduled task enables the automated data transfer directely into Columbus on a regular basis. **D:** If the Columbus system cannot be accessed, the Export Data function can be used to store data intermediately before importing manually into Columbus.

Using **Columbus Transfer** is recommended if Columbus is available in the network. It will be faster and easier than the **Export Data** function because the data is transferred directly.

4.1.11 Job status

Any type of data management operation in the Harmony database, e.g. write archive, columbus transfer, delete data etc., will run in the background. The status of each job can be checked in the **Job Status** dialog (***Figure 4-7***). This dialog displays a list of background jobs which are running, which are queued to be run or which have been processed in the last 30 days.

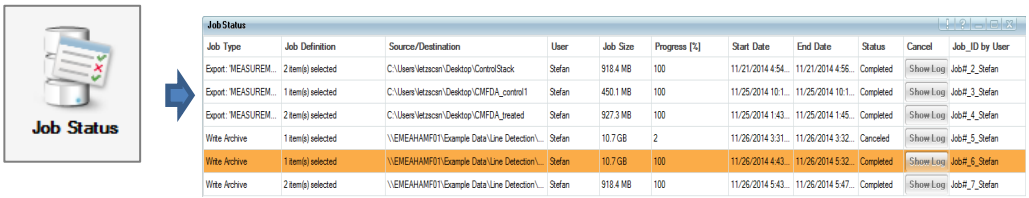


Figure 4-7: The Job Status dialog displays a list of background jobs. Each job can be canceled and viewed in detail within the respective log file.

If a job fails you can check the log file to see what caused the problem. The log file also includes all information about data which should be processed so that it is possible to reproduce the same job when the problem is solved.

Columbus transfer: Please note that Columbus has a Job Status display as well. If the data transfer failed due to an issue with Columbus, you will find detailed information in the respective Columbus Job Status table or log file.

The Job Status table only shows jobs which have been started on that respective computer. For example, a job started on the Harmony PC will not be displayed in the Job Status table on an office version of Harmony although the same database may be involved.

Log files for jobs which have been processed will be automatically deleted after 30 days. Save them separately if you wish to store them for a longer period.

4.2 Harmony Data Management – Recommendations

4.2.1 How to organize the Harmony archive

Over time, the Harmony archive will comprise large amounts of data so reliable data management is crucial if the original data are to be retrieved from an archive after some months or years.

In principal there are two ways to organize the Harmony archive on a secondary storage device.

One option is to create one large archive folder that contains all archived data. New archives can easily be added to this folder. If one object has to be imported into the Harmony database again, the whole folder can be browsed through with the **Read Archive** dialog. This dialog opens the **Database Browser** where extensive sorting and search options are available. The navigation time may increase with the folder size.

The second option is to create several individual folders on the archive server. These folders could for instance be named according to the project, application or the time span in which the project was executed. This will reduce the size of individual archive folders, may increase the overview in the archive and make it easier to change the location of the archive folder if necessary (***Figure 4-8***).

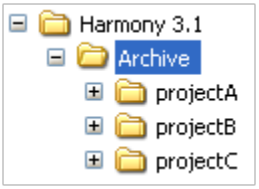


Figure 4-8: Example for a Harmony archive organized in project-related folders. Project-related folders should contain all evaluations and unevaluated measurements for individual projects.

4.2.2 How to restore database objects from evaluation and measurement archives

The inheritance of object information and keywords in the Harmony database and Harmony archives allows all database objects to be restored out of an object which inherited this information. In case database objects have been accidentally deleted and need to be restored, the inheritance structure of Harmony provides a solution to restore implicit object information to explicit database objects which are re-usable and searchable within the **Database Browser**.

If for instance an experiment has to be repeated and all the database objects needed (e.g. channels, plate type, assay layout and experiment) are not available in the Harmony database, they can be restored using the corresponding measurement or evaluation archive. In the same way, an unusual evaluation can be rechecked by restoring all used objects (measurement, analysis sequence and evaluation) from an evaluation archive.

After reading the archived measurement or evaluation and restoring them back into the Harmony database (using the **Read Archive** function), it is possible to go back to the **Image Analysis** or **Setup** tabs to assess the inherited information and restore it to re-usable database objects by clicking the corresponding **Save…** button.

The assay layout can be re-saved as an object in the Harmony database by opening the **Assay Layout Editor** and actively saving the assay layout there (***Figure 4-9***).

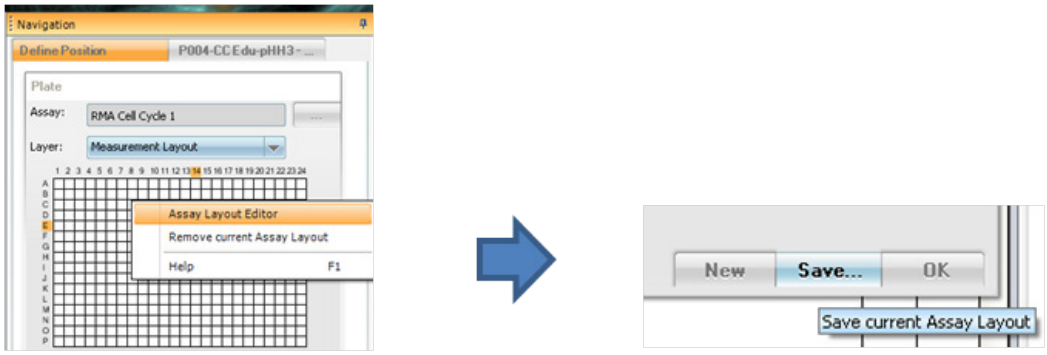


Figure 4-9: How to restore an assay layout that is implicit within an evaluation but not available as a distinct object in the Harmony database. Open the assay layout editor and save the assay layout actively.

The plate type can be saved as an object in the Harmony database by right clicking in the Global Control section and selecting **Plate Type Details** from the context menu (opens the **Plate Type Definition Wizard**) (***Figure 4-10***).

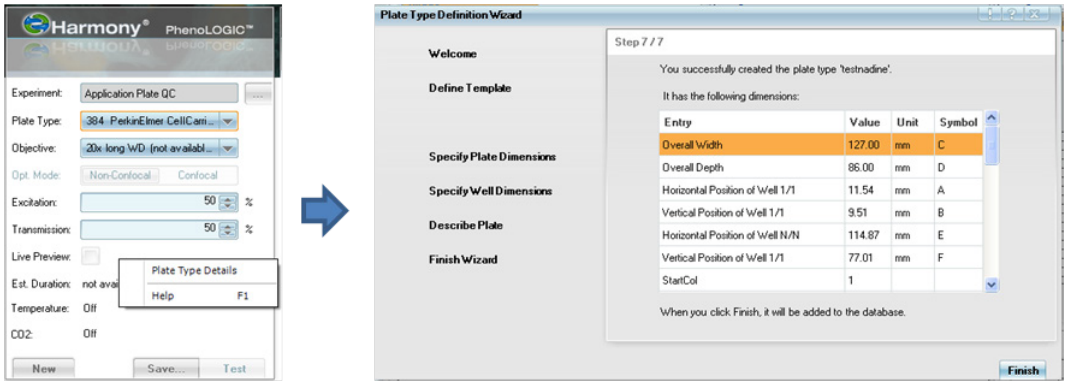


Figure 4-10: How to restore a plate type that is implicit within an evaluation and not available as a distinct object in the Harmony database. After opening the Plate Type Definition Wizard by right clicking the Global Control, the plate type can be restored by going through the wizard and actively saving it.

Restoring an object to the database also works if the measurement/ evaluation is still in the Harmony database (i.e. not archived yet). The object can be restored by opening the measurement/evaluation in the Harmony interface and actively saving it by going back in the Harmony workflow.

An evaluation without its associated measurement is meaningless. Therefore, evaluations always contain the corresponding measurement. After reading an evaluation archive, the inherited measurement will immediately be a searchable object in the Harmony database.

If the Harmony database suffers serious damage and there are no archives or no backup, there is no way to restore your data (please see section 4.1.1 for further information).

Measurements that have not been evaluated have to be archived as well. They cannot be restored from evaluation archives because without being evaluated, their object information has not been passed on to an evaluation.

4.2.3 How to create a backup

To avoid any loss of active data in the Harmony database, it is recommended to regularly create a backup of the active data in the Harmony database (see also chapter 4.1.1). As Harmony's **Write Archive** function generates a data copy on a secondary device, it is possible to use this function as a basic backup option.

All Harmony data including image files, XML files and database metadata can be accessed by 3rd party backup software. Therefore, we recommend backing up all Harmony data on the Harmony PC daily using 3rd-party backup software so that it can be restored if there is a crash (see also section "Backup all Data on Harmony PC" in the Harmony Help or User Manual).

If you don't have 3rd-party software available, it is possible to use the **Write Archive** function as a basic backup option to generate a data copy on a secondary device.

To create such a backup, a folder on the secondary device should be created for use as the backup storage location. Next, copy all data currently in the Harmony database into this backup folder by using **Write Archive**.

Then, a **Scheduled Task** should be created to copy the data that has changed since the last copy process ("incremental"). We recommend running the **Write Archive** process daily with the following criteria in the **Scheduled Tasks** dialog: younger than two days but older than one day. Please see ***Figure 4-11*** and ***Figure 4-12*** for an explanation of these criteria.

Using these criteria on a daily basis ensures that the amount of daily transferred data does not become too large. Ideally, the task should be run during the night to avoid interference of the copy process with running measurements.

To allow a **Write Archive Scheduled Task** to run during the night make sure that the Harmony PC is running and the backup folder on the secondary device can be accessed.

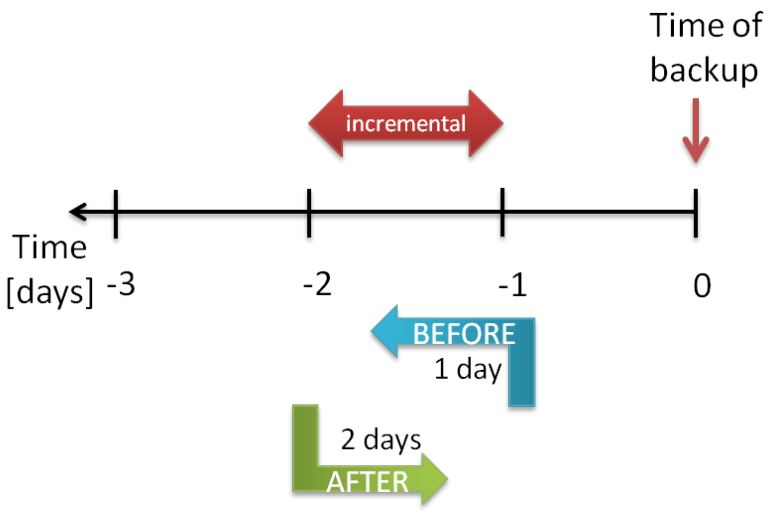


Figure 4-11: Recommendations to select data for a Scheduled Write Archive Task as a basic backup method. After creating a backup, the data copied by Write Archive are older than one day AND not older than two days.

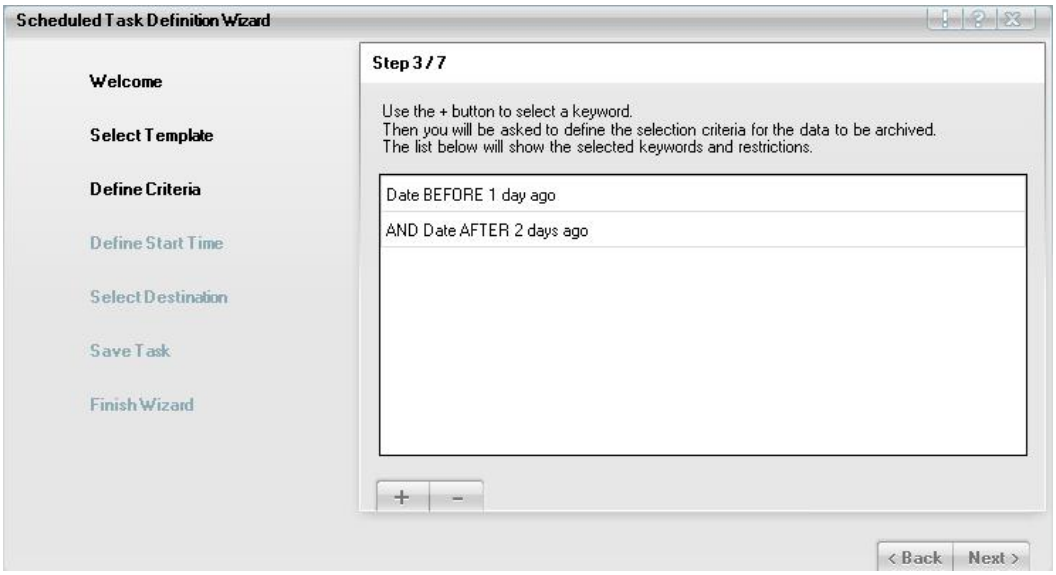


Figure 4-12: Scheduled Task Definition Wizard for a daily Scheduled Write Archive Task. Criteria to define the Scheduled Task can be added to the list (using the Add/Remove keyword buttons). Here the keyword "Date" is used twice so that data older than one day and younger than 2 days will be archived.

If you created an archive and a backup, consider renewing your backup periodically. To do this, create a new backup folder and copy all data in the Harmony database into it (using **Write Archive**). Then, delete the old backup folder.

The **Write Archive** only adds data to a folder. Therefore the backup folder continues to grow and will contain data no longer present in the primary storage because they have been archived and deleted for long-term retention.

Archiving can also be automated as a Scheduled Task. When using this option please note that accessing archives with a large number of objects will take much longer than archives with less objects. As a guideline archives should not exceed 5000 database objects. Therefore, it is best to use every month a new archive as destination for a Scheduled Task.

4.2.4 Example data workflow

This example workflow presents one possible solution for data management and data safety. It may have to be adapted to fit individual needs and environments. Here we combine the recommendations given above into a workflow.

Data generated on the Operetta, Operetta CLS or Opera Phenix are stored in the Harmony database on the Harmony PC. These data can be reached, analyzed and managed either by Harmony on the Harmony PC or via Harmony installed on an office PC. With **Write Archive** and **Scheduled Task**, Harmony provides all the functionality required to archive finished projects and create a basic backup for the active data in the database (please see sections 4.1.7 and 4.2.2). To secure the archive from any data losses make sure that a secondary archive is created (Media backup) by your local IT department.

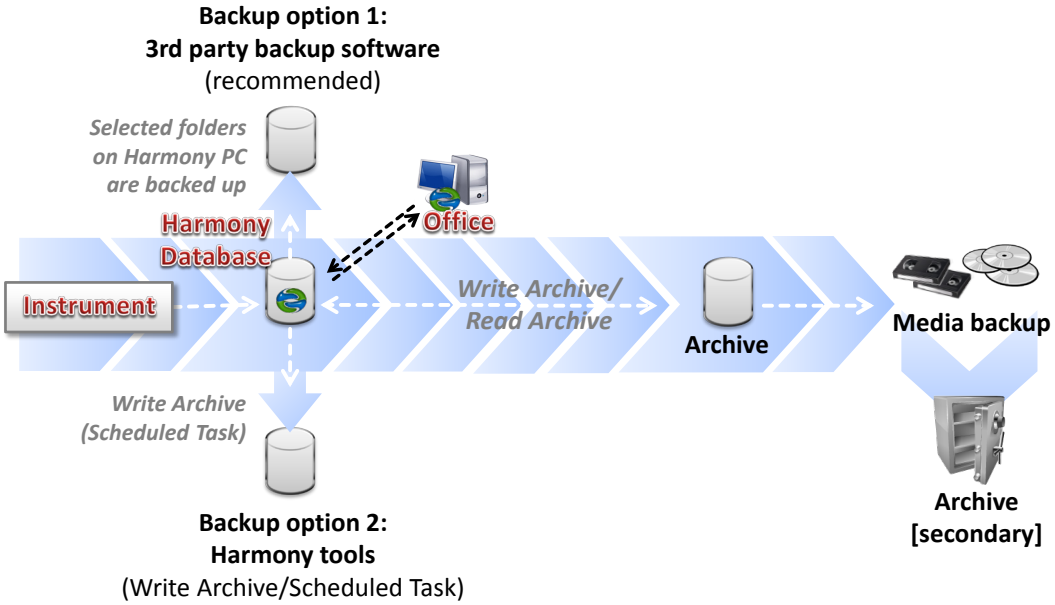


Figure 4-13: Example data workflow for data created on the Operetta, Operetta CLS or Opera Phenix. A backup of the Harmony database on the Harmony PC can be done daily using 3rd party backup software. The Harmony tools Write Archive and Scheduled Task provide the functionality required to create an archive and a basic data backup. A backup is a copy of the active data in the Harmony database. It should be used as a short-term solution for disaster recovery. An archive is a long-term storage of finished projects. To avoid any data loss from the archive, a media backup should be created. The structure and safety of the media backup and secondary archive should be determined by your local IT department.

4.2.5 Example data workflow combined with a Columbus server

If a Columbus server is integrated into the data workflow, the recommendations for data management and safety are slightly different. Again, this example workflow presents one possible solution for data management and data safety. It may have to be adapted to fit individual needs and environments.

Columbus offers the option to store and analyze data from a range of different microscopes and high content screening devices, including Operetta, Operetta CLS and Opera Phenix. To load Operetta, Operetta CLS or Opera Phenix data from the Harmony database into Columbus, the data can be directly transferred using **Columbus Transfer** if Columbus is available in the network. Otherwise the data has to be exported using **Export Data** (method “Measurement – incl. associated files”) before importing them manually into Columbus.

If Columbus is to be used exclusively for data analysis, it is possible to delete the original data from the Harmony database, but with caution:

Exported or transferred data is being modified and converted to another format which **cannot be re-imported into the Harmony database**.

If the original measurement is needed or an experiment will be repeated with the same settings, it can only be restored from a Harmony compliant archive and not from an export. Therefore, we recommend creating archives of experiment data and, if needed, of measurement data (using **Write Archive**).

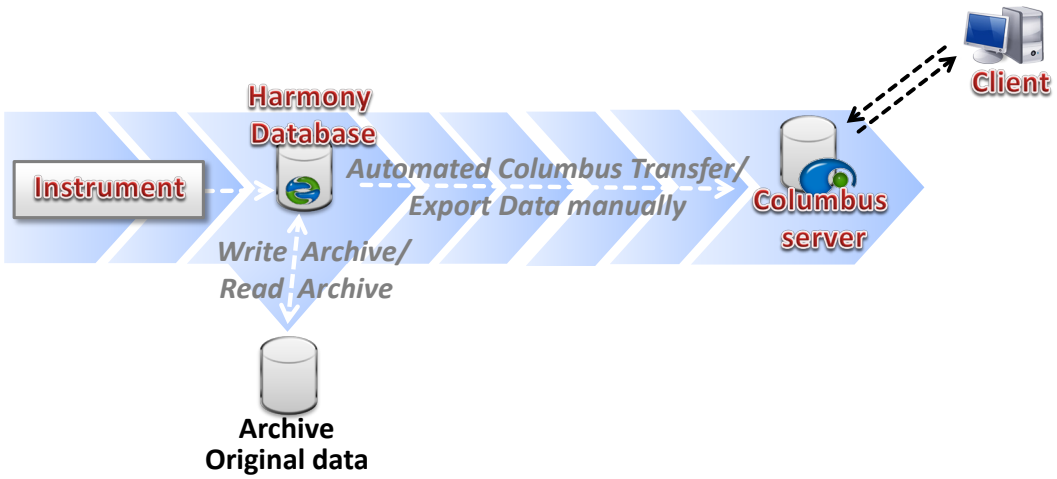


Figure 4-14: Example Harmony data workflow with Columbus server integration. Data tranferred to Columbus is modified and lack experiment related information. To be able to re-import data into the Harmony database, it is recommended that the original data is kept by creating an archive (using the Write Archive function of Harmony).

Appendix

Fluorescence Excitation Options

Depending on your choice, your Opera Phenix is equipped with four or five different laser lines. The UV range is covered by a 405 nm laser or a combination of 375 and 425 nm lasers. For the red range, a 640 nm laser is installed. The following ***Table 5-1*** lists all options and states their usability with different fluorophores. Please note that only a selection of excitation options can be included in one instrument.

Part No	Excitation/ Laser wavelength [nm]	Optimal for	Can also be used for
HH14000101 HH14000121 (high power laser)	405	Sapphire	DAPI, Fura-2, HOECHST 33342, UV-fluorescent proteins (Cerulean, CFP, mTurquoise2)
HH14000102 HH14000122 (high power laser)	425	UV fluorescent proteins (e.g. Cerulean, CFP, mTurquoise2)	
HH14000102 HH14000122 (high power laser)	375	CellMask Blue, DAPI, HOECHST 33342	Sapphire, Fura-2
HH14000101 HH14000121 (high power laser) HH14000102 HH14000122 (high power laser)	488	Alexa 488, Calcein, green fluorescent proteins (e.g. EGFP), Fluorescein, Rhodamine 110	Alexa 532, YFP
HH14000101 HH14000121 (high power laser) HH14000102 HH14000122 (high power laser)	561	Alexa 568, dsRed, Cy3, MitoTracker Orange, red fluorescent proteins (e.g. mCherry, dTomato, mStrawberry)	
HH14000101 HH14000121 (high power laser) HH14000102 HH14000122 (high power laser)	640	Alexa 633, Alexa 647, CellMask DeepRed, Cy5, MitoTracker Deep Red, VivoTag 645	Cy5.5, VivoTag 680, VivoTag 680 XL

Table 5-1: Fluorescence excitation options of the Opera Phenix

Fluorescence Emission Options

The following ***Table 5-2*** lists all emission options for the Opera Phenix. Optional filters that have to be ordered separately are shaded gray. For a list of all of the preconfigured channels in Harmony and their respective emission, please refer to ***Table 5-3***.

Part No.	Emission band	Typical application / dye
Comes with part no. HH14000101 HH14000121 HH14000102 HH14000122	435-480	Standard UV emission filter. Suitable for CellMask Blue, DAPI, HOECHST 33342. Can also be used for Fura-2, Cerulean, CFP and mTurquoise2.
Comes with part no. HH14000101 HH14000121 HH14000102 HH14000122	435-550	Filter with extended range to capture complete emission of UV-fluorophores. Please note that this filter cannot be used simultaneously with 488nm excitation. Optimal for UV-Fluorescent proteins (Cerulean, CFP, mTurquoise2) as well as CellMask Blue, DAPI, Fura-2, HOECHST 33342.
HH14000341	435-515	Optional filter for FRET applications. Optimal for capturing donor emission of FRET pairs consisting of UV-fluorescent proteins (Cerulean, CFP, mTurquoise2) and e.g. YFP.
Comes with part no. HH14000101 HH14000121 HH14000102 HH14000122	500-550	Standard blue emission filter. Suitable for Alexa 488, Calcein, CellTracker Green CMFDA, EGFP, Fluo-4, Fluorescein (FITC), Rhodamine Green, YFP and Sapphire. Can also be used for Alexa 532.
Comes with part no. HH14000101 HH14000121 HH14000102 HH14000122	570-630	Standard yellow emission filter. Suitable for Alexa 568, Cy3, dsRed, MC Tracker Orange and red fluorescent proteins like mCherry, dTomato and mStrawberry.
Comes with part no. HH14000101 HH14000121 HH14000102 HH14000122	650-760	Standard red emission filter, only available in instruments with 640nm excitation. Suitable for Alexa 633, Alexa 647, CellMask Deep Red, Cy5, DRAQ5, MC Tracker DR, VivoTag645. Can also be used for Cy5.5, VivoTag-S 680 and VivoTag 680XL.

Table 5-2: Emission filter options of the Opera Phenix. Optional filters that have to be ordered separately are shown in gray.

Fluorophores and their pre-configured filter settings

In ***Table 5-3*** you will find a list of all the preconfigured channels in the Harmony Setup Screen, and their respective filter settings. Channels using one of the optional emissions are shaded gray.

Channel name in Harmony®	Excitation [nm]	Emission [nm]	Comment
Alexa 488	488	500-550	
Alexa 532	488	500-550	
Alexa 568	561	570-630	
Alexa 633	640	650-760	
Alexa 647	640	650-760	
Brightfield	Trans-mission	650-760	
Calcein	488	500-550	
CellMask Blue	375	435-480	
CellMask Blue – extended	375	435-550	Filter with extended range
CellMask Deep Red	640	650-760	
CellMask Green	488	500-550	
CellTracker Green	488	500-550	
Cerulean	405	435-480	
Cerulean	425	435-480	
Cerulean – extended	405	435-550	Filter with extended range
Cerulean – extended	425	435-550	Filter with extended range
Cerulean – extra	405	435-515	Optional emission filter for FRET applications
Cerulean – extra	425	435-515	Optional emission filter for FRET applications
CFP	405	435-480	
CFP	425	435-480	
CFP – extended	405	435-550	Filter with extended range
CFP – extended	425	435-550	Filter with extended range
CFP – extra	405	435-515	Optional emission filter for FRET applications
CFP – extra	425	435-515	Optional emission filter for FRET applications
Cy3	561	570-630	
Cy5	640	650-760	
Cy5.5	640	650-760	
DAPI	375	435-480	
DAPI	405	435-480	
DAPI – extended	375	435-550	Filter with extended range
DAPI – extended	405	435-550	Filter with extended range

Digital Phase Contrast	Trans-mission	650-760	
DRAQ5	640	650-760	
dsRed	561	570-630	
dTomato	561	570-630	
EGFP	488	500-550	
Fluo-4	488	500-550	
Fluorescein (FITC)	488	500-550	
Fura-2	375	435-550	
Fura-2	405	435-550	
HOECHST 33342	375	435-480	
HOECHST 33342	405	435-480	
HOECHST 33342 – extended	375	435-550	Filter with extended range
HOECHST 33342 – extended	405	435-550	Filter with extended range
mCherry	561	570-630	
MitoTracker Deep Red	640	650-760	
MitoTracker Orange	561	570-630	
mStrawberry	561	570-630	
mTurquoise2	405	435-480	
mTurquoise2	425	435-480	
mTurquoise2 – extended	405	435-550	Filter with extended range
mTurquoise2 – extended	425	435-550	Filter with extended range
mTurquoise2 – extra	405	435-515	Optional emission filter for FRET applications
mTurquoise2 – extra	425	435-515	Optional emission filter for FRET applications
Rhodamine 110	488	500-550	
Sapphire	375	500-550	
Sapphire	405	500-550	
TRITC	561	570-630	
VivoTag 645	640	650-760	
VivoTag 680 XL	640	650-760	
VivoTag-S 680	640	650-760	
YFP	488	500-550	

Table 5-3: Pre-configured filter settings of Harmony channels. Optional filters that have to be ordered separately are highlighted in gray.

Table 5-4 lists the channels that are used for FRET measurements. FRET channels using one of the optional emissions are shaded gray.

Channel name in Harmony®	Excitation [nm]	Emission [nm]	Emission [nm]	Comment
FRET: Cerulean / YFP	405	435-480	500-550	
FRET: Cerulean / YFP	425	435-480	500-550	
FRET: Cerulean – extended / YFP	405	435-550	500-550	
FRET: Cerulean – extended / YFP	425	435-550	500-550	
FRET: Cerulean – extra / YFP	405	435-515	500-550	Optional emission filter for FRET applications
FRET: Cerulean – extra / YFP	425	435-515	500-550	Optional emission filter for FRET applications
FRET: CFP / YFP	405	435-480	500-550	
FRET: CFP / YFP	425	435-480	500-550	
FRET: CFP – extended / YFP	405	435-550	500-550	
FRET: CFP – extended / YFP	425	435-550	500-550	
FRET: CFP – extra / YFP	405	435-515	500-550	Optional emission filter for FRET applications
FRET: CFP – extra / YFP	425	435-515	500-550	Optional emission filter for FRET applications
FRET: mTurquoise2 / YFP	405	435-480	500-550	
FRET: mTurquoise2 / YFP	425	435-480	500-550	
FRET: mTurquoise2 – extended / YFP	405	435-550	500-550	
FRET: mTurquoise2 – extended / YFP	425	435-550	500-550	
FRET: mTurquoise2 – extra / YFP	405	435-515	500-550	Optional emission filter for FRET applications
FRET: mTurquoise2 – extra / YFP	425	435-515	500-550	Optional emission filter for FRET applications

Table 5-4: Pre-configureded filter settings of Harmony FRET channels. Optional filters that have to be ordered separately are shown in gray.

Abbreviations:

CFP: Cyan Fluorescent Protein

DAPI: 4',6-Diamidino-2'-phenylindol

EGFP: Enhanced Green Fluorescent Protein

Cerulean: A cyan fluorescent protein

Sapphire: Mutant of Green Fluorescent Protein

TRITC: Tetramethylrhodamine isothiocyanate, a rhodamine derivative

YFP: Yellow Fluorescent Protein

Objectives

A list of all available Opera Phenix objectives is shown in ***Table 5-5***. Three air and three water immersion objectives can be mounted at the same time in the objective turret for automated (software controlled) selection.

Part No.	Objective	NA	Working distance [mm]	Correction collar	Corrected for plate thickness [mm]	Field of view [mm]	Depth of focus** [µm]	Effective xy resolution*** [µm]		Recommended minimum distance between planes**** [µm]
								Binning 1	Binning 2	
HH14000401	1.25x Air	0.03	4	No	0.17	10.33 × 10.33	1501.0	10.6	21.2	682.3
HH14000402	5x Air	0.16	12.1*	No	0.17	2.58 × 2.58	58.2	2.6	5.3	26.5
HH14000403	10x Air	0.3	5.2	No	0.17	1.29 × 1.29	16.2	1.3	2.6	7.4
HH14000404	**20x Air**	**0.4**	**8.28***	**Yes**	**0 – 1.5**	**0.65 × 0.65**	**8.0**	**0.79**	**1.32**	**3.6**
HH14000407	20x hNA Air	0.8	0.55	No	0.17	0.65 × 0.65	2.6	0.66	1.32	1.2
HH14000421	20x Water	1.0	1.7	No	0.17	0.65 × 0.65	1.8	0.66	1.32	0.8
HH14000405	40x Air	0.6	3.28	Yes	0 – 1.5	0.32 × 0.32	3.3	0.53	0.66	1.5
HH14000408	40x hNA Air	0.75	0.71	No	0.17	0.32 × 0.32	2.2	0.42	0.66	1.0
HH14000422	40x Water	1.1	0.62	No	0.17	0.32 × 0.32	1.2	0.33	0.66	0.5
HH14000423	63x Water	1.15	0.6	No	0.17	0.21 × 0.21	1.0	0.28	0.42	0.5

Table 5-5: Available Opera Phenix objectives. The default objective which is always included in the instrument is highlighted in gray.

* The working distance of the objective is so large that it will not be completely used due to the given limitation of the z-drive.

** Depth of focus = 1.4 λ n / NA^2 with λ = 500 nm, n = 1.33, NA = numerical aperture. Eventually with a reduction factor due to undersampling on the camera.

*** Calculated at 520 nm. Optical resolution = 0.61 λ / NA. Eventually with a reduction factor due to undersampling on the camera.

**** Calculated as “Depth of focus / 2.2” (according to Nyquist’s theorem). Please note that using smaller distances will not improve the results.

The working distance given here is the total distance from lens to focal point. Please note that manufacturers of objectives often use the maximum working distance for historical reasons.
The maximum working distance is defined by objective manufacturers as the distance from lens to focal point minus 0.11 mm (0.11 mm equals the thickness of a typical cover slip reduced by its refractive index).

The 10x Air objective is best suited for plates with a bottom thickness of 170 µm. As it has no correction collar, a much thicker plate bottom will affect the optical image quality.

On Opera Phenix the allowed maximal setting for the correction collar is 1 mm. Please do not set the correction collar to values higher than 1 mm as automatic image alignment is only possible for correction collar settings ≤ 1 mm.

Plates

For a list of compatible PerkinElmer plates, please refer to ***Table 5-6*** and ***Table 5-7***. For a selection of plates from various brands see ***Table 5-8***. Please ensure that plates and objectives are compatible.

In ***Table 5-6***, ***Table 5-7***, and ***Table 5-8***, the comment “Restricted plate area” is shown as a footnote for some objectives. This means that with the selected combination of plate type and objective and regardless of the focus height, some edge wells cannot be selected for the measurement layout. For other objectives, restrictions may also apply when a focus height above, for example, 50 µm is used. This depends on the individual characteristics of each objective / plate combination and should be checked using the Harmony software even before a decision on a plate / objective combination is made.

See also Harmony Help or User Manual (section “Restrictions of Measurable Plate Area”).

Plates with an optical bottom thickness of greater than 1 mm are not recommended for use on Opera Phenix. The use of such plates might result in inferior image quality and an increased number of focusing errors.

Plate type	ViewPlate-96 F TC	Glass-Bottom ViewPlate-96 F TC	CellCarrier-96	CellCarrier-96 Ultra	CellCarrier Spheroid ULA 96	GravityTRAP 96
Well area [mm^2]	33	28	34	34	40 (whole well, u-shaped); 0.4 (well center, flat)	0.8
Recommended working volume Maximum working vol. [µl]	50 – 300 360	50 – 200 / 400	25 – 300 / 392	25 – 300 / 425	50 – 200 / 350	40 – 70 / 100
Bottom material	Polystyrene, TC-treated	Glass	Polystyrene, TC-treated	Cycloolefin, TC-treated	Polystyrene	Polystyrene
Optical thickness of plate bottom* / Thickness of plate bottom [µm]	570 / 900	116 / 175	120 / 190	124 / 190	645/ 1020	684/ 1080
Bottom height [mm]	2.47	0.3	3.31	0.21	3.1	1.45
Refractive index of plate bottom material	1.58	1.51	1.58	1.53	1.58	1.58
Does bottom material support adherence of cells?	✓	✗ Coating necessary	✓	✓	✗ Ultra low attachment “ULA” coating	✗ Ultra low attachment “ULA” coating
Suitable for the following objectives	1.25x Air** 5x Air 10x Air 20x Air 20x Air hNA 20x Water** 40x Air 40x Air hNA	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water 63x Water	1.25x Air** 5x Air 10x Air 20x Air 20x Air hNA** 20x Water** 40x Air 40x Air hNA 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water 63x Water	5x Air 10x Air 20x Air 40x Air	5x Air 10x Air 20x Air 40x Air
Part No.	6005182 (case of 50)	6005430 (case of 40)	6005550 (case of 40), 6005558 (case of 160)	6055300 (case of 50), 6055308 (case of 160)	6055330 (case of 10) 6055334 (case of 40)	ISP-09-001 (case of 10)

Table 5-6: Available 96-well plates from PerkinElmer, their characteristics, and their compatibility with different objectives.

* Optical thickness is calculated as: plate bottom thickness / refractive index of plate bottom material.

** Restricted plate area

Plate type	ViewPlate-384 F TC	CellCarrier-384	CellCarrier-384 Ultra	CellCarrier-1536	ViewPlate-1536
Well area [mm^2]	11	11	11	2.3	2.3
Recommended working volume Maximum working vol. [µl]	10 – 100 / 135	10 – 80 / 118	10 – 110 / 145	3 – 8 10	3 – 8 10
Bottom material	Polystyrene, TC-treated	Polystyrene, TC-treated	Cycloolefin, TC-treated	Polystyrene, TC-treated	Polystyrene, TC-treated
Optical thickness of plate bottom* / Thickness of plate bottom [µm]	120 / 190	120 / 190	122 / 190	120 / 190	57 / 90
Bottom height [mm]	2.9	0.3	0.21	0.4	1.9
Refractive index of plate bottom material	1.58	1.58	1.53	1.58	1.58
Does bottom material support adherence of cells?	✓	✓	✓	✓	✓
Suitable for the following objectives	1.25x Air** 5x Air 10x Air 20x Air 20x Air hNA** 20x Water** 40x Air 40x Air hNA** 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water 63x Water	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water 63x Water	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA** 20x Water** 40x Air 40x Air hNA** 40x Water** 63x Water**
Part No.	6007460 (case of 40)	6007550 (case of 40), 6007558 (case of 160)	6057300 (case of 50) 6057308 (case of 160)	6004430 (case of 20) 6004439 (case of 100)	6004460 (case of 40)

Table 5-7: Available 384 and 1536-well plates from PerkinElmer, their characteristics, and their compatibility with different objectives.

* Optical thickness is calculated as: plate bottom thickness / refractive index of plate bottom material.

** Restricted plate area

Plate type	96 Well Corning Special Optics	384 Well Greiner µClear®	384 Well Greiner SensoPlate™ Plus	384 Well Corning FlatBottom	1536 Well Greiner SCREENSTAR
Well area [mm^2]	32	8.5	8.4	10.3	2.1
Recommended working volume / Maximum working volume [µl]	50 – 300 / 360	10 – 130 / 138	10 – 130 / 145	10 – 100 / 112	3 – 15 / 17.8
Bottom material	Polystyrene, TC-treated	Polystyrene, TC-treated	Glass	Polystyrene, TC-treated	Cycloolefin, TC-treated
Optical thickness of plate bottom* / Thickness of plate bottom [µm]	80 / 127	120 / 190	116 / 175	400 / 635	124 / 190
Bottom height [mm]	3.05	2.72	0.36	2.14	0.5
Refractive index of plate bottom material	1.58	1.58	1.51	1.58	1.53
Does bottom material support adherence of cells?	✓	✓	✗ Coating necessary	✓	✓
Suitable for the following objectives	1.25x Air** 5x Air 10x Air 20x Air 20x Air hNA 20x Water** 40x Air 40x Air hNA 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA** 20x Water** 40x Air 40x Air hNA** 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water 63x Water	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA** 20x Water** 40x Air 40x Air hNA 40x Water** 63x Water**	1.25x Air 5x Air 10x Air 20x Air 20x Air hNA 20x Water 40x Air 40x Air hNA 40x Water** 63x Water**
Part No.	Corning #3614	Greiner #781091	Greiner #781856	Corning #3712	Greiner #789866

Table 5-8: Available plates from various brands, their characteristics, and their compatibility with different objectives.

* Optical thickness is calculated as: plate bottom thickness / refractive index of plate bottom material.

** Restricted plate area

Comparison Operetta, Operetta CLS and Opera Phenix

Table 5-9 shows a comparison of the Operetta, Operetta CLS and Opera Phenix fluorescence excitation options. For details of the Opera Phenix light sources please see ***Table 5-1***.

Dyes	Opera Phenix light source	Operetta CLS Part No. and Excitation band	Operetta Part No. and Excitation band
UV nuclear dyes (HOECHST 33342, DAPI), UV dyes (HCS CellMask™ Blue), UV fluorescent proteins (Sapphire) Cyan fluorescent proteins (CFP, Cerulean)	375/425 nm laser 405 nm laser	HH16000201 HH16000202 355 – 385 nm LED HH16000202 390 – 420 nm LED 435 – 460 nm LED	HH12000301 360 – 400 nm HH12000302 410 – 430 nm
Green fluorescent proteins (e.g. EGFP), green dyes (Alexa Fluor® 488, Fluorescein, Calcein, Rhodamine 110, CellTracker® Green) Yellow fluorescent proteins (e.g. YFP), green dyes (e.g. Alexa Fluor® 532)	488 nm laser	HH16000201 HH16000202 460 – 490 nm LED HH16000202 490 – 515 nm LED	HH12000303 460 – 490 nm HH12000304 490 – 510 nm
Yellow fluorescent proteins (e.g. DsRed), yellow dyes (MitoTracker® Orange, Cy™3) Red fluorescent proteins (mCherry, dTomato), orange dyes (e.g. Alexa Fluor® 568)	561 nm laser	HH16000201 HH16000202 530 – 560 nm LED	HH12000305 520 – 550 nm HH12000306 560 – 580 nm
Red dyes (CellMask™ Deep Red, Alexa Fluor® 633) Far red dyes (Cy™5, DRAQ5™, CellMask™ DeepRed, MitoTracker® Deep Red, Alexa Fluor® 647)	640 nm laser	HH16000201 HH16000202 615 – 645 nm LED HH16000202 650 – 675 nm LED	HH12000307 600 – 630 nm HH12000308 620 – 640 nm

Table 5-9: Comparison of the Opera Phenix, Operetta CLS and Operetta excitation options.

Table 5-10 shows a comparison of the Operetta, Operetta CLS and Opera Phenix fluorescence emission / detection options.

Application / dye	Opera Phenix Emission band	Operetta CLS Emission band Part No.	Operetta Emission band Part No.
Standard UV emission filter. Suitable for CellMask Blue, DAPI, Hoechst 33342. Can also be used for Fura-2, Cerulean, CFP and mTurquoise2.	435 – 480 nm	430 - 500 nm (Default)	410 – 480 nm (HH12000401)
Emission filter with extended range to capture complete emission of UV-fluorophores. Please note that this filter cannot be used simultaneously with 488nm excitation. Optimal for UV fluorescent proteins (Cerulean, CFP, mTurquoise2) as well as CellMask Blue, DAPI, Fura-2, Hoechst 33342.	435 – 550 nm		410 – 530 nm (HH12000402)
Optional filter for FRET applications. Optimal for capturing donor emission of FRET pairs consisting of UV-fluorescent proteins (Cerulean, CFP, mTurquoise2) and e.g. YFP. Optional filter with extended range, suitable to capture Cyan fluorescent proteins (Cerulean, CFP, mTFP1), Coumarin 6.	435 – 515 nm (HH14000341)	460 - 515 nm (HH16000300) 470 – 515 nm (HH16000301) 470 – 540 nm (HH16000302)	460 – 500 nm (HH12000403) 460 – 540 nm (HH12000404)
Standard blue emission filter. Suitable for Alexa 488, Calcein, CellTracker Green CMFDA, EGFP, Fluo-4, Fluorescein (FITC), Rhodamine Green, YFP. Can also be used for Alexa 532 and Sapphire.	500 – 550 nm	500 – 550 nm (Default) 525-580 nm (HH16000304)	500 – 550 nm (HH12000405) 500 – 530 nm (HH12000406) 520 – 560 nm (HH12000407) 530 – 590 nm (HH12000408)
Optional filter for FRET applications. Optimal for capturing acceptor emission of FRET pairs consisting of Cyan-fluorescent proteins (Cerulean, CFP, mTurquoise2) and yellow fluorescente proteins (Venus, YFP, Citrine).		515 – 580 (HH16000303)	
Standard yellow emission filter. Suitable for Alexa 568, Cy3, dsRed, MitoTracker Orange and red fluorescent proteins like mCherry, dTomato and mStrawberry.	570 – 630 nm	570 – 620 nm (HH16000305) 570 – 650 nm Default)	560 – 630 nm (HH12000410) 590 – 640 nm (HH12000412)
Standard red emission filter. Suitable for Alexa 633, Alexa 647, CellMask Deep Red, Cy5, DRAQ5, MitoTracker Deep Red, VivoTag 645. Can also be used for Cy5.5, VivoTag-S 680 and VivoTag 680 XL. Transmission mode (Brightfield, Digital Phase Contrast)	650 – 760 nm	655 – 705 nm (HH16000306) 655 – 760 nm (Default)	640 – 680 nm (HH12000414) 650 – 700 nm (HH12000417) 650 – 760 nm (H12000416)
Optional filter, suitable for VivoTag 680, VivoTag 680 XL, Cy5.5, Alexa 660, Alexa 680, ATTO 680, Cy5.5		685 – 760 nm (HH16000307)	

Table 5-10: Comparison of the Opera Phenix, Operetta CLS and Operetta emission options. Please note that only the closest match is listed.

Standard Protocols

Materials

CellCarrier-384 (PerkinElmer #6007550); SilverSeal aluminium foil (greiner-bioone #676090); 37 % Formaldehyde (Sigma-Aldrich #25,254-9); PBS (Gibco #14040-091); Triton X-100 (Promega #H5142); Tween® 20 (Sigma-Aldrich #P7949); BSA (Sigma #A3059).

Adherent growing cell line; cell culture medium with additives (FBS, L-Glutamine); trypsine; compounds.

Methods

Cell seeding in 384 microplates

Example protocol for seeding of adherent growing cells in CellCarrier-384 tissue culture plates:

- Grow cells in a cell culture flask until they reach sub-confluence.
- Detach cells by trypsin incubation and seed out 5 x 10^3 cells per well (50 µl).
- Incubate at 37 °C and 5 % CO_2 for 18 to 24 h.
- Continue with compound treatment or fixation.

Compound treatment

Example protocol for compound stimulation of cells growing in CellCarrier-384 tissue culture plates:

- Prepare a compound dilution series in full culture medium.
- Remove the medium.
- Add 50 µl compound-dilution per well.
- Incubate cells for the required period (from 15 min up to 3 days).
- Continue with fixation.

Fixation

Example protocol for fixation of cells in CellCarrier-384 tissue culture plates:

- Prepare 4 % formaldehyde solution.
- Discard supernatant.
- Wash cells with PBS.
- Add 35 µl 4 % formaldehyde per well.
- Incubate 20 min at RT.
- Discard supernatant.
- Wash twice with 35 µl PBS.
- Continue with staining or immunofluorescence staining.

Staining of Cells

Example protocol for staining fixed cells in CellCarrier-384 tissue culture plates:

- Discard supernatant.
- Add 35 µl staining solution.
- **NOTE: Some dyes cannot be co-stained and require separate staining.**
- Incubate for the recommended period (1 min to several hours).
- Discard supernatant.
- Wash once with 35 µl PBS.
- Add 50 µl PBS.
- Seal plate tightly with sealing foil.

You may store plates at 4 °C for several days prior to measurement.

Immunofluorescence staining

Example protocol for immunofluorescence of cells in CellCarrier-384 tissue culture plates:

- Prepare:
  - 0.5 % Triton X-100 solution in PBS.
  - 0.05 % Tween® 20 solution in PBS.
  - 3 % BSA solution in PBS.
  - Primary and secondary antibody dilutions in 3 % BSA (1:400 – 1:1000).
- Discard supernatant.
- Add 35 µl 0.5 % Triton X-100 to the wells.
- Incubate for 5 min at RT.
- Discard supernatant.
- Wash cells twice with 35 µl PBS.
- Add 35 µl 3 % BSA.
- Block for 20 min at RT or over night at 4 °C.
- Discard supernatant.
- Add 35 µl primary antibody dilution.
- Incubate for 1 h at RT.
- Discard supernatant.
- Wash three times with 0.05 % Tween® 20 in PBS.
- Add 35 µl secondary antibody dilution.
- Incubate for 1 h at RT in the dark.
- Discard supernatant.
- Wash three times with 0.05 % Tween® 20.
- Perform nuclei staining (see Staining of Cells).
- Seal plate tightly with sealing foil.

You may store plates at 4 °C for several days prior to measurement.

Please note that these protocols presented here are only a selection of a wide range of fixation and staining protocols. All these methods depend on the cell line, the dye, the antibody, and especially on the target, and therefore they have to be adapted for optimal results.

Protocol for Coating of Cell Carrier with Collagen I

Example protocol for coating CellCarrier-384 tissue culture plates with Collagen I.

Materials

CellCarrier-384 (PerkinElmer #6007550); PBS (PAA #H15-002); Collagen (rat) (Roche #11179179001); 0.2 % acetic acid (v / v), sterile.

Methods

Preparation of collagen stock solution

- Dissolve the vial of lyophilized collagen with 5 ml sterile 0.2 % acetic acid (v / v) – concentration: 2 mg / ml.
- Pour the acetic acid onto the lyophilizate and let it stand for several hours until it has dissolved.
- Reconstituted collagen solution is stable at 4 °C until the expiration date.

Coating of CellCarrier-384 plates

- Dilute the reconstituted collagen stock solution to 22 µg / ml with sterile 0.2 % acetic acid (v / v).
- Add 25 µl of the collagen solution per well – Final concentration: **5 µg collagen per cm^2.**
- Incubate for 60 min at RT in the laminar flow hood.
- Remove the collagen solution.
- Wash three times with 50 µl PBS.
- Use plates immediately or leave the plates in the hood to dry and store under sterile conditions.

Plates can be stored for up to two weeks at 4 °C prior to usage.

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.

Binning: A digital image processing term, describing the integration of adjacent pixels into a single larger pixel. For example, in 2x2 binning, 4 pixels are combined to one single larger pixel and their intensities are summed up. This results in higher signal intensity, improved signal to noise ratio, shorter exposure times and thus increased acquisition speed. Binning 2 quarters the image file size (i.e. the effective resolution of the camera will be 1080 x 1080 px), that way saving data space and speeding up the image analysis. However, image resolution will be half the resolution of an unbinned image (i.e. the effective resolution of the camera will be 1080 x 1080 px) in case of correct Nyquist sampling. With binning 1 the full resolution of the camera(s) is used (2160 x 2160 px):1 camera pixel corresponds to 1 pixel in the final image. In order to avoid non-square pixels only symmetrical binning (identical in horizontal and vertical direction) is allowed.Brightfield correction: A flatfield correction exclusively for brightfield images. The bigger the field of view of an objective lens, the higher is the chance that vignetting effects are visible at the image borders. This intensity reduction at the image’s periphery can be corrected by applying a flatfield correction. The Harmony algorithm removes the background profile, which greatly improves the visibility of all cells in a monolayer and especially cells at the well border. In addition it smoothens the visualization in montages and global images. This is also resulting in higher quality analyses of all brightfield images. The correction algorithm requires only one single image.

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).

$$Y = (\text{Bottom}) + \frac{(\text{Top}) - (\text{Bottom})}{1 + \left(\frac{X}{EC_{50}}\right)^{-(\text{Hill coefficient})}}$$

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).

Flatfield correction: Flatfield correction is improving the quality of high content images. The intensity distribution in microscopic images is often not perfectly homogeneous because of vignetting effects (intensity reduction at the image border/corner). This effect mainly depends on the choice of objective, channel and image acquisition mode (confocal/widefield). Vignetting effects may affect visualization of the images, segmentation and analysis of absolute intensities. In the image, each pixel consists of a background and an object intensity part. Both of which are superimposed but need to be corrected separately to achieve optimal results. Hence, two flatfield correction profiles (for background and object intensities) are calculated. Whereas normally only a few images are required for the background correction algorithm (i.e. "basic flatfield correction"), the object intensity correction needs to be more sophisticated as object intensities vary strongly due to e.g. different expression levels. The information from many images is needed to extract the underlying coordinate-dependent illumination profile. Therefore the "advanced flatfield correction" which corrects both, background and object intensities, will not always be available. It is only offered if the inherent quality control is passed. All flatfield correction profiles are derived automatically and simultaneously to each measurement, resulting in homogenously illuminated images, beneficial for both visualization and analysis.

Gamma Correction: Nonlinear intensity enhancement of dark image areas in order to make the structures inside these areas visible.

Global Image: A global image is a montage of all fields in a well. Depending on how many fields are used to form the global image, the resolution experiences a dynamic, software-driven adjustment (binning). Dynamic binning allows the global image to fit into the computer memory while preserving the highest possible level of detail. Both resolution-reduced global images and original full resolution images can be part of one analysis sequence (see Multiscale Analysis).

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 Alignment: The Opera Phenix' fully automated image alignment procedure is correcting any image offsets that might impair the quality of global images, well or plate overviews and PreciScan applications. The correction algorithm is calculated on basis of a proprietary alignment sample attached below the scan table. Images from this durable sample are acquired each time a (test) measurement (not snapshot) is started.

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 Opera Phenix's transmission light source (740 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 multiple wells, e.g. 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.

MultiScale Analysis: MultiScale Analysis is an analysis technique that uses different levels of resolution or "scales" from one measurement. I.e. larger objects (such as colonies) are detected on a binned, low detail, montage image of all image fields in the well (global image). These detected large objects can be used as a region of interest (ROI) for the detailed analysis on the original full resolution images, e.g. nuclei within the colonies. The ROI can spread over multiple image fields. Before Harmony 4.5, image analysis was limited to one image field at a time.

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 on an →overlay, which defines 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: Defines the positions of individual objects in an image. Overlays are used to refer to object positions in further analysis steps and for visualization of detected objects.

Parameter Tuning: Adjustment of the →analysis parameters in order to optimize the image analysis results.

PhenoLOGIC™: PhenoLOGIC is a Harmony plug-in enabling machine learning for image segmentation and cell classification. Instead of relying on user-selected properties to recognize phenotypes or regions, the user can train the software by selecting positive/negative examples in the image. Once the training has been finished, PhenoLOGIC will determine the best parameters to distinguish cellular phenotypes or detect image regions.

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.

PreciScan™: The PreciScan feature offers a fully automated workflow to scan a sample at a low magnification and to automatically rescan only the desired objects/areas with a second measurement at a higher magnification. The selection of fields for the rescan depends on a user-defined analysis sequence which runs as online analysis in the prescan experiment.

Prescan/Rescan: A manual procedure for sequential measurements at different magnifications. It can be used for example to image one whole well using a low-magnification objective (e.g. 1.25x) and visually identify areas of interest in this overview. In a second step a high-magnification objective (e.g. 40x) is selected and based on the low-magnification image, which is displayed in the well layout, the area of interest can be systematically covered with fields for the rescan.

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 of Interest (ROI): A defined region inside objects in which measurements are made in subsequent image analysis steps, e.g. measuring the intensity of a fluorescent marker inside the region. Also just called **region**.

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*]

$$Z' = 1 - \frac{3 * (stddev(\text{Negative Control}) + stddev(\text{Positive Control}))}{\left| mean(\text{Negative Control}) - mean(\text{Positive Control}) \right|} = 1 - \frac{3}{SNR}$$

Contributors

Sebastian Bösch, Karin Böttcher, Matthias Fassler, Angelika Foitzik, Nadine Fricke, Gabriele Gradl, Jörg Hennek, Chris Hinnah, Peet Kask, Lisa Lamb, Emma Langford, Stefan Letzsch, Mandy Malle, Katharina May, Ülo Mets, Sarah Piper, Thora Pommerencke, Hartwig Preckel, Simone Schicktanz, Alexander Schreiner, Elmar Thews and Arndt Zengerling.

References

Opera Phenix™ User Manuals

- Harmony Help Function (built into the Harmony Software)
- Opera Phenix User Manual (including Software Installation Guide)
- Opera Phenix Safety Manual
- Image Analysis Guide

Literature

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs