# HoloMonitor® M4

## Setup and Operation Manual

February 2018, Hstudio 2.7.3, rev. 8



Line measure: 283 µm

Profile Analysis

Peak
21,4 µm

0.0 µm

0    71    142    213    283 µm
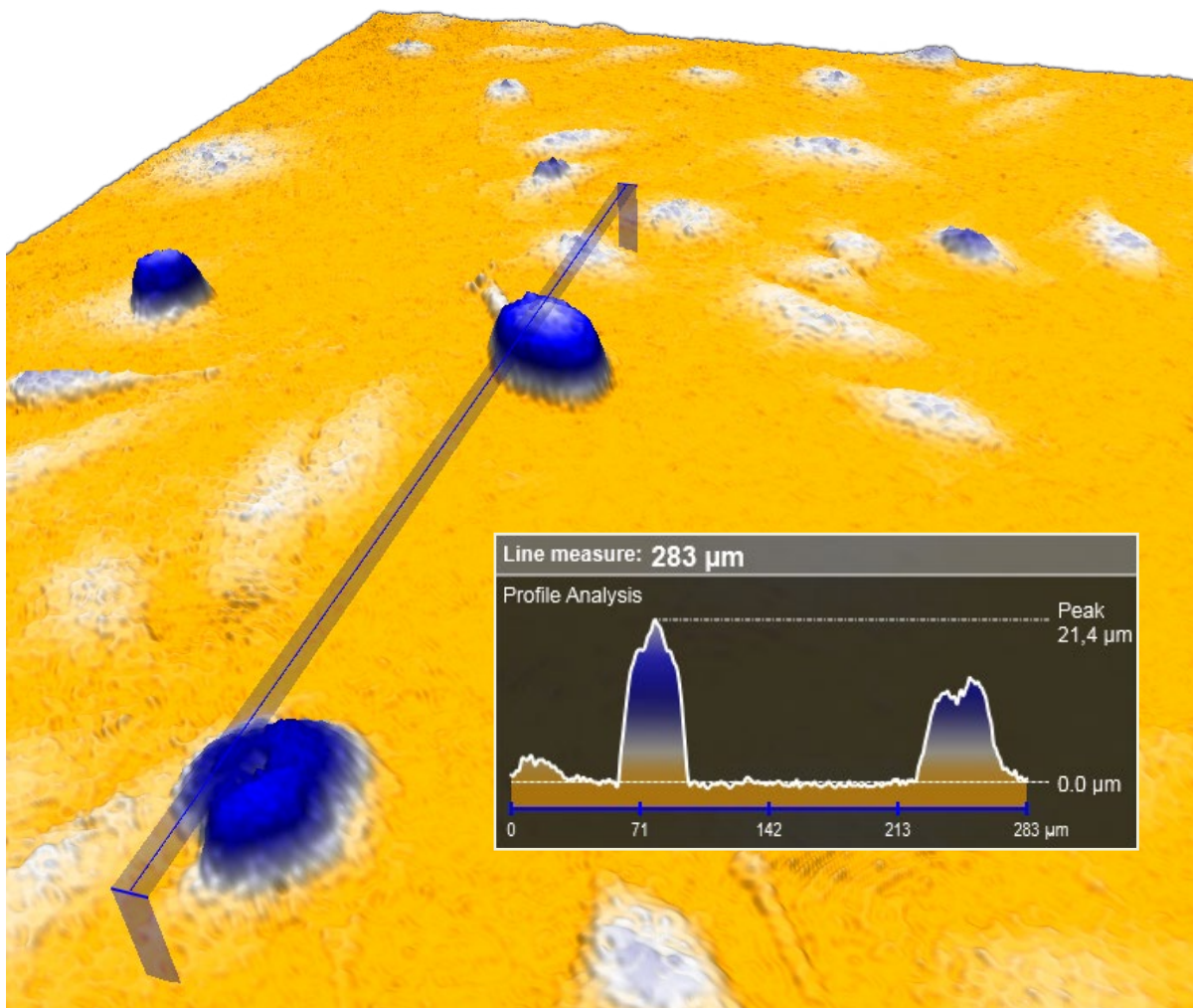
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phi PHASE HOLOGRAPHIC IMAGING

# Contents

# The manual

In the first part of the manual, Instrument, there is information on instrument safety and handling.

In the second part of the manual, Software, there are detailed guides of how to perform different procedures using Hstudio, such as how to capture an image and how to analyze the images.

In the third part of the manual, there are trouble shooting guides for the software and the hardware.

The PDF version of the manual is interactive, with internal links. Clicking the link will refer you to the section of the manual the link refers to. The symbol below is found throughout the manual. **Clicking it will bring you back to the previous view.**

Interactive button for previous view.

# Instrument safety

CE

HoloMonitor M4 was designed and tested in compliance with low voltage directive 2006/95/EC and electromagnetic compatibility 2004/108/EC. References to relevant harmonized standards used, or references to the specification in relation to which conformity is declared, are EN61010-1:2001 safety requirements for electrical equipment for measurement and EN60825-1:2007 safety for laser products.

This manual includes information and warnings which must be observed by the user. It contains information notes as well as information of importance to safety of personnel and property. If the equipment is used in a manner not specified in the manual, the protection provided by the equipment may be impaired.

Symbols are categorized as follows:

Important information.

Risk for physical damage on person or property.

Risk for electrical shock that might cause damage to person or property.

Risk for laser light causing damage to person or property.

# Warranty

The manufacturer guarantees that the instrument has no material or production defects when delivered, according to EU directive 99/44/EC sale of consumer goods and associated guarantees. If the manufacturer is informed within 6 months after delivery, the manufacturer is obliged to rectify such defects. It is the manufacturers decision whether to repair the instrument or replace the instrument with an instrument free of defects. No guarantee is provided for defects caused by natural wear (wearing parts in particular) or improper use.

The instrument manufacturer is not liable for damage caused by faulty operation, negligence or any other meddling with the instrument, particularly the removal or replacement of instrument components, or the use of accessories from other manufacturers. This forfeits all the claims against warranty.

**With the exception of the procedures specified in this manual, no maintenance or repair of HoloMonitor may be performed by users. Repairs may only be performed by specially authorized personnel.**

# Introduction to HoloMonitor® M4

HoloMonitor M4 is a live cell imaging time-lapse cytometer, providing imaging and quantification of unstained living adherent cells directly in their culture vessels. It can be used for a wide range of applications, including:

- Cell count and proliferation

- Cell culture quality assessment

- Cell morphology

- Cell motility and migration

- Cell division/mitosis

- Toxicology

HoloMonitor is based on digital holography microscopy. This technique is based on measurements of how the cells shift the phase of light that passes through the cells, and does not require any kind of labeling or staining.

HoloMonitor is incubator compatible, allowing experiments to be performed in a cell culture incubator or a hypoxia chamber. The cells can be analyzed while growing undisturbed in a range of different cell culture vessels, see www.phiab.se/support/vessel.

HoloMonitor is equipped with a fixed sample stage, or with a computer-controlled motorized XYZ-stage.

**Applications are described in application notes, such as cell motility, cell death, cell cycle and toxicology. Application notes are available at www.phiab.se/publications/application-notes and associated protocols at www.phiab.se/support/protocol.**
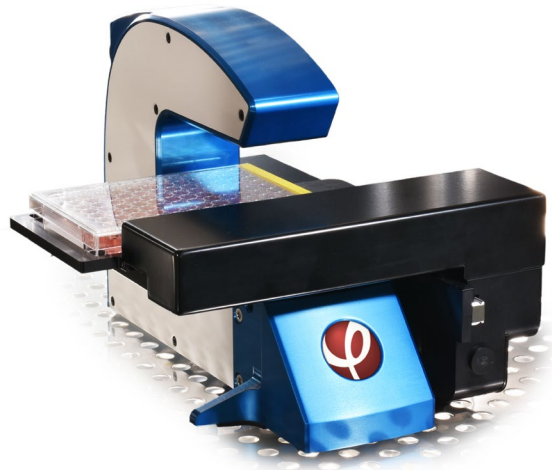
# Computer requirements

HoloMonitor is controlled by the proprietary Hstudio software running on an external computer. Hstudio needs to be installed prior to installation of the HoloMonitor base unit. Make sure the computer fulfills the minimum requirements:

| | |
|---|---|
| Operating system: | Windows 10, 64-bit |
| Processor: | Intel Core i7 |
| Memory: | 16 GB RAM (8 GB minimum) |
| Hard drive: | 556 GB SSD (256 GB minimum) and external USB3 hard drive for backup and transfer |
| Display: | Full HD (1920x1080) or higher |

The files associated with Hstudio are stored in the folder C:\PHI, which must not be used for anything else. User created data is stored under C:\PHI as well. However, the user data location can be changed at any time.

# Hstudio installation

### 1 BACKUP
If Hstudio already is installed, make a backup according to the instructions in Database handling and backup (page 93).

### 2 UNINSTALL PREVIOUS VERSION
Uninstall previous Hstudio version prior to installation. Previously created user data will not be removed. Go to

*Control Panel → Uninstall*
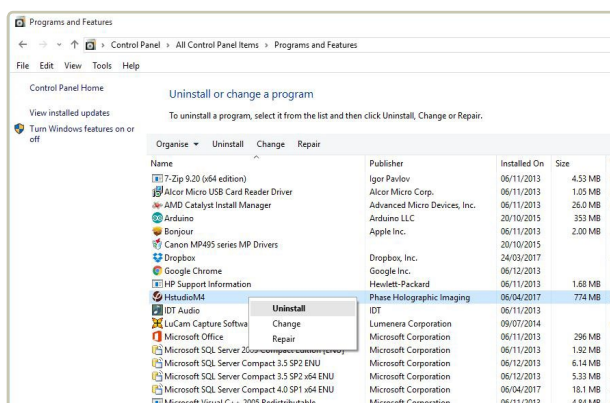


### 3 INSTALLATION
Locate the installation executable file **HstudioM4setup.exe** on the PHI USB memory and double click it to begin installation.

Accept the user account control question by clicking **Yes**.
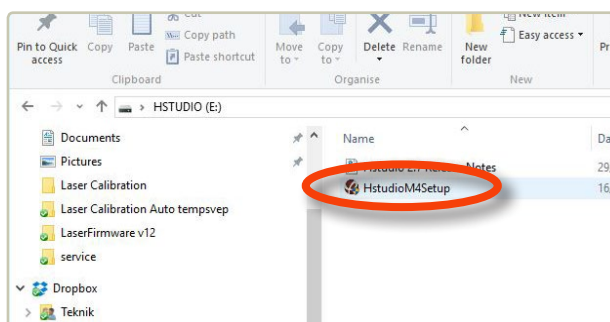
## 4 PREREQUISITES INSTALLATION

The installation will start with prerequisites installation which launches separate installation programs from third-party vendors. Depending on the computer configuration this step may not occur.

Click *Install* to continue and wait for the installation to complete.
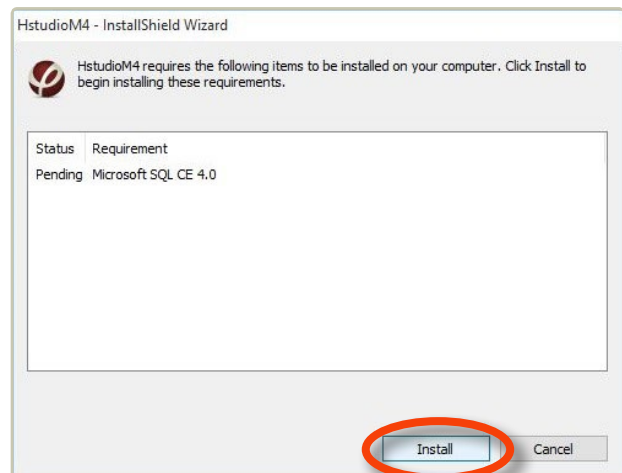
## 5 HSTUDIO INSTALLATION

Progression of installation.

Click *Next* to continue.

Enter your information. Click *Next* to continue.

Click *Install* to start the installation process.
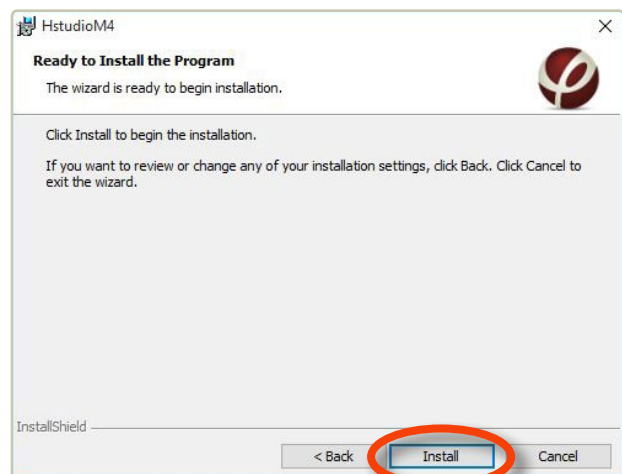
Click *Finish* to complete the installation.
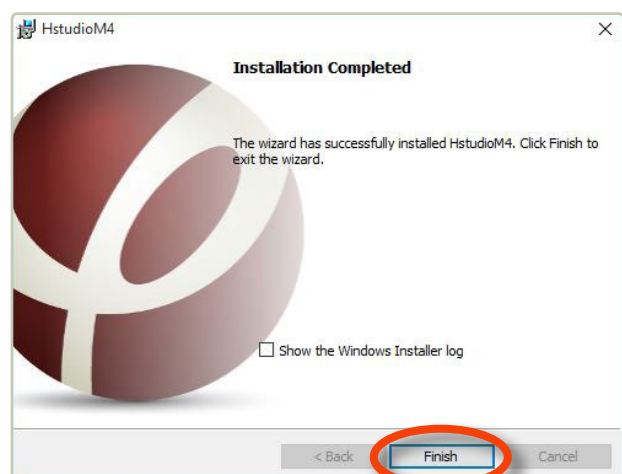
# HoloMonitor installation

HoloMonitor is controlled by a computer running the proprietary Hstudio cell analysis and tracking software, further described in [Introduction to Hstudio 2.7](#) (page 23).



HoloMonitor M4 with a motorized stage contains the following parts:

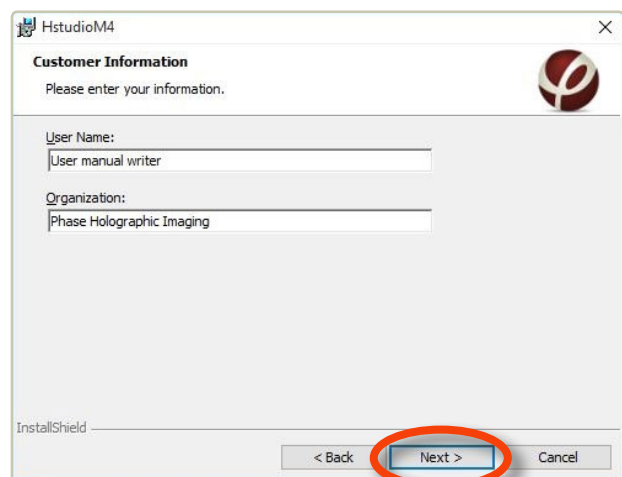| | | | |
|---|---|---|---|
| 1 | HoloMonitor with motorized stage | 8 | Main unit power supply |
| 2 | Objective protective cap | 9 | Laser unit power supply |
| 3 | Laser unit with optical fiber | 10 | Laser unit power adapters |
| 4 | Vessel holders for motorized stage | 11 | HoloLid Petri |
| 5 | HoloLid 6-well | 12 | Cotton swabs |
| 6 | Transportation plate | 13 | USB cables |
| 7 | USB memory with Hstudio | 14 | Spacer plates for fixed stage (not shown) |

## 1  REMOVAL OF TRANSPORTATION PLATE

- Turn HoloMonitor upside down. Let it rest on the protective foam it came in.

- Remove the six screws holding the plate.

- Turn the instrument back (right side up) and remove the tape and protective foam.
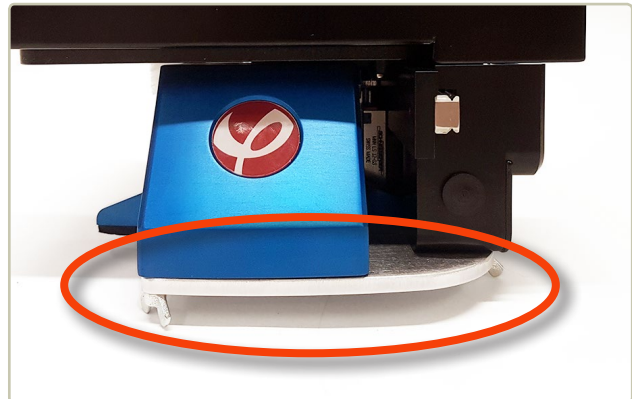


## 2  REMOVING OBJECTIVE PROTECTIVE CAP

- Remove the cap protecting the objective.



## 3  CLEANING OPTICAL SURFACES

- The top of the objective (A) and the object beam window (B) are cleaned with a fresh cotton swab dipped in 70% ethanol.

- Use a dry cotton swab to remove ethanol residues.

## 4 CONNECTING THE LASER UNIT

The optical fiber must be handled gently. **Do not forcefully stretch or bend the fiber. Do not touch the fiber tip.**
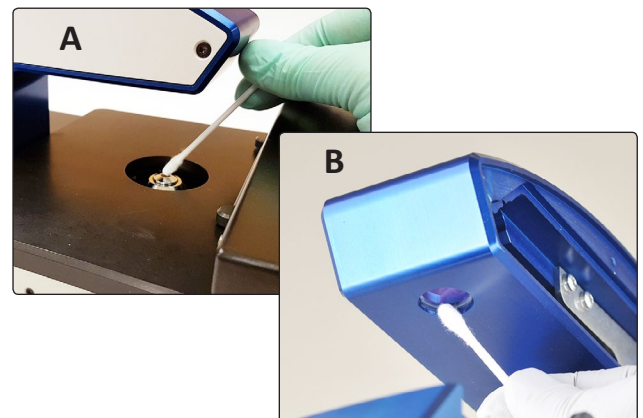
The laser needs approximately 15 seconds to warm up before the laser light is visible. An additional 15 minutes is required before it is fully stabilized.

The HoloMonitor laser unit is a Class 2M laser. A Class 2M laser is safe because of the aversion response, if viewed by a naked eye. However, to avoid any unnecessary discomfort when handling the disconnected optical fiber, **make sure that the laser beam emerging from the fiber does not radiate directly into somebody's eye.**

- Carefully remove the protective cap from the *Optical fiber* tip. Save the protective cap for future use.

- Gently insert the optical fiber tip into the fiber connector. Make sure that the metal protrusion (ridge, yellow arrow) is aligned with the guiding notch in the connector socket (red arrow) before tightening the lock ring.

- Connect the *Laser unit power supply* to the *Laser unit*.

- Connect the laser unit power supply to a power outlet, using one of the *Laser unit power adapters*.

## 5 CONNECTING THE STAGE
- Make sure the motorized stage is connected.
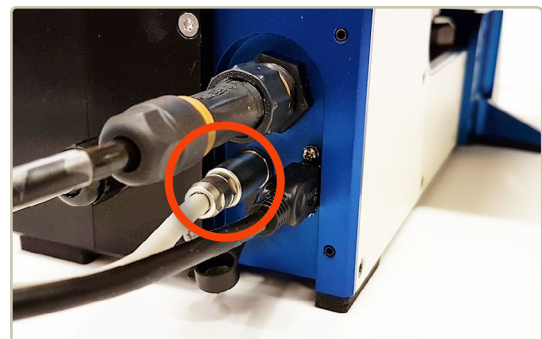
## 6 CONNECTING THE USB CABLE

- Connect a *USB cable* in the micro USB connector.

- Connect the USB cable to your computer.
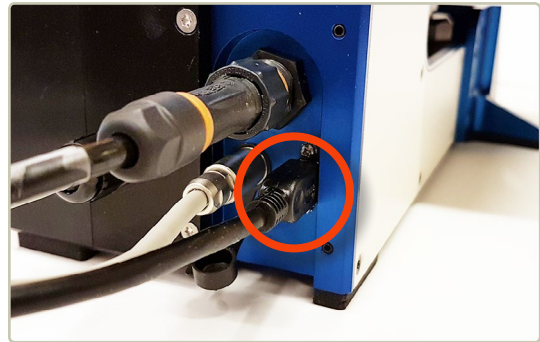
## 7 CONNECTING POWER

- Connect the *Main unit power cable* to the power connector. Make sure the connector is in the correct position before tightening the lock ring.

- Connect the power cable to a power outlet.
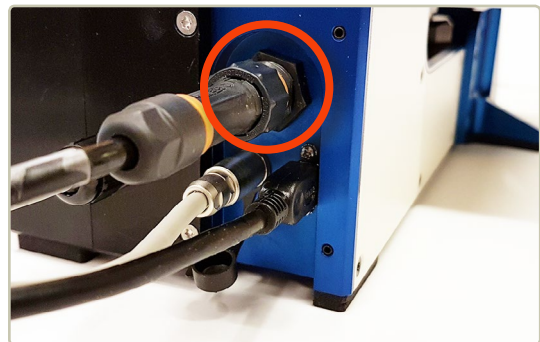
## 8 ATTACHING THE VESSEL HOLDER

HoloMonitor with motorized stage is delivered with three different vessel holders, for microscope slides, standard multi-well plates and for standard Ø 35 mm Petri dishes.

To ensure that the vessel is correctly fitted on HoloMonitor; first attach the cell culture vessel to the vessel holder, then attach the vessel holder to the stage by clicking it on from the side, as seen in the image blow.

# Starting the instrument for the first time

## 1   CONNECT THE INSTRUMENT

Connect the fully assembled instrument to the computer using one of the included USB cables. Drivers will be installed automatically when the USB cable is attached and power is turned on.
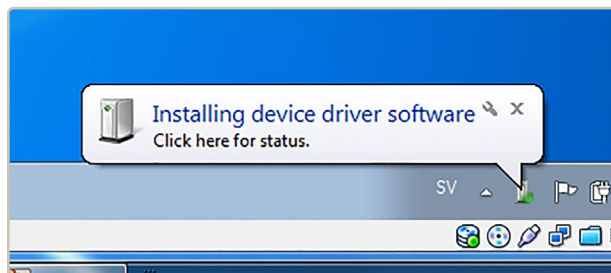
## 2   START HSTUDIO CALIBRATION

Whenever the *Live capture tab* is selected for the first time after starting Hstudio, it will automatically suggest a recalibration.

Select the *Live capture tab*, remove any sample from the stage and click *Continue* to enter the *Calibration wizard*. Follow the Calibration procedure (page 19). The calibration is performed to optimize the image quality and is finalized in approximatively one minute.
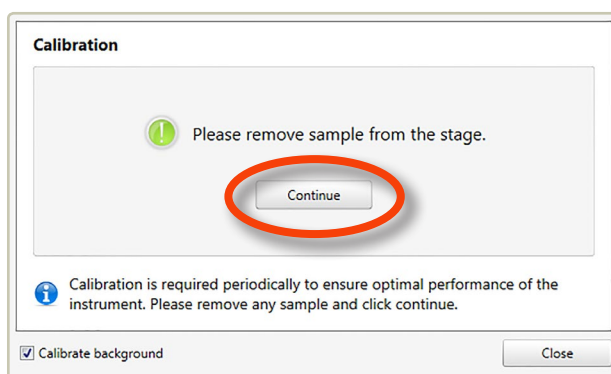
## 3   VIEW LIVE IMAGES

If the calibration is in the green area, the instrument is ready to use. Follow the instructions in section View live images (page 26)

If the calibration is in the red area, go to Troubleshooting (page 98).

  www.phiab.se | support@phiab.se

# Placing HoloMonitor inside a cell incubator

- Clean HoloMonitor according to the Cleaning instructions (page 18).

- Disconnect the *Laser unit power supply* from the power outlet.

- Disconnect the *Optical fiber*, the *USB cable* and the *Main unit power supply* cable from the instrument.

- Put on the protective cap on the *Optical fiber* tip. **Do not touch the fiber tip.**
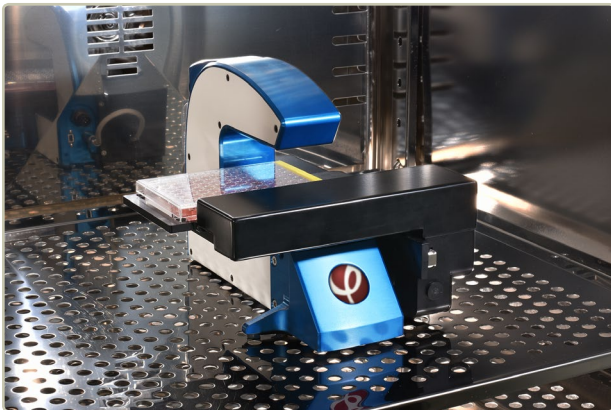


- Place HoloMonitor inside the cell incubator. Make sure there is space for the *Motorized stage* to operate.

- Pull the *Optical fiber*, the *USB cable* and the *Main unit power supply* cable through the access port of the incubator and reconnect them to the instrument.

- Finally, reconnect the *Laser unit power supply* to the power outlet.

- After at least three hours of acclimatization, calibrate HoloMonitor according to the Calibration procedure (page 19) to ensure that the instrument operates optimally before proceeding with any experiment.

Be careful when handling the optical fiber so it is **not stretched, pulled, bent or harmed in any way.**

To avoid build-up of internal condensation, **the power must be switched on at all times while HoloMonitor is inside the incubator.**

**Do not lift HoloMonitor by holding the motorized stage.** Only lift holding the blue and white metal of the instrument.

# Cleaning instructions

## 1 CLEANING OPTICAL SURFACES



- The top of the objective (A) and the object beam window (B) are cleaned with a fresh cotton swab dipped in 70% ethanol.

- Use a dry cotton swab to remove ethanol residues.

- To validate that the optical surfaces are clean, calibrate HoloMonitor according to the Calibration procedure (page 19), to ensure that the *Diagnostic Values* are within the green ranges.

- Repeat cleaning, if the *Diagnostic Values* are not in the green ranges.
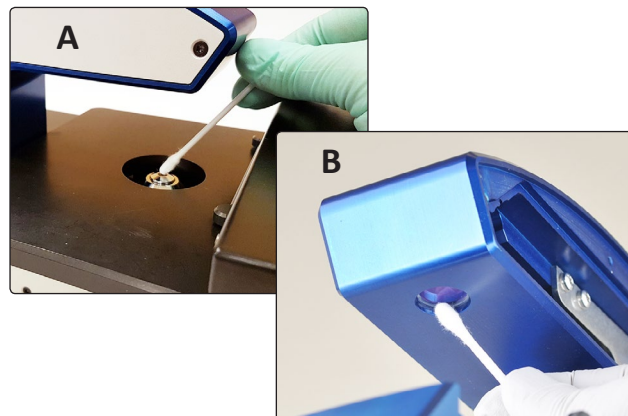
**An instruction video of how to clean the instrument is available at www.phiab.se/support.**

## 2 CLEANING INSTRUMENT EXTERIOR

- The instrument can be wiped down with a damp cloth (ethanol or water). Cleaning must be performed so that no liquid enters the interior of the instrument.

# Calibration procedure

HoloMonitor should be re-calibrated before use. Image calibration gives a background image that is used by Hstudio to lower background disturbances and increase the image quality. The calibration procedure takes approximately one minute.

## 1  PERIODIC IMAGE CALIBRATION

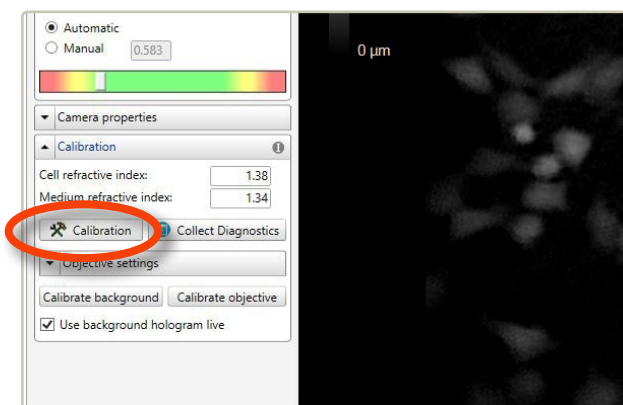Start the calibration procedure by clicking the *Calibration button* in the *Calibration side panel* in the *Live capture tab* in Hstudio.



## 2  CALIBRATION WIZARD

The three *Diagnostic values* measure the image quality. All values should be in the green range. If this is not the case, please follow the [Cleaning instructions](#) (page 18). After cleaning, click *Recalibrate* to refresh the *Diagnostics values*.
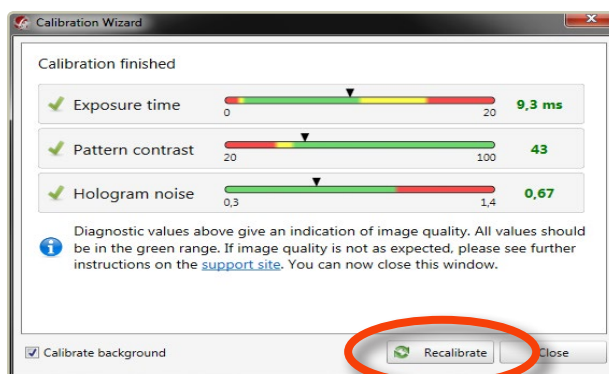


## 3  CALIBRATION WARNING

If Hstudio fails to perform a calibration, a warning will appear. After verifying that HoloMonitor is properly installed and cleaned, click *OK*.
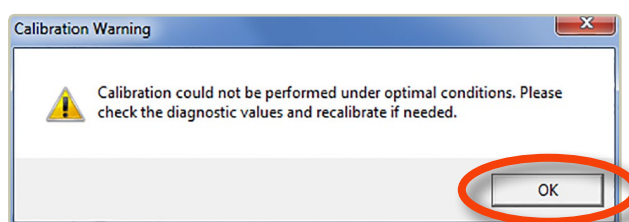


## 4  FURTHER PROBLEMS

If any of the *Diagnostic values* remain outside the green range after several cleaning attempts and overnight drying (see [Troubleshooting](#)), contact the technical support at [support@phiab.se](mailto:support@phiab.se) for further assistance.

# Long-term storage

- Long-term storage of HoloMonitor should be at room temperature outside the incubator. Use a dust cover, if the instrument is not in use for a longer period of time.

- All cords and the laser fiber can be kept inside the incubator at all times if it is inconvenient to remove them. When detaching the laser fiber, use the protective cap of the laser fiber.

- Before re-installing HoloMonitor into the incubator, run the **Calibration procedure** (page 19) to assure that the optical surfaces are clean.

**If the cables are left inside the incubator for a longer period of time, remember to wipe them off regularly with 70% ethanol to reduce the risk of incubator contamination.**

# Sample requirements

## Cells

Mono-layer of adherent eukaryotic cells seeded in any of the recommended cell culture vessels. Eukaryotic suspension cells need to be mounted on a microscope slide, a Nexcelom counting chamber, a single use hemocytometer or a cell culture vessel similar to IBIDI slides.

## Cell culture vessels

For the best imaging quality, use

- 35 mm Sarstedt Petri dishes with HoloLid,

- IBIDI plastic ware (channel slides, chemotaxis slides, wound healing inserts),

- Sarstedt 6-well plates with HoloLid or

- Sarstedts Lumox 24 or 96 well plates with HoloLid.

Glass-bottom Petri dishes are highly recommended. For a complete list of recommended cell culture vessels, see www.phiab.se/support/vessel.

# HoloLids™

The PHI HoloLids have been especially designed to eliminate image disturbances caused by surface vibrations and condensation inside the cell culture vessel.



HoloLids are available in number of different formats. They are made of poly methyl methacrylate (PMMA, Plexiglas), a non-toxic material often used in medical surgery implants, dentures etc. For more information see www.phiab.se/products/hololids.

# Introduction to Hstudio 2.7

Hstudio™ is a specially designed software for capture and analysis of holographic microscopy images using HoloMonitor. Hstudio enables the user to capture both single images and time-lapses. With images captured at regular intervals, time-lapses can be used to study slow cell processes. The procedure is simple. The user captures images of the sample using HoloMonitor, which are analyzed in the Hstudio software. The imaging procedure does not affect the cells in any measurable way.

To simply count cells takes less than a minute. Confluence measurements and morphology measurements are performed simultaneously. To further analyze the cell morphology, either the built in analysis functions can be used, or data can be exported as XML-files for further work in Excel or similar programs. Cell movement and morphology can be tracked over time through a time-lapse sequence for both individual cells and cell populations.

The raw data will remain intact through all analyses, as all changes made by the user only concern how the results are displayed.

Hstudio is divided into seven functional parts that are represented in seven different tabs:
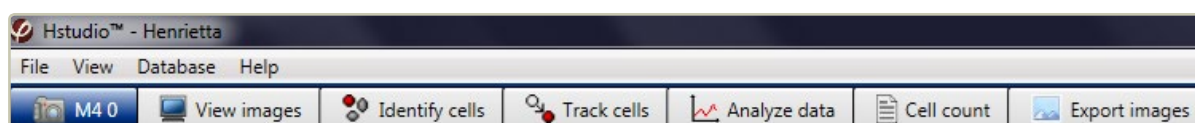


| Live capture | Live cell viewing and capturing of images and time-lapses. |
| --- | --- |
| View images | Viewing of captured images and time-lapses. |
| Identify cells | Image and time-lapse segmentation, resulting in the identification and outlining of the cells. |
| Track cells | Tracking of individual cells in time-lapses. |
| Analyze data | Analysis of cells in images and time-lapses, as well as display and export of the results. |
| Cell count | Counting of cells in images. |
| Export images | Visualization and export of images and time-lapse movies. |

## View area and side panels

The *View area* is the main viewing area where cell images and cell data are shown. Basic functions controlling the *View area* are found in the *Side panels* to the left and right of it:
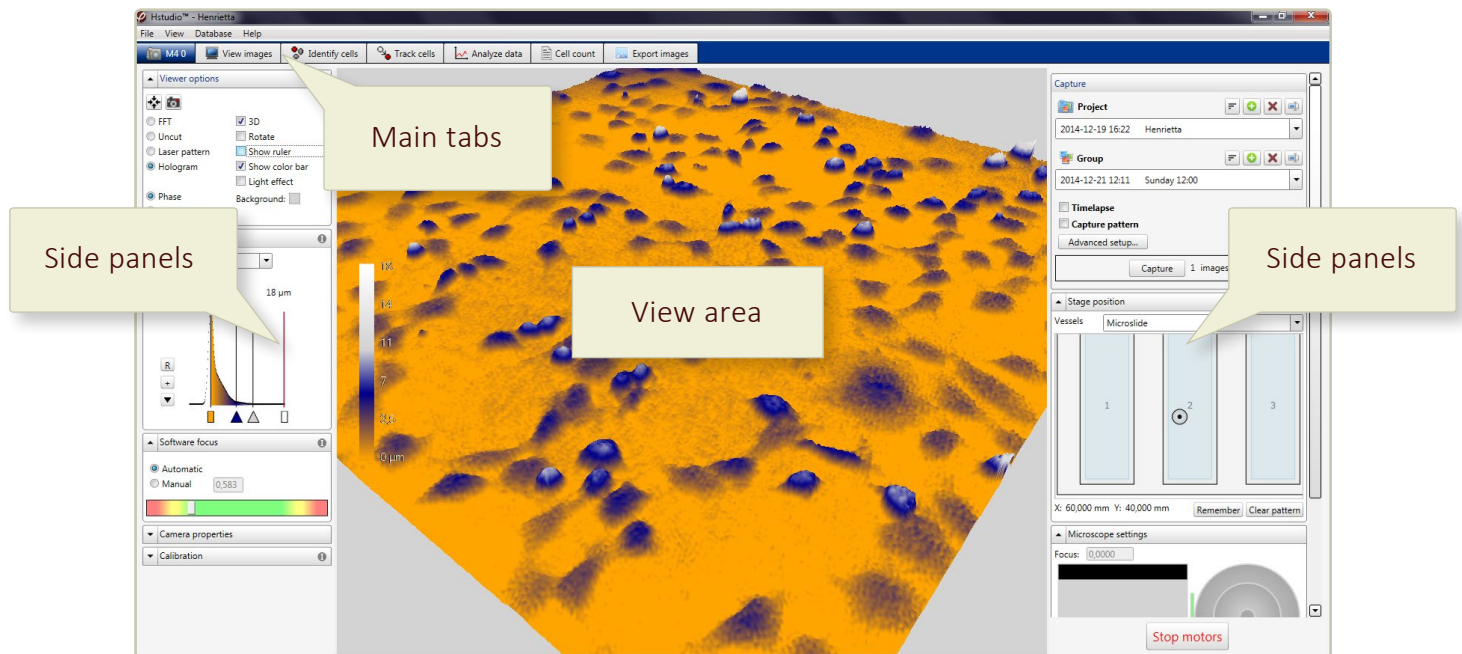


If the side panels are collapsed, they can be expanded by clicking the black arrow tip found in every side panel header:
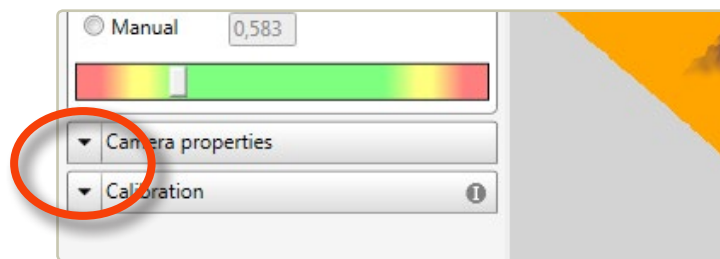


**Information concerning the different side panels can be found by clicking the *Information button* which are placed in the side panel headers:**
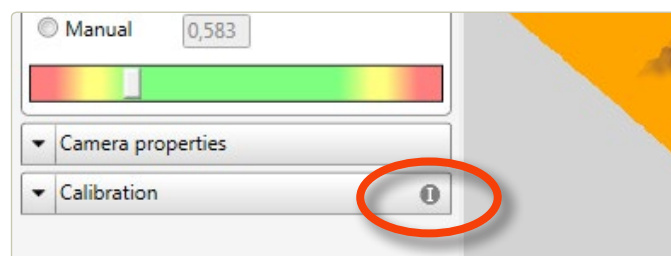
## The Image frame panel

In all the *Main tabs*, except the *Live capture tab*, the right side panel is the *Image frame list*:
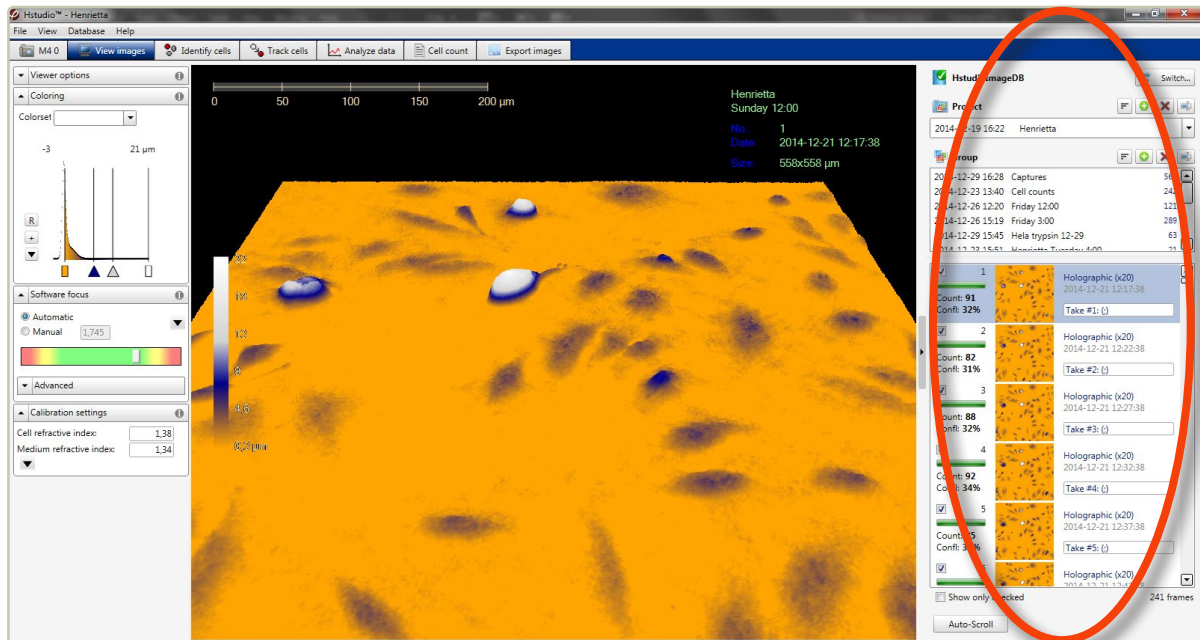


It controls which *Group* of images that is currently being viewed or analyzed. A group of images may either be images captured at various cell culture positions or it may be a time-lapse sequence captured at the same position. Image *Groups* are organized into *Projects* which in turn are stored in a selectable *Database*.

# View live images

## THE THEORY OF FOCUSING

Select the Live Capture tab:



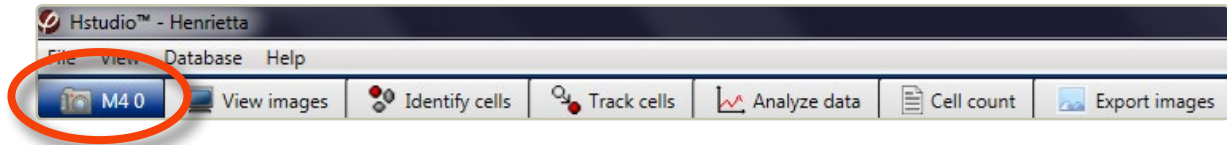HoloMonitor has both a mechanical and a software focus. They need to cooperate in order for the image to be in focus. With the mechanical focus, the user moves the cells to the approximately correct distance from the objective. The software focus then finds and calculates the cell images. The *Software focus panel* indicates how well the computer is able to focus an image, based on the light information that reaches the sensor. When the focus bar is in the middle of the green field, the focus is optimal.
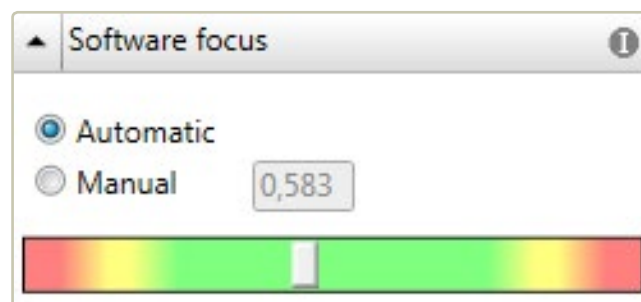


The software focuses the images only when the cell culture vessel is at approximately the correct distance from the objective. The aim is to have the cell surface of the cell culture vessel approximately 1 mm above the object tip.
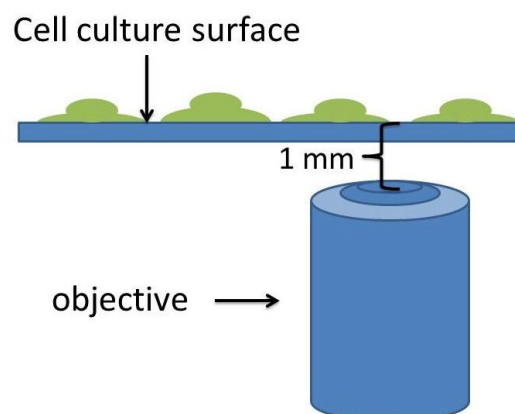
Below, three holographic phase images are shown that are in focus, out of focus or completely out of focus:
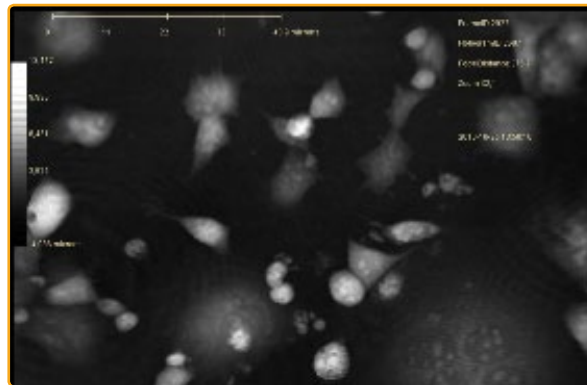


*A holographic phase image that is in focus*



*A holographic phase image that is out of focus*



*A holographic phase image that is totally out of focus*

# PRACTICAL FOCUSING

Put the cell sample on the stage. An image will appear in the *View area*. Ensure that the software focus is set to automatic. If the image is correctly focused, the white marker bar in the *Software focus panel* will be in the green area.
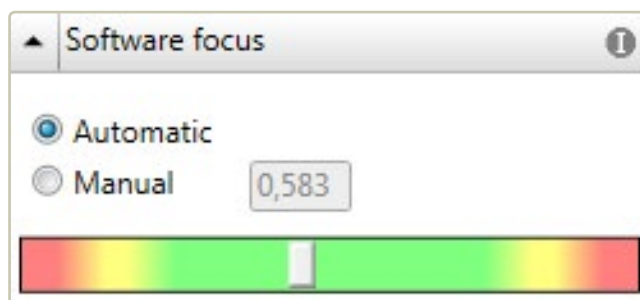


If the image is not correctly focused, the white bar will be in the red. In that case the mechanical focus needs to be adjusted in order for the software focus to be able to work.
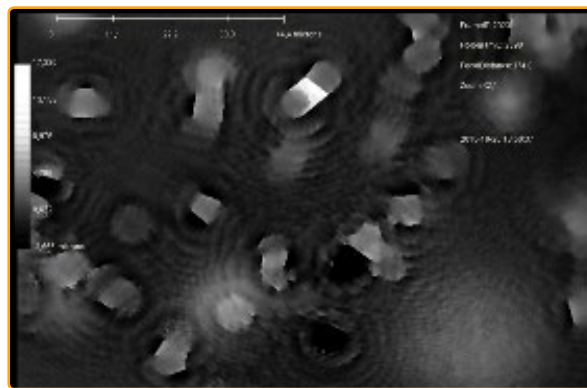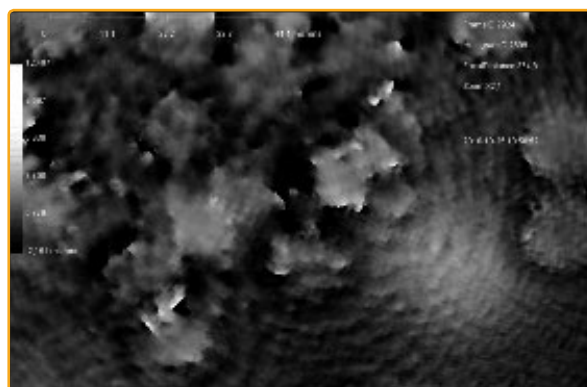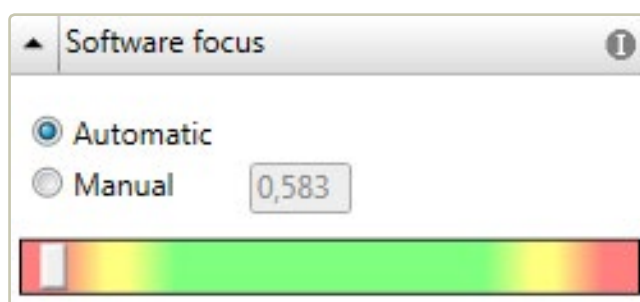


## Focus with a fixed sample stage

One or more spacer plates are used to vertically position the cell culture vessel within the auto focus range. Four spacer plates with varying thickness are provided, 1, 1.5, 2 and 2.5 mm marked with 1 – 4 respectively.

When placing a spacer plate on to the stage, make sure that the two pins on the table are positioned in the holes in the spacer plate.

Choose the correct spacer plate for the presently used cell culture vessel, see www.phiab.se/support/vessel. Then place the cell sample on the stage. An image will appear in the *View area*. If the image is correctly fo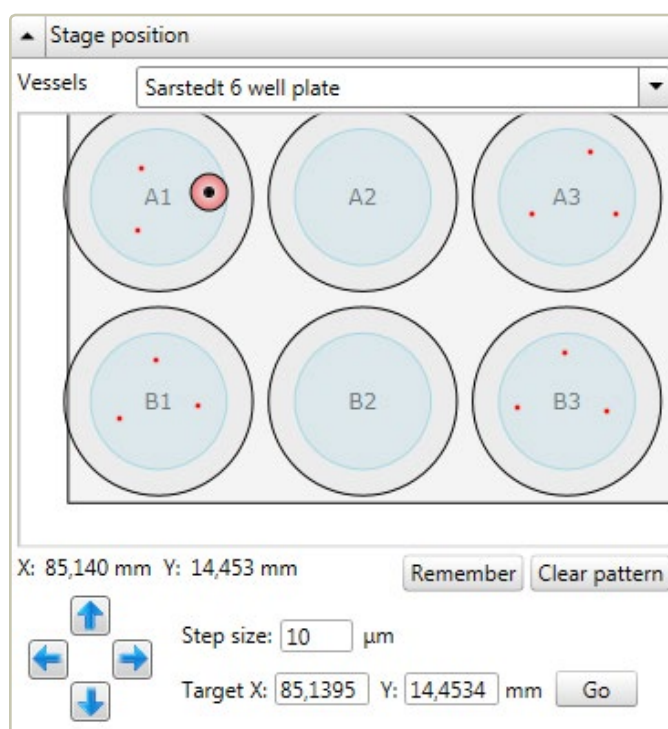cused, the slide bar in the *Software focus panel* will be in the green area. Essentially, the image is focused if the correct distance plate is used. If the image is out of focus, try a different distance plate or a different combination of plates until the software focus is in the middle of the green. The thicker the plate, the further away from the objective the sample will be, and the thinner the plate, the closer to the objective the sample will be.

## Focus with a motorized XYZ-stage

The motorized XYZ-stage is delivered with holders for Sarstedt cell culture vessels.

- Choose the correct vessel holder, insert the sample and snap it on to the stage as described in Attaching the vessel holder (page 15).

- Select the correct vessel template from the *Vessels list* in the *Stage Position side panel*. An image of the selected vessel will appear. The blue area represents the area of the vessel available for image capture:

- Wait for the image to stabilize.

- Make sure that *Automatic* is activated in the *Software focus panel*:
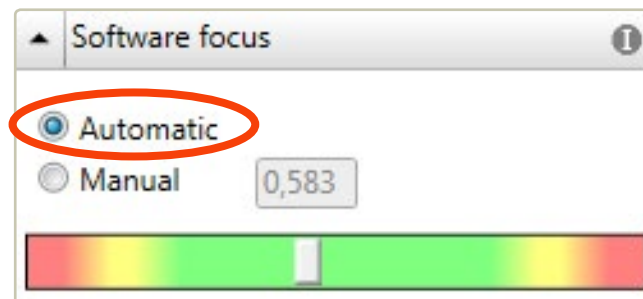


Adjust the mechanical focus using the *Microscope settings panel* which is found to the right of the *View area*. Provided that the cell culture vessel has been correctly selected, focus is within the range shown by the green vertical bar.
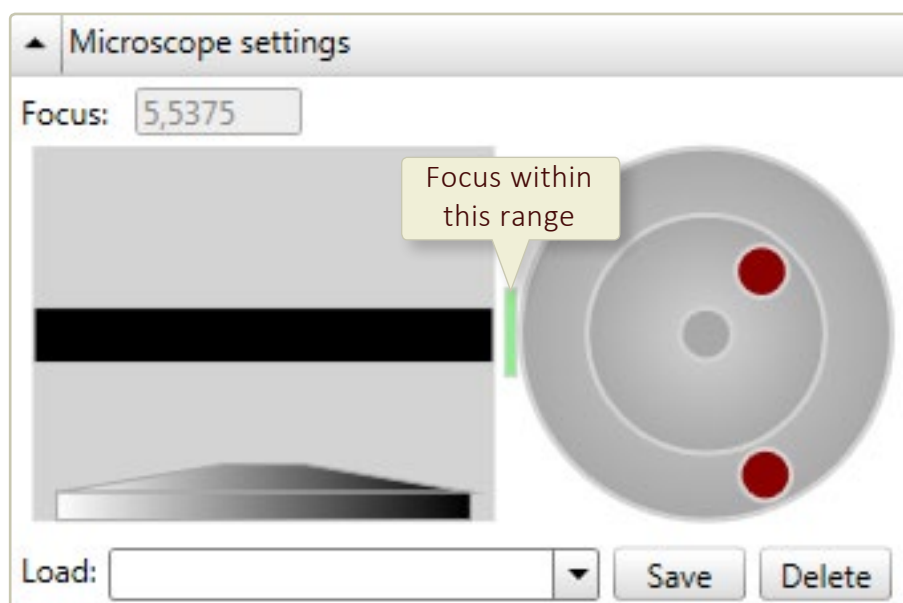


To the left in the *Microscope settings panel* there is an objective representation and a black horizontal bar that represents the sample stage. The mechanical focus distance can be adjusted in large steps by left-clicking, holding and moving the black bar with the mouse cursor. This allows the stage to be moved up and down. The distance between the objective and the sample is shown in the *Focus text box*.

To the right in the *Microscope settings panel* there is a double gray wheel with red knobs which is used for intermediate (outer wheel) and small (inner wheel) focus adjustment steps. The wheels are moved by left-clicking, holding and moving the red knobs.

Keep adjusting the focus until the white rectangle in the color bar in the *Software focus panel* is placed in the center of the green area of the color bar. The mechanical focus is now set to allow the software focus to operate optimally.

### Manual software focusing

Automatic focusing mostly results in well focused images. Some cell samples, e.g. very thin cells, are more demanding and may need to be focused manually.

- Activate *Manual* in the *Software focus panel*:
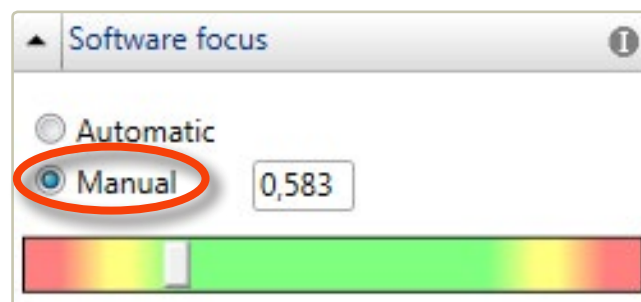


- First the software focus settings must be adjusted. The text box next to the *Manual* button shows the current software focus distance in mm. That distance can be set to a different value by using the colored slide bar beneath the text box. Move the white rectangle until it is in the middle of the green area:
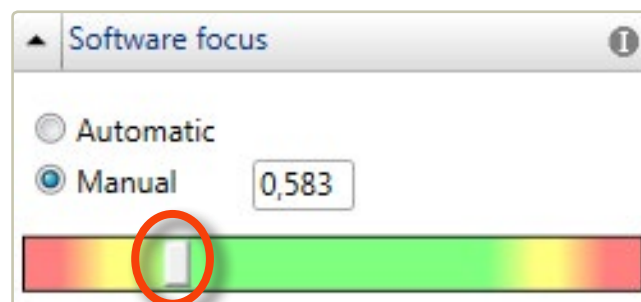


- Thereafter adjust the mechanical focus using the *Microscope settings panel*. As the computer updates the image with small intervals, it is recommended to await the results of one change before making further focus changes. Keep adjusting the mechanical focal distance until the cells are in focus.

## MOVING THE MOTORIZED SAMPLE STAGE

The *Stage position panel*, which is found to the right of the *View area*, is used to move the sample stage:



First select the kind of cell culture vessel currently used, by making a selection in the drop-down *Vessels list*. An image of the selected vessel will appear. The blue area r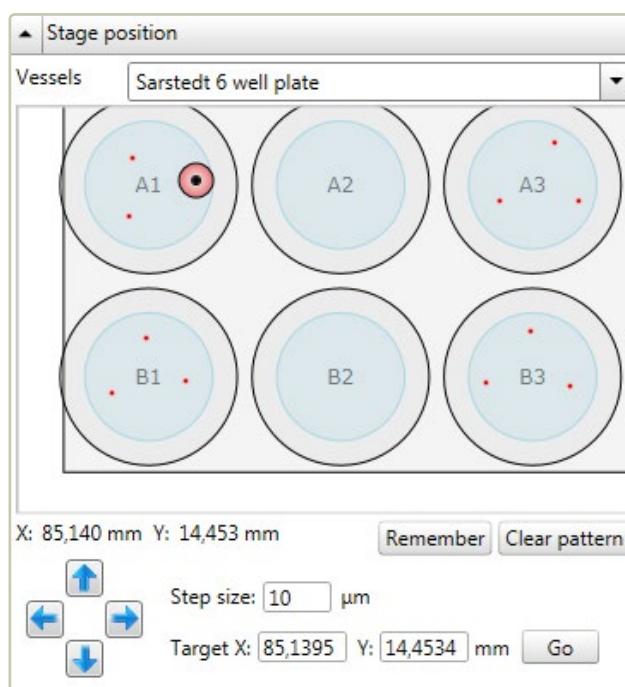epresents the area of the vessel available for image capture. In the vessel representation above, the vessel pattern is made for a 6-well plate.

- The stage is moved in large increments by clicking and dragging the objective representation (i.e. the small double circle) in the *Stage position panel*. Alternatively, place the mouse cursor on the desired position and then left-click. If needed, use the scroll wheel to zoom in on a particular well.

- The stage is moved in *Step size* increments by using the arrow buttons or the arrow keys on the numerical pad.

- The stage is moved in 10 × *Step size* increments by pressing the shift key simultaneously with using the arrow keys on the numerical pad.

- Additionally, the stage can be moved to an absolute position by entering the target xy-coordinates and pressing the Go button.

Every time the stage is moved the image might need a second or two to stabilize.

# CHANGE HOW LIVE IMAGES ARE DISPLAYED

To change how the live holographic image is displayed, use the *Viewer options panel* in the *Live capture tab*:
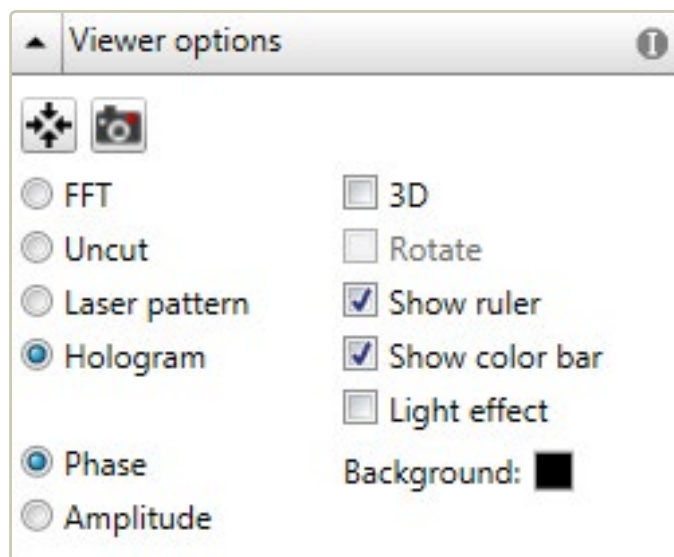


By clicking the ✛ button the image will be centered in the *View area*. By pressing the 📷 button an image of the current view will be saved as an image. The different image display options in the *Viewer options panel* can be activated by checking the boxes. Some boxes can be checked in combination, thus making it possible to combine functions.

| | |
|---|---|
| *FFT* | displays the Fourier transform. |
| *Uncut* | displays the image as it is first created. |
| *Laser pattern* | displays the interference pattern resulting from the merging of the object and reference laser beams. |
| *Hologram* | displays the holographic image which is created from the interference pattern. Either the phase or bright-field image can be displayed: |
| *Phase* | displays the phase image. |
| *Amplitude* | displays the less used bright-field image. |
| *3D* | displays the holographic image in 3D. |
| *Rotate* | automatically rotates the image. |

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*Show ruler*        displays a horizontal scale bar representative of the distance in X and Y.

*Show color bar*      displays a vertical scale bar representative of the height in Z.

*Light effect*        applies an artificial light source to the image which may sometimes render an improved image.

*Shiny surface*      shows the rendered surface with a reflective visual effect. *Shiny surface* is only visible when *Light effect* is checked.

The image below shows a holographic image with cells displayed in 2D and 3D. The color bar represents the height in Z, with yellow showing the background and white the thickest part of the cells.
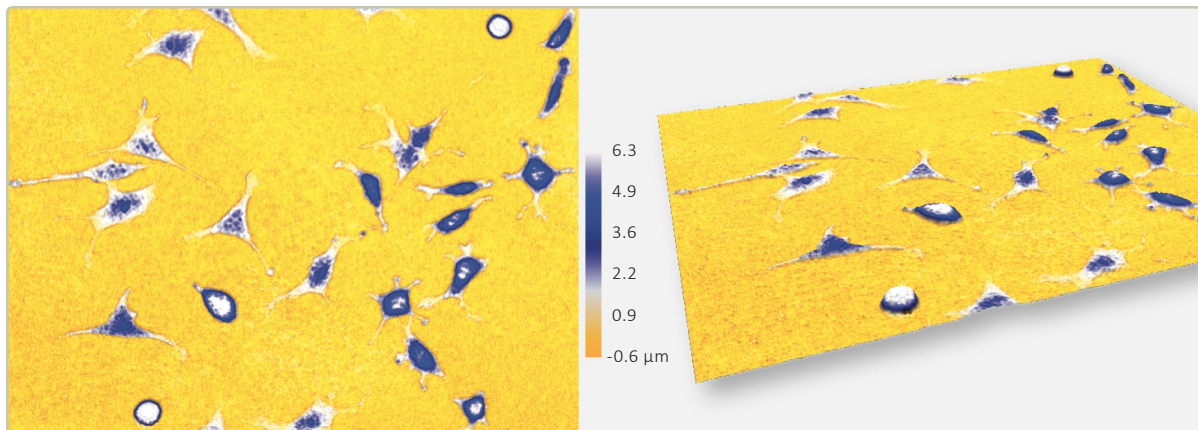


## Move, flip or zoom the holographic image in the view area

- To zoom the live image, left-click the image and then use the mouse scroll button.

- To move the live image, click, hold and drag the image using the left mouse button.

- To flip and move the live 3D image, click, hold and drag the image using the right mouse button.
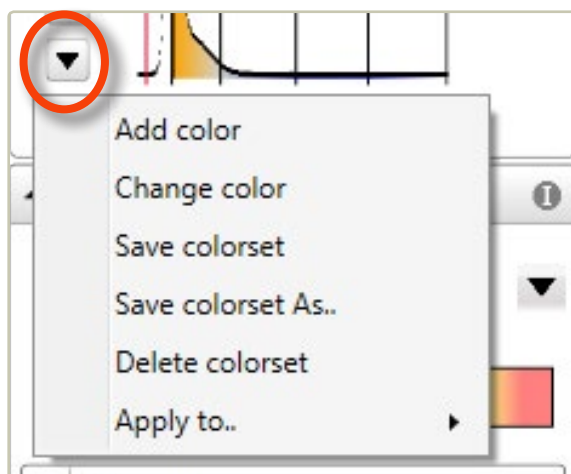
## Change image coloring

All images originally appear in gray scale. If colors are needed or wanted, use the *Coloring panel* to the left of the *View area*. A set of colors that are saved together is called a *Colorset*. A previously saved *Colorset* can be used with the current image by making a selection in the drop-down *Colorset list* which is found at the top of the *Coloring panel*:
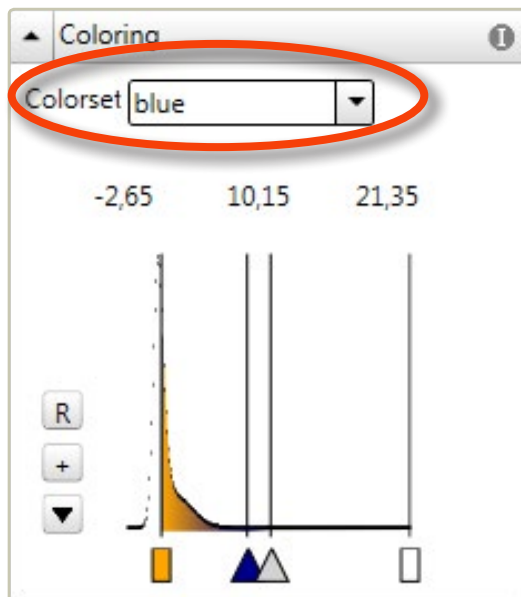


Additional functions are found by clicking the ▼ button in the *Coloring panel*:



- By left-clicking the R button the coloring in the image is rescaled to better utilize the dynamic range of the image. This button needs to be operated every time the image coloring is mis-adjusted.

- A new color can be added to a *Colorset* by clicking the + button and selecting a new color. A colored triangle representing the new color will appear beneath the histogram. Alternatively, right-click on the x-axis and select *Add color*.

- Change the color by right-clicking on a colored triangle beneath the histogram and select a new color, alternatively left-click the ▼ button and select *Change color*.

- To change the color span, left-click and move the desired *Color triangle* ▲▲ beneath the histogram.

- To save a new *Colorset* with the current color settings, left-click the ▼ button and choose *Save as*.

- To save the current color settings to a previously saved *Colorset*, left-click the ▼ button and choose *Save*. Note that this will overwrite the settings previously saved.

- To delete a *Colorset*, select it in the drop-down *Colorset list*. Then left-click the ▼ button and select *Delete colorset*.

## CHANGE DEFAULT CELL AND MEDIUM REFRACTIVE INDEX
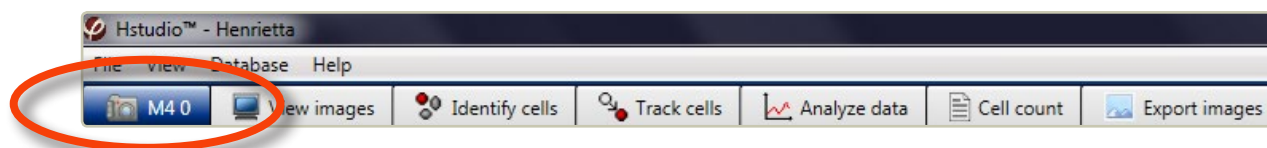
As described in Morphological parameters (page 106), Hstudio uses the difference in refractive between the cells and the medium to calculate cell thickness in µm. The default refractive index values are changed in the *Calibration side panel*:
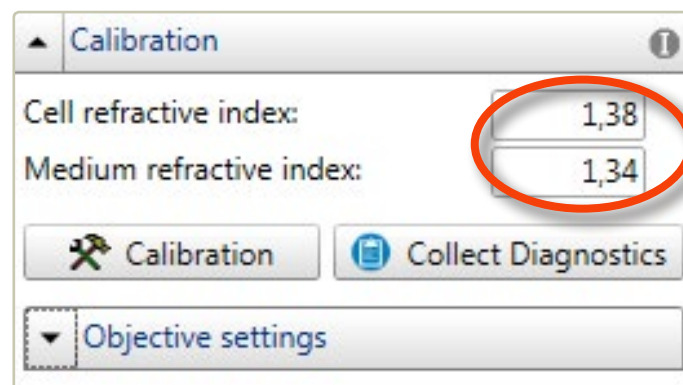
# Capture images

Select the *Live capture tab*:



Before images can be captured, a place of storage must be prepared. The images must be stored in a *Group* within a *Project*. Go to the *Capture side panel*:
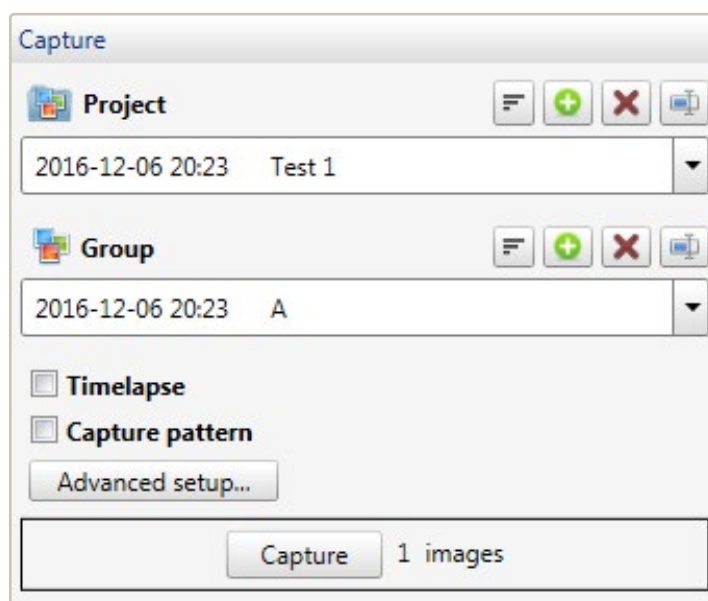


Either open an existing project in the drop-down *Project list* or create a new project by using the ⊕ button. Then open an existing group or create a new group where the images will be saved. When a new project or group is created, the date and time are automatically included in the name. Projects and groups can be sorted by name or date, using the ⊟ button.

## CAPTURE A SINGLE IMAGE

- Place a cell sample on the stage. A live image will appear in the *View area*.

- Make sure that you are satisfied with the quality of the live image.

- Select or create a *Project* and *Group*.

- Click Capture .

## CAPTURE A TIME-LAPSE SEQUENCE AT ONE POSITION

To enable slow events to be recorded and studied, a movie can be created from images captured at intervals, i.e. a time-lapse movie. To capture a time-lapse sequence check ☑ *Time-lapse*:



- Place a cell sample on the stage. A live image will appear in the *View area*.

- Make sure that you are satisfied with the quality of the live image.

- Select or create a *Project* and *Group*.

- Enter the total time for the time-lapse and select the desired time unit (seconds, minutes or hours).

- Enter the interval between the capture time points and select the desired time unit (seconds, minutes or hours). The minimum interval between captures is given to the right of the interval time unit.

- The number of time-points will be given when total time and interval are entered.

- Click Capture . While the experiment is running, the capture progress will be displayed. The experiment can be paused or canceled at any time.

| Capture progress | |
|---|---|
| Capture complete: | |
| 1 % | |
| Remaining Captures | 120 |
| Remaining Timepoints | 120 |
| Time to next capture | 4 min, 53 sec |
| Pause | Cancel |
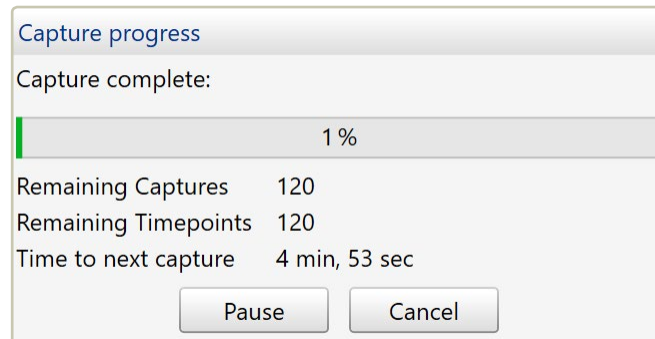
**Imaging speed**

HoloMonitor can take a maximum of 240 images per hour. HoloMonitor does not impair the normal growth of your cells as long as the maximum speed is not exceeded.

## CAPTURE TIME-LAPSES AT SEVERAL POSITIONS IN PARALLEL

When HoloMonitor is equipped with a motorized stage, multiple time-lapses may be captured in parallel by combining a ☑ *Timelapse* with a ☑ *Capture pattern*:

| Capture | | |
|---|---|---|
| 🖼 **Project** | ⊟ ⊕ ✖ 🖳 |
| 2016-12-06 20:23    Test 1 | ▾ |
| 🖼 **Group** | ⊟ ⊕ ✖ 🖳 |
| 2016-12-06 20:23    A | ▾ |
| ☑ **Timelapse** | |
| Total time | 2 | hr ▾ |
| Interval | 10 | min ▾  (Min interval 0,6) |
| Number of timepoints  13 | |
| ☑ **Capture pattern** | |
| Selection | Clear pattern |
| Advanced setup... | |
| Capture    169  images | |
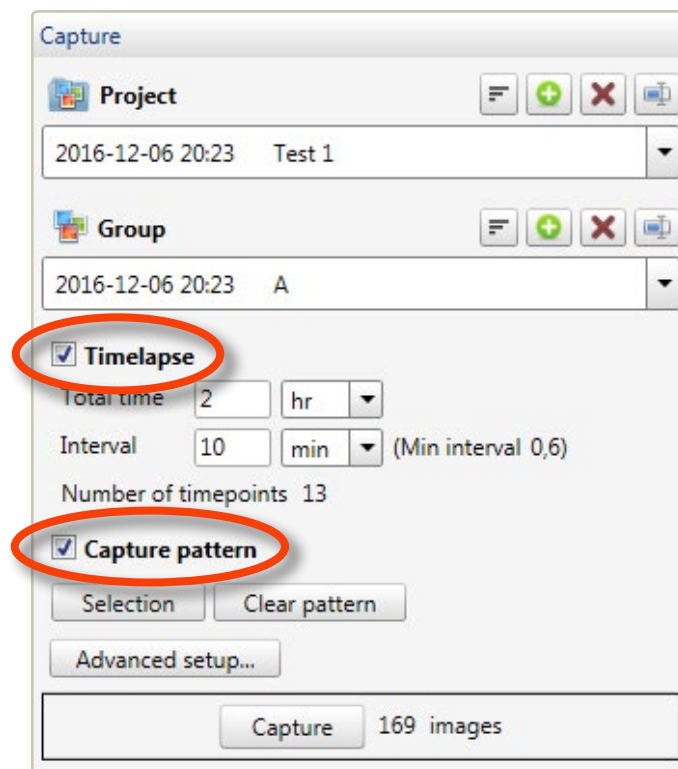
## Creating a capture pattern

- Start by selecting the appropriate cell culture vessel in the drop-down *Vessel list* in the *Stage position panel*:
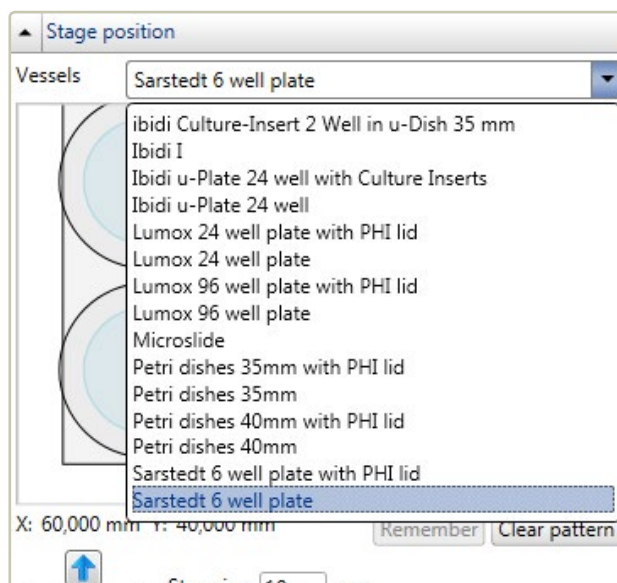


- Select and move the stage to the desired capture position by left-clicking in a blue area.

- Ensure that the cells are in focus. If the cells are not in focus, adjust the focus as described in The theory of focusing (page 26). When a capture position is selected, the current Z-position of the stage will be saved and used when the position is imaged. It is therefore important that the cells are in focus when saving a capture position.
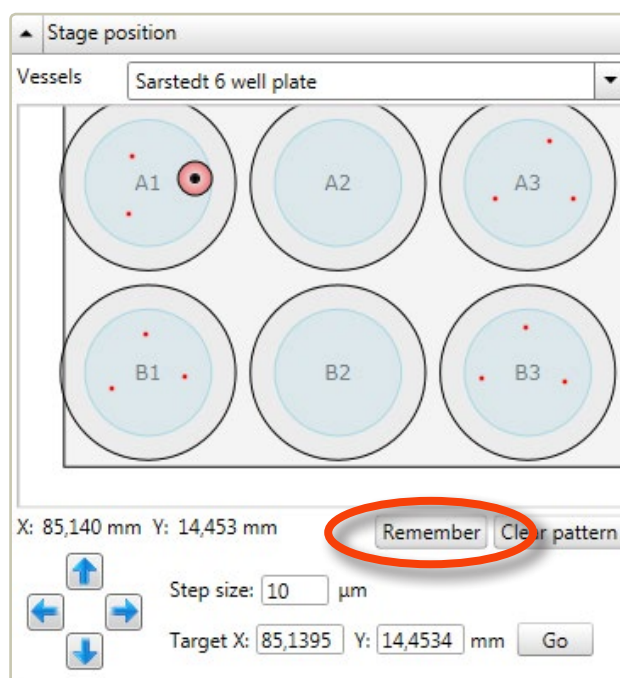
- Click the [ Remember ] button to save the current position and focus to the *Capture pattern*. A saved capture position is indicated by a small red dot.



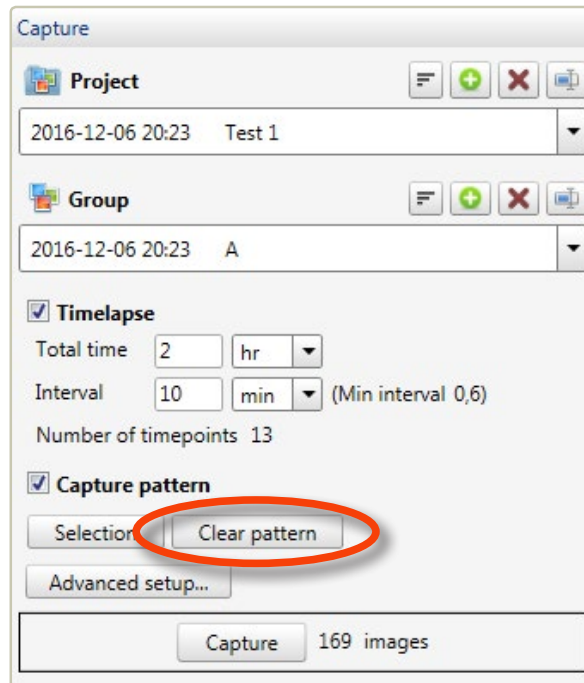- Repeat selecting capture positions until all desired positions have been selected.
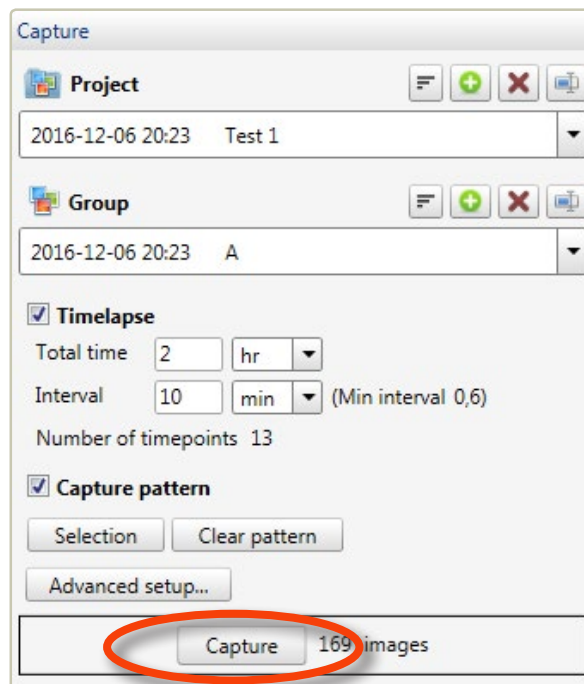
## Clearing capture positions

To delete a created *Capture pattern*, click  Clear pattern  :



## Capture images

Click the  Capture  button to start the image capturing sequence:



Once capture begins, the stage will go through the selected positions one by one and repeatedly capture an image at each position.

## SAVING TIME-LAPSES IN SEPARATE GROUPS

By default, all time-lapse images from the selected *Capture positions* are saved in the same *Group*. However, by clicking  Advanced setup…  it is possible to specify in which group a specific time-lapse should be saved. In the example below, the two time-lapses captured in each well will be saved in the same group. Well A1 and A2 are saved in new groups while the other four wells are saved in existing groups.
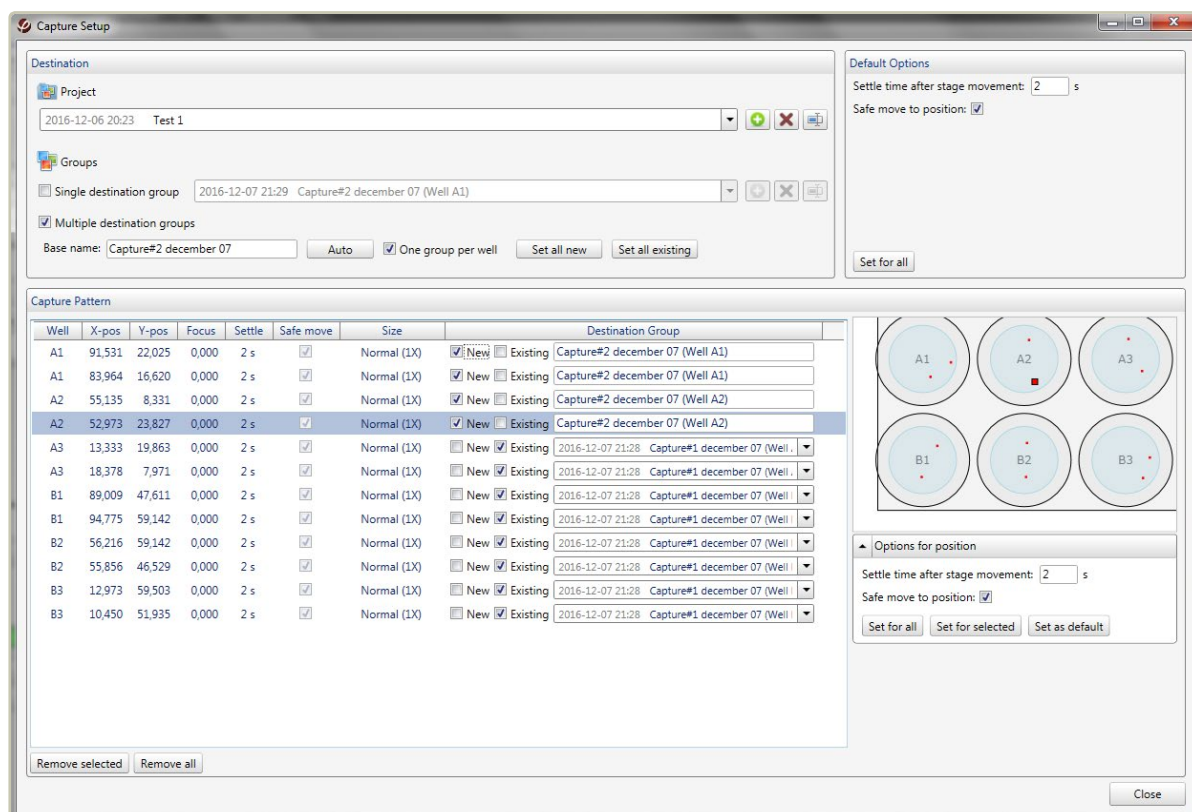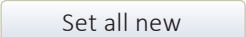


When checking ☑ *Multiple destination groups*, time-lapses will be saved in separate groups. When ☑ *One group per well* is checked as well, time-lapses from the same well are set to be saved in the same group.

The suggested new group names may be changed by changing the *Base name* or by individually changing the group names. By checking ☑ *Existing* it is possible to select an existing group from the drop-down list.

The group names are collectively controlled by five buttons:

| Set all existing | sets all time-lapses to be saved in existing groups. |

| Set all new | sets all time-lapses to be saved in non-existing groups. |

| Remove selected | removes selected capture position. |

| Remove all | removes all capture positions. |

| Auto | brings back the auto-generated names. |

## Movement Settings

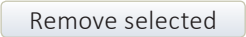*Settle time*  Surface vibrations degrade image quality. After stage movement, the media surface is therefore allowed to settle down before image capturing. The needed time depends on the cell culture vessel type. Typically, 2 seconds are sufficient. However, some vessels may require longer time. Petri dishes, for example, need a settle time of 5 seconds.

*Safe move*  Some cell culture vessels require that the stage is lifted when moving to a new position.

These settings are controlled in the *Default options panel* and in the *Options for position panel*, which appears when a capture position is highlighted in the *Capture pattern window*.

## Close

To save all the settings, click  Close  .

# AUTOMATICALLY GENERATE RANDOM CAPTURE POSITIONS

Instead of selecting each position individually, it is possible to automatically generate *Capture positions* by clicking <kbd>Selection</kbd> and activating *Select wells* in the *Capture panel*:
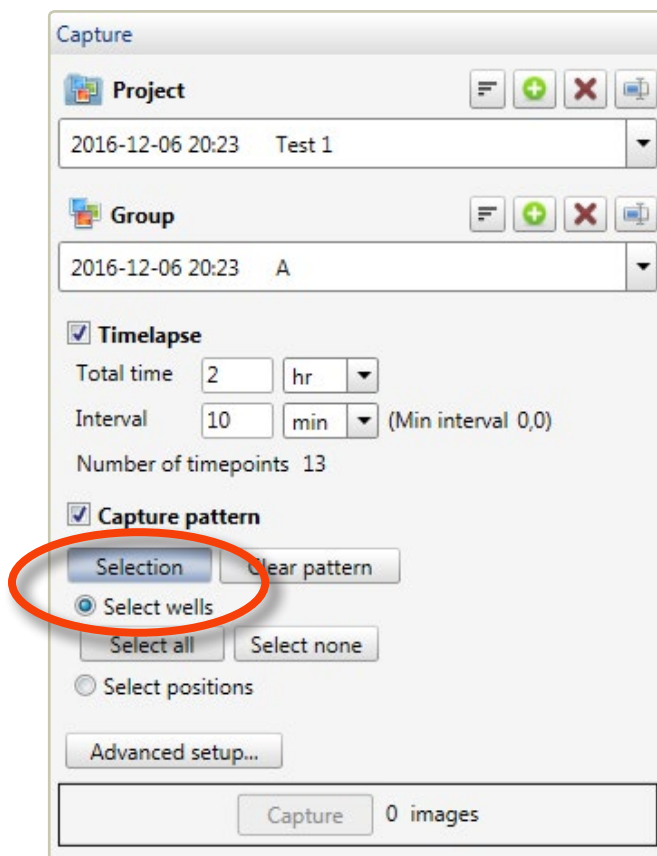


## Select wells
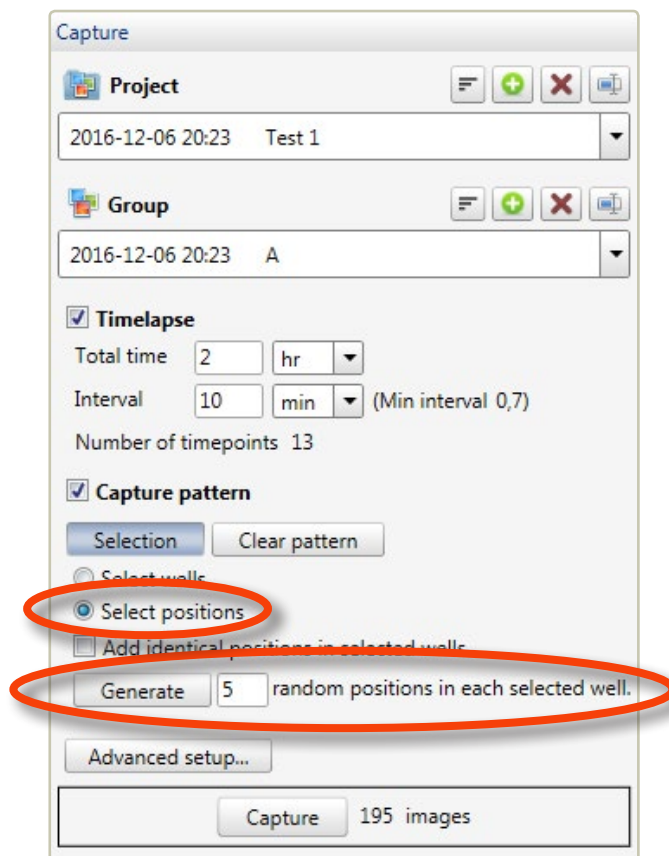
First the relevant wells need to be selected. This is done by clicking on the wells in the graphical representation of the wells, which appears to left of the *Capture panel*. In the example below, wells A1-A3 are selected.

## Generate Random capture positions

To randomly generate *Capture positions,* activate *Select positions* and click Generate :



This will generate the specified number of random positions in the selected wells:



When ☑ *Add identical positions to selected wells* is checked, the same random positions will be generated in each well. The graphical representation is closed by clicking the Selection button again.

**When positions are generated randomly, the current Z-position of the stage will be saved and used for imaging of all positions. It is therefore important to verify focus.**

# ADD CAPTURE POSITIONS MANUALLY

Activate *Select positions* in the *Capture panel*:



Once *Select positions* has been activated, capture positions can be generated by simply clicking in the blue areas in the graphical representation of the wells which appears to the left of the *Capture panel*:
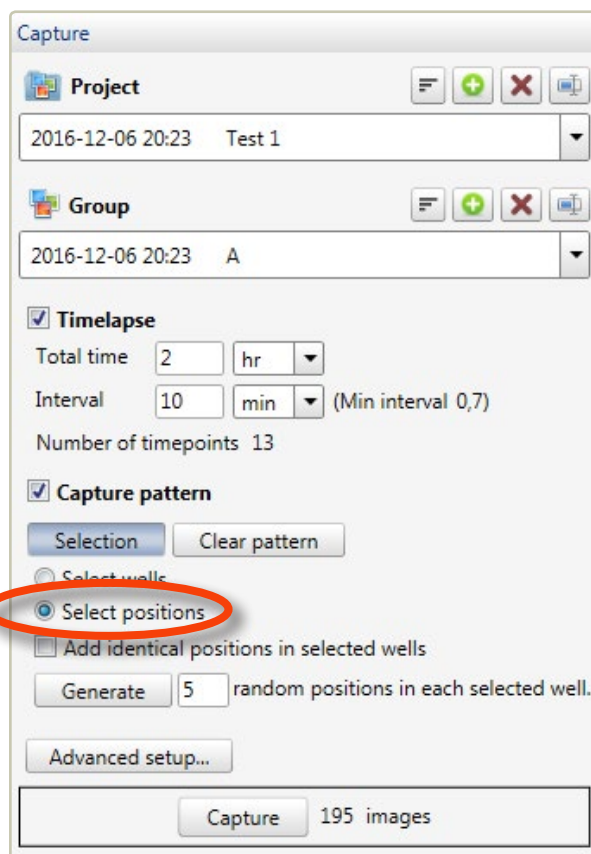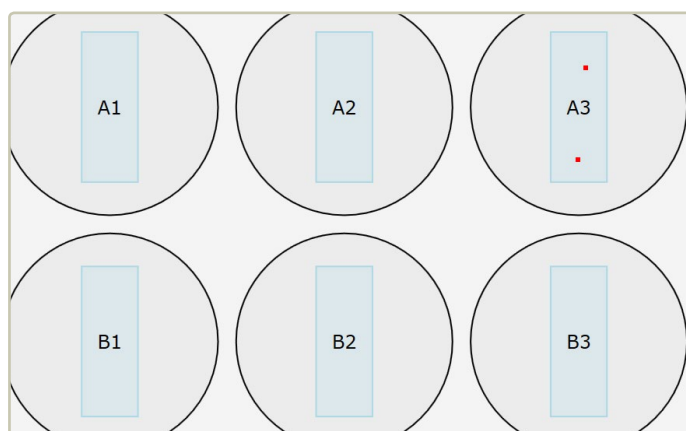


**When positions are generated by clicking, the current Z-position of the stage will be saved and used for imaging of all positions. It is therefore important to verify focus.**

# FOCUS VERIFICATION

Before capturing images, its important to verify focus. When hovering over a capture position, the dot expands to a square:
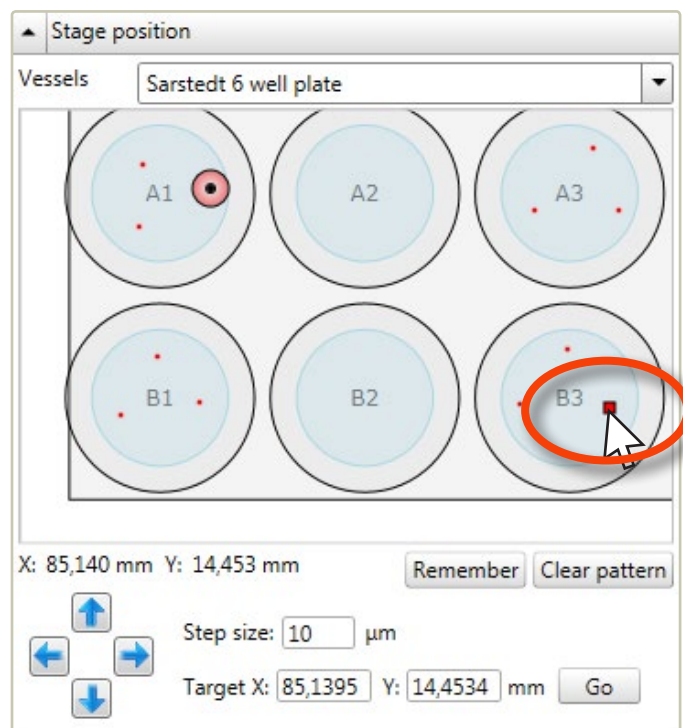


When the square is clicked, the stage moves to that position. It will be imaged and displayed in the *View area* at the Z-position that will be used when images are captured. Verify a sufficient number of capture positions to ensure that all capture positions are in focus.

# View and adjust captured images

## VIEW AN IMAGE

Go to the *View images tab*:
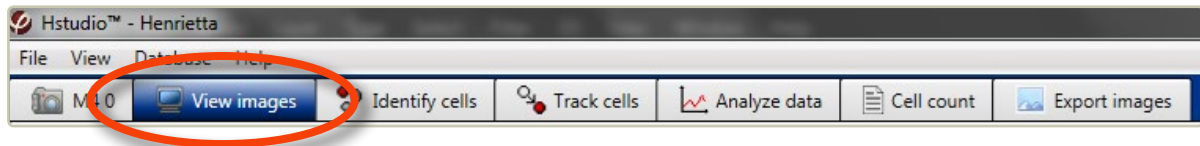


Select a *Project* and a *Group* in the *Image frame list*, found to the right side of the screen:
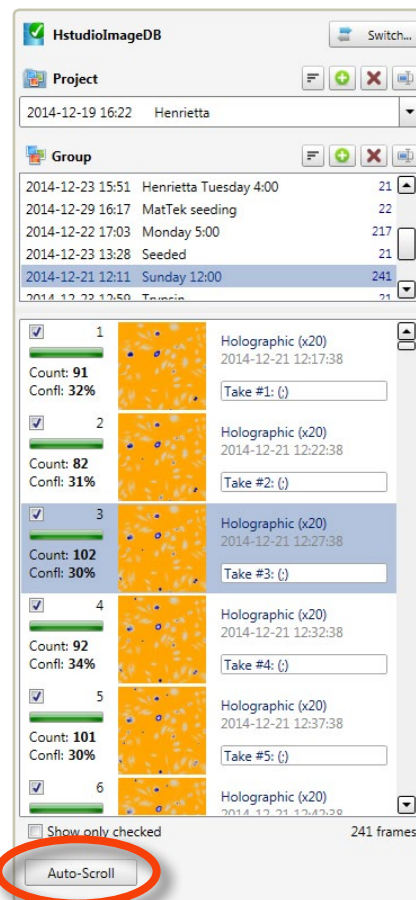


An image frame list for the selected group will appear. Images, as well as information pertaining to the images, are presented in the list. Select an image to view it in the *View area*. Click Auto-scroll to show the image frames as a movie.

# VIEW ONE POSITION OF SEVERAL IN PARALLEL TIME-LAPSES

When several parallel time-lapses have been captured at different positions and then saved in the same *Group*, it is still possible to view the time-lapse for each position separately. The images in the *Image frame list* will be presented in order of capture. If for example 7 positions have been selected, every seventh frame will belong to the time-lapse of one of the positions.

- Select the first of the images at the chosen position.

- Right-click in the *Image frame list* select *Check* and thereafter *Check every X frame*:
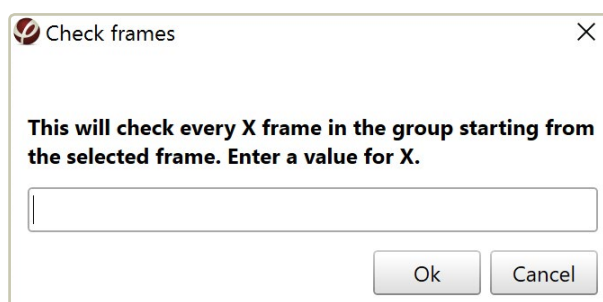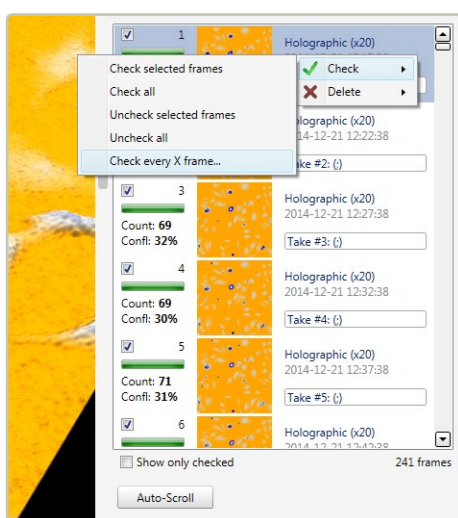




- In the example above, 7 would then be entered in the *Check frames window*.

- After clicking OK, check the ☑ *Show only checked* found below the *Image frame list*. Now, only the checked images will be displayed, and they will belong to the same time-lapse.

# CHANGE HOW CAPTURED IMAGES ARE DISPLAYED

The holographic image display can be modulated using the *Viewer options panel,* found to the left of the *View area*:



By clicking ⊹ the image will be centered in the *View area*. Clicking 📷 saves the currently viewed image. Checking the boxes will activate different options. Most boxes can be checked in combination:
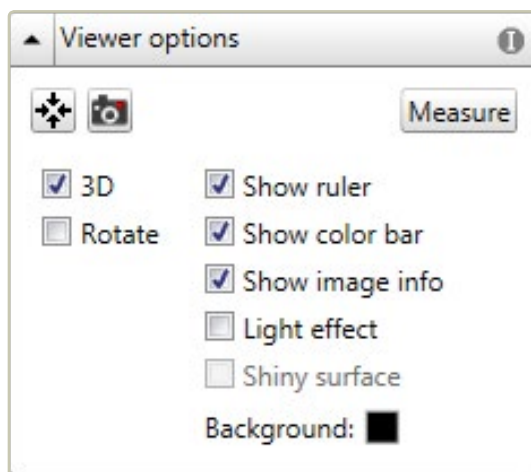
☑ *3D*                     displays the image in 3D.

☑ *Rotate*                 automatically rotates the image. Rotate is only active when 3D has been checked.

☑ *Show ruler*            displays a horizontal scale bar representative of the distance in X and Y.

☑ *Show color bar*        displays a vertical scale bar representative of the height in Z.

☑ *Show image info*      displays additional information associated with the image:

| | |
|---|---|
| *First row* | specifies in which *Project* the image is located. |
| *Second row* | specifies in which *Group* the image is located. |
| *Nbr* | specifies which number the image has in the *Group*. |
| *Date* | specifies the capture date and time of the image. |
| *Channel* | specifies if the image is holographic or phase contrast. |

| ***Size*** | specifies image width and height in μm. |

☑ ***Light effect*** — applies an artificial light source to the image which may sometimes render an improved image.

☑ ***Shiny surface*** — shows the rendered surface with a reflective visual effect. ***Shiny surface*** is only active when ***Light effect*** is checked.

## Change the image coloring

All holographic images will appear in gray-scale. Use the ***Coloring panel***, which is found to the left of the ***View area***, to add pseudo coloring to the images. The colors that are applied will color the image relatively to the thickness of the cells. For additional information on how to set the coloring see Change image coloring (page 35).

## Move, flip or zoom the cell image

- To zoom the cell image in the ***View area***, move the mouse cursor to the view area, click and scroll using the scroll button on the mouse.

- To move the cell image to a desired location, click, hold and drag the image using the left mouse button.

- To flip and move a holographic 3D image, click, hold and drag the image using the right mouse button on the image.

## RE-FOCUS A CAPTURED IMAGE

After image capture most images are well focused. Some images may need to be re-focused manually after capture. If an image is not correctly focused, as in the example below, re-calculating the software focus may restore focus.



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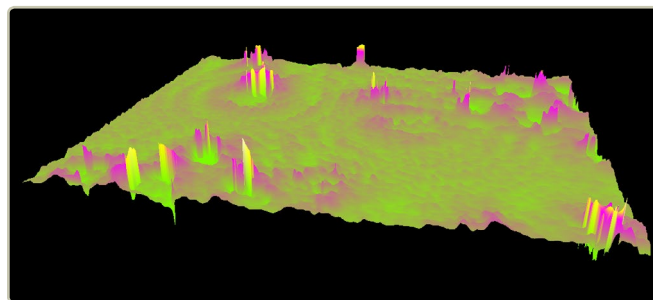
### Recalculate focus manually

Activate *Manual* in the *Software focus side panel*:
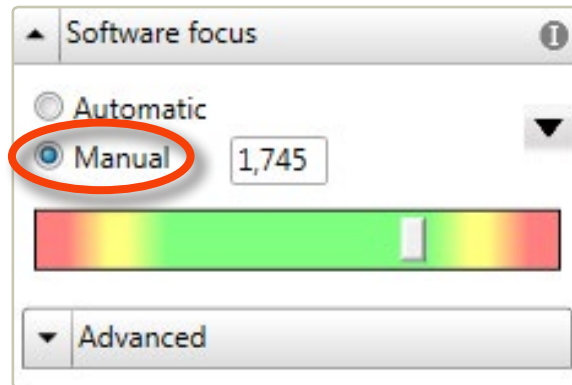


The software focal distance in mm can be changed either by entering a value manually or by using the slider beneath the text box.

- To find a starting value, choose an image that was captured at the same time and that is well focused and note the focal distance of that image.

- Select the image that needs to be re-focused in the *Image frame list*. Activate *Manual* in the *Software focus side panel*.

- Focus the image using the slider and the value from the well focused image.

- Use the ▼ button to apply the update to either the current frame, all frames or all checked frames.

### Recalculate focus automatically

To replace the manual changes with the automatic focus, activate *Automatic* in the *Software focus panel* and then use the ▼ button to apply the update to either the current frame, all frames or all checked frames.

# Cell identification

Choose the *Identify cells tab*:
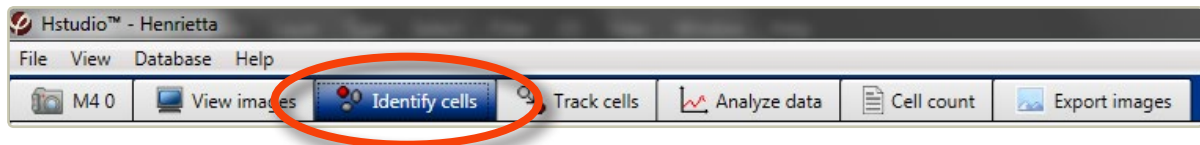


In the *Image frame list panel,* select the *Project*, *Group* and the image of interest to make it appear in the *View area*:

The software will then automatically suggest a cell identification:



## CHANGE HOW IDENTIFIED CELLS ARE DISPLAYED

In the *Options panel*, it is possible to control how cells are displayed and how identification adjustments are saved:

☑ *Show threshold*          displays non-background pixels in red.

☑ *Show cell markers*       displays the blue marker that identifies a cell.

☑ *Show outline*           outlines the cells.

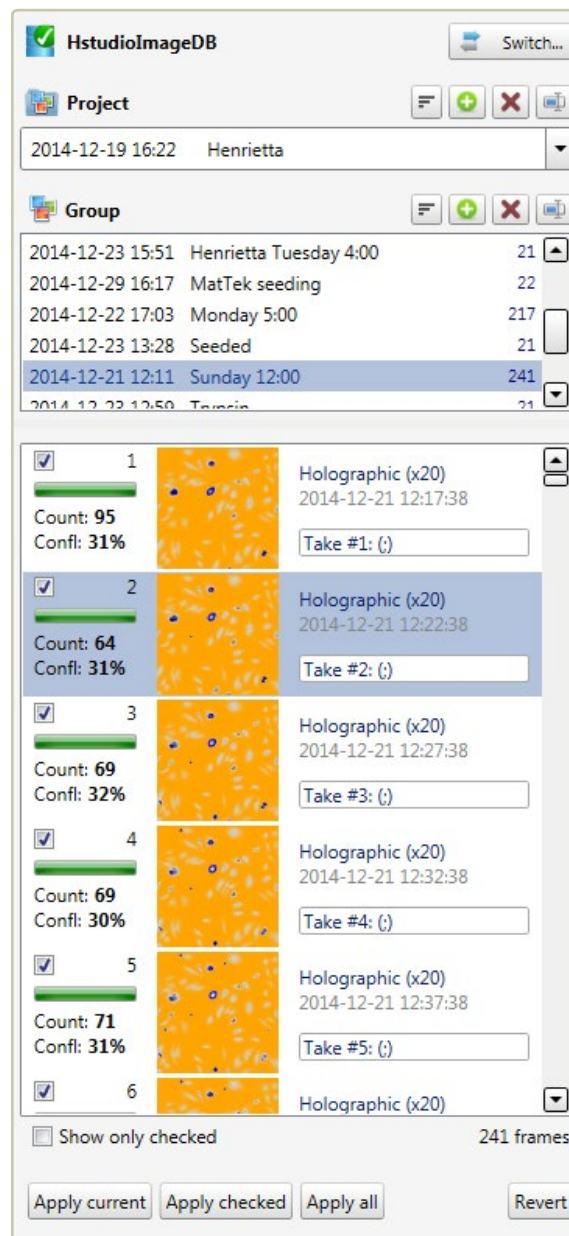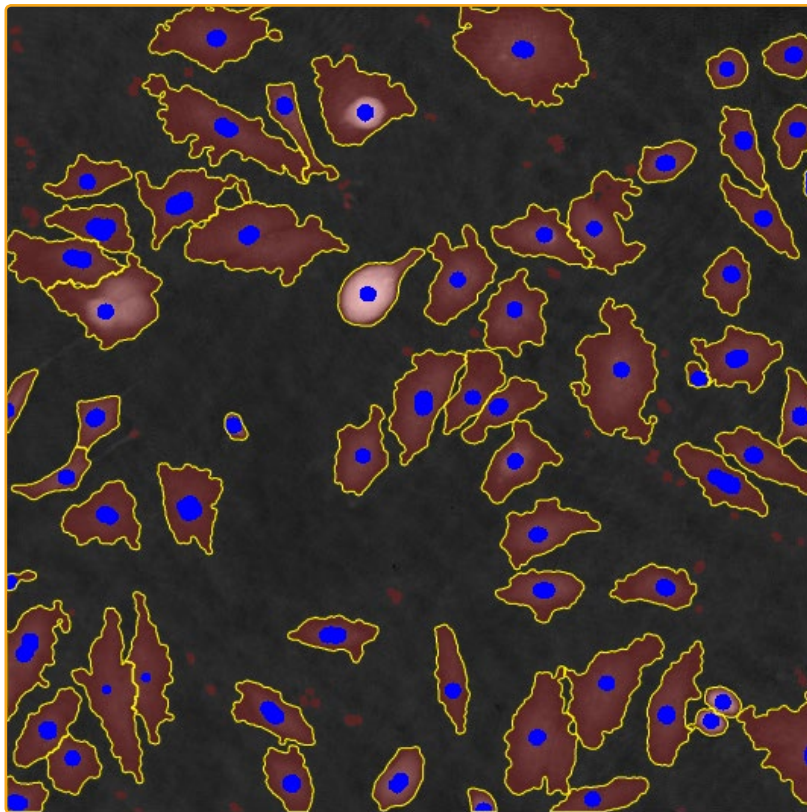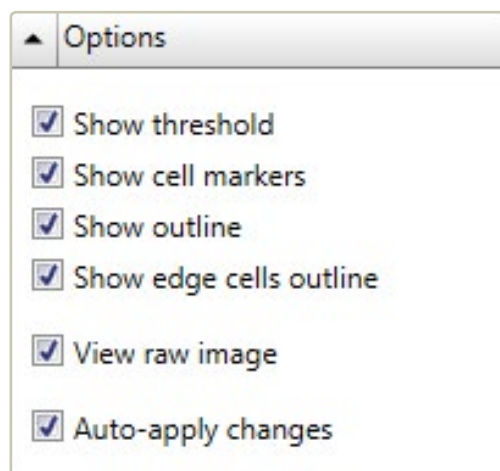☑ *Show edge cells outline*  outlines also cells touching the image edges.

☑ *View raw image*         displays the image without using pseudo coloring, see [Change image coloring](#) (page 35).

☑ *Auto-apply changes*      adjustments are automatically applied when switching to another image in the *Image frame list*.


## ADJUST CELL IDENTIFICATION

There are several methods to automatically calculate the *Background threshold* in the *Adjustments tab*. The background threshold determines which objects that will be identified as cells in the image. The software uses the *Minimum error* method by default. The different *Background threshold methods* will result in slightly different thresholds. It is advisable to try out which method that works best for every type of cell sample.
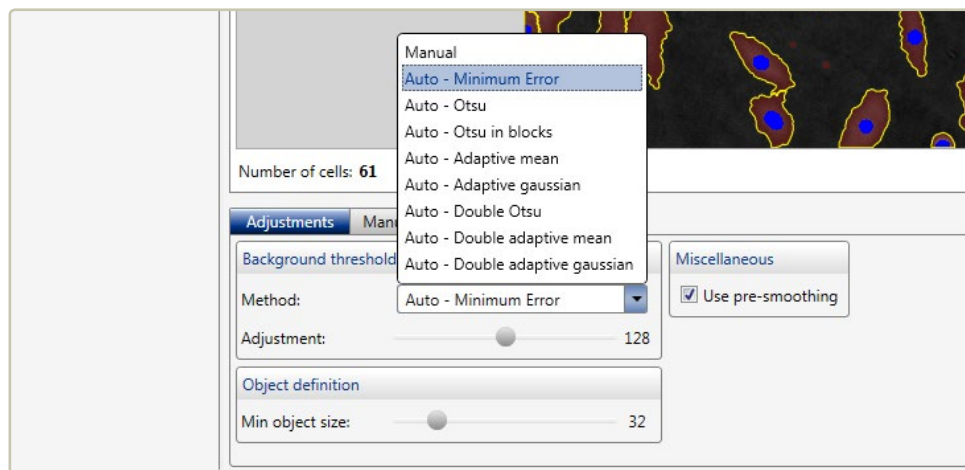


*Manual*               allows the user to directly set the threshold level using the slider.

*Minimum error*         sets the threshold level using the minimum error histogram-based threshold method.

*Otsu*                 sets the threshold level using the Otsu method.

| | |
|---|---|
| ***Otsu in blocks*** | splits the image into blocks which are thresholded separately using Otsu method. This is a form of adaptive threshold. |
| ***Adaptive mean*** | sets an adaptive threshold using a mean filter. |
| ***Adaptive gaussian*** | sets an adaptive threshold using a Gaussian filter. |
| ***Double Otsu*** | is a threshold method where both a wide and a narrow threshold mask are used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. ***Double Otsu*** uses double thresholding with Otsu global threshold as mid-level threshold. |
| ***Double adaptive mean*** | is the same as ***Double Otsu***, but with two adaptive mean threshold masks. |
| ***Double adaptive gaussian*** | is the same as ***Double Otsu***, but with two adaptive Gaussian threshold masks. |

In addition to the different automatic calculation methods for cell identification, the user can adjust the settings in several ways using the ***Adjustments tab***, which is found below the ***View area***:
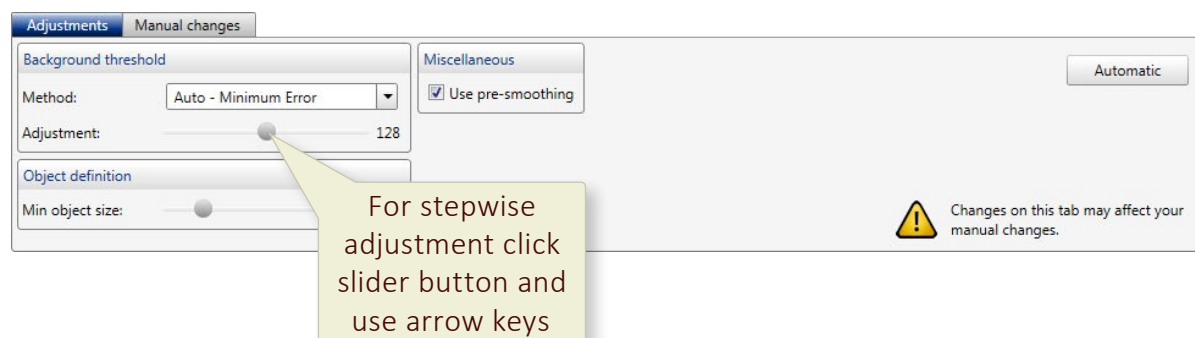


The threshold between cells and background can be adjusted by using the ***Adjustment slider***, thus adjusting the cell area. Additionally, the minimum cell size is adjusted by using the ***Min object size slider.*** When ☑ ***Use pre-smoothing*** is activated, the cell edges will be smoothed. ⬚ Automatic ⬚ sets the default values for automatic cell identification.

In other words, the identification adjustments are performed in three steps:

• Determine which threshold calculation method is optimal for the current cell culture.

• If needed, use the adjustment slider to adjust the threshold setting.

- If needed, use the *Min object size slider* to ensure that every cell has a cell marker.
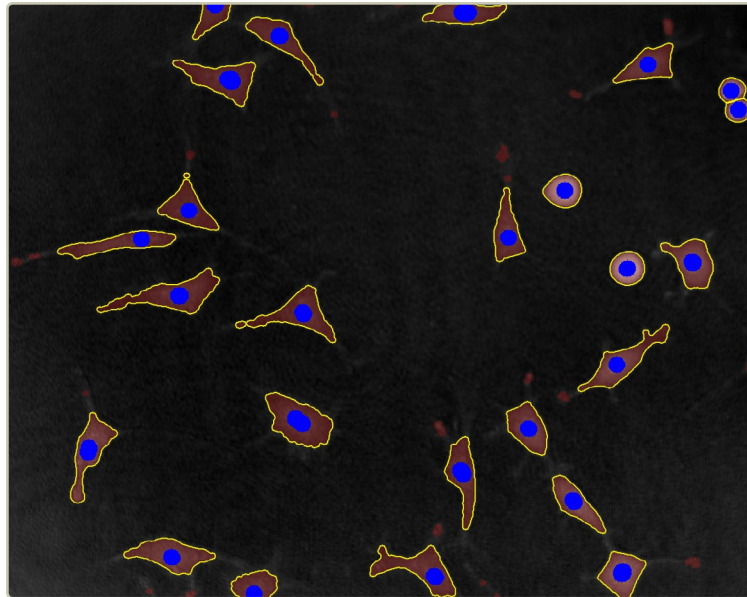


*Every identified cell has a border and a blue cell marker.*

## Save cell identification adjustments

When ☑ *Auto-apply changes* is activated, adjustments will be saved automatically for the image frame display in the *View area*. To apply the same adjustments to other image frames, use the [ Apply ] buttons below the *Image frame list*:
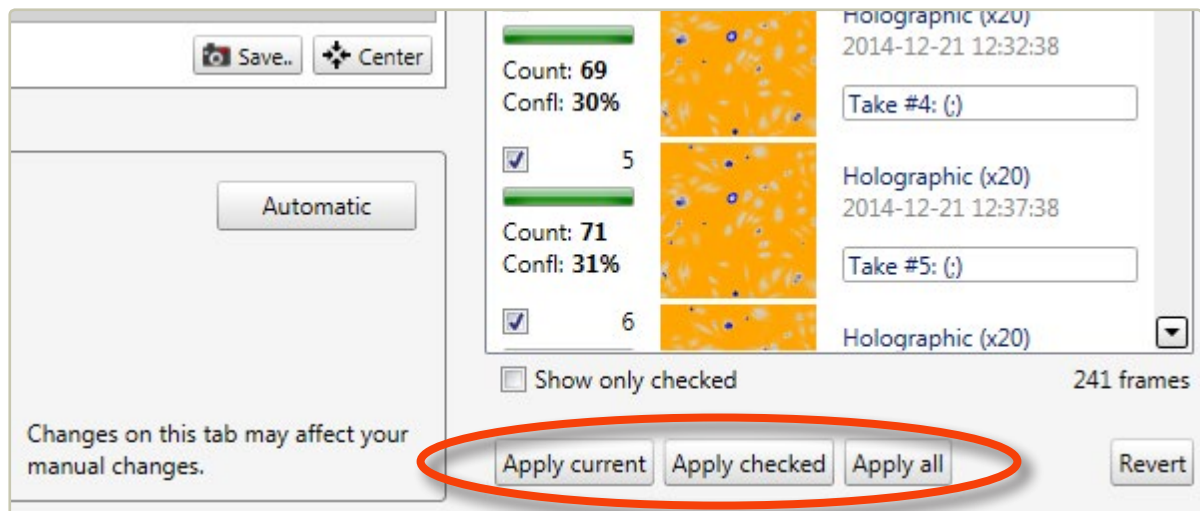
## MAKE ADJUSTMENTS FOR INDIVIDUAL CELLS

The identification of individual cells may be manually changed. It is possible to add, remove and delete as well as enlarge or shrink identified cells. These functions are found in the *Manual changes tab* below the *View area*:
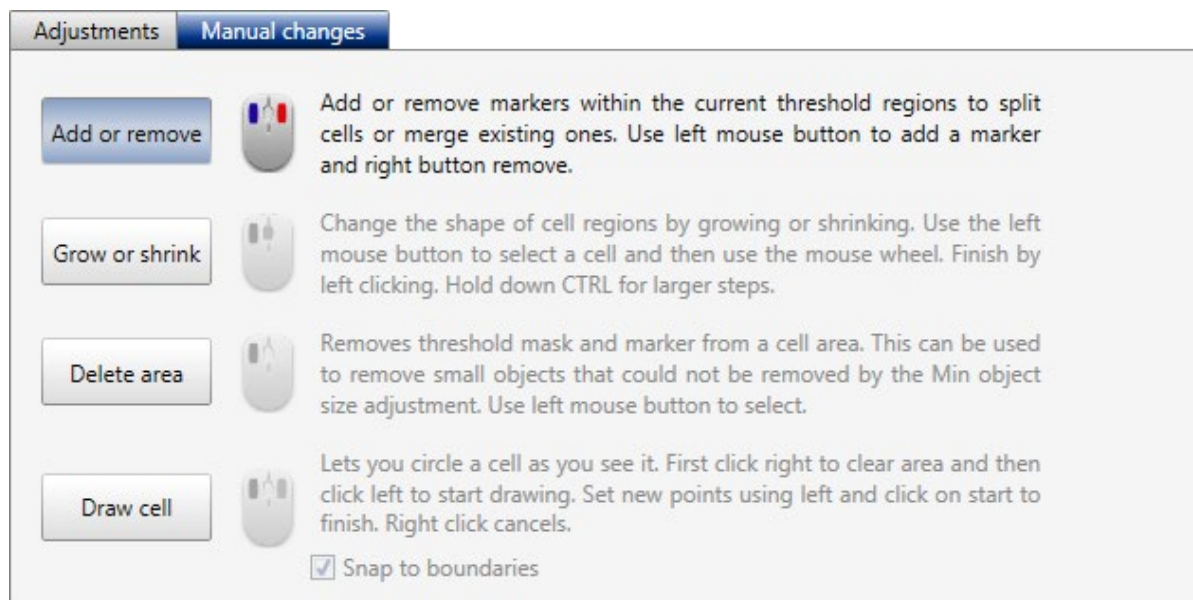


*Add or remove*    the blue cell markers that identify a cell. This results in mergers or splits of identified cell areas.

*Grow or shrink*   the identified cell regions.

*Delete area*      identified cell areas.

*Draw cell*        boundaries manually.

To the right in the *Manual changes tab* there are buttons that enable the user to edit the changes:
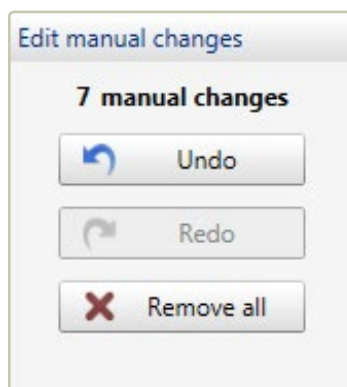
It is possible to undo the last adjustment step, to redo the undone step and to remove all adjustments.

**Note that settings made using the *Manual changes tab* are applied only to individual cells. These changes cannot be applied to other image frames.**

# Cell tracking

## BASIC CELL TRACKING

Cells can be tracked through a time-lapse sequence and analyzed both for movement and for morphology changes over time. Go to the *Cell Tracking tab*:
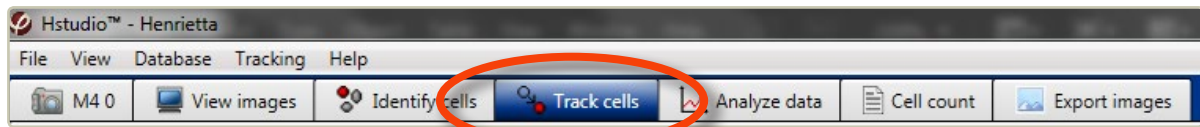


## START TRACKING

### Start a new tracking

Choose the *Track cells tab*. If tracking has not been performed previously, a tab will appear with the choices of starting a new analysis or opening a previous.
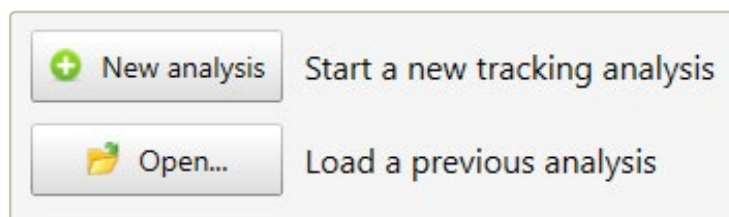


After clicking [ New analysis ] a message will appear:



> Start by adding frames from the frame list to the right to create a timeline.

## Add image frames to the analysis

Select a *Project* and a *Group* in the *Image frame list*, found to the right of the *View area*:
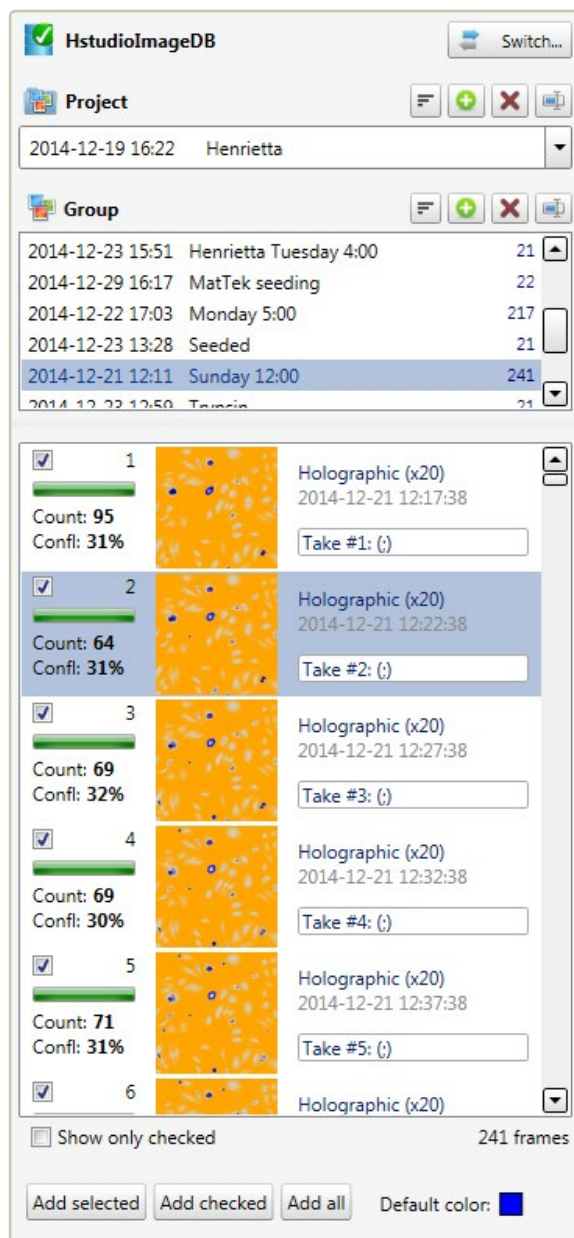


To track cells, select and add a series of image frames by using the [Add selected] or the [Add all] button, found below the *Image frame list*. Several images can be added simultaneously if they are all selected. The shift key can be used to select several consecutive images and the Ctrl-key to select non-consecutive image frames.

## Select cells to be tracked

The added image frames will be shown in the *Source frames tab*:
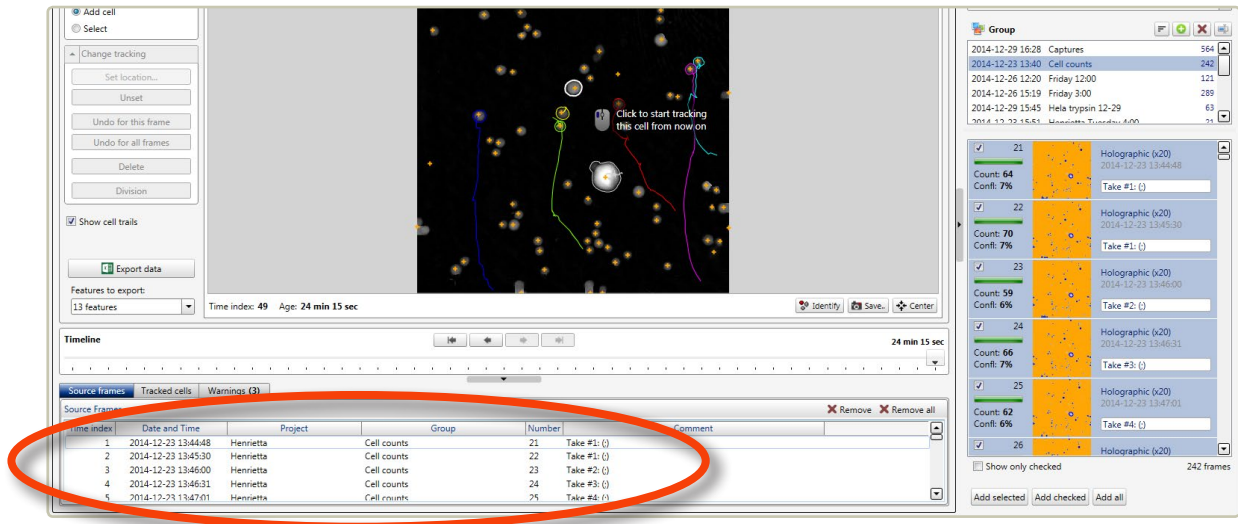


In order to follow a cell through the added images, the cell needs to be added to the tracking. Activate *Add cell* in the *Select mode panel*:
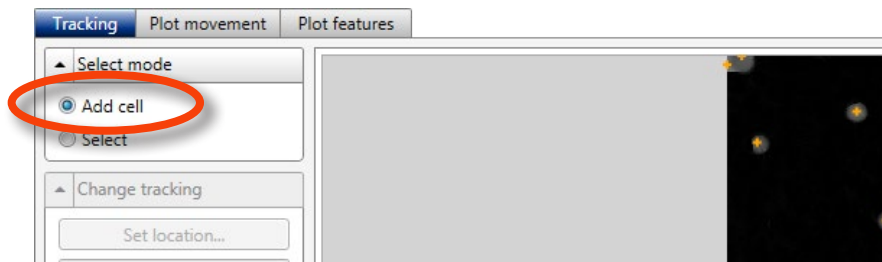


The center of each identified cell is marked with a small orange +. Click the + to add the cell to the tracking. Click each cell to be tracked:
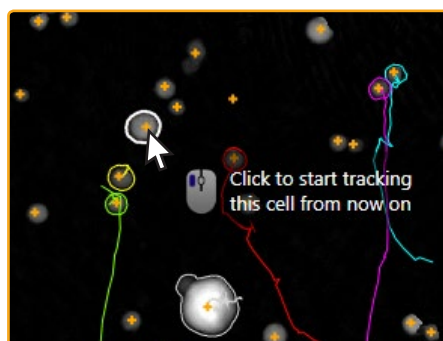


The cell will be tracked from the image where it was added. If the identification is not satisfying, go to the *Identify cells tab*, and adjust the Cell identification (page 53).

# CELL TRACKING WARNINGS

When the cell tracking for some reason deviates from the previous image, the software will give warnings. The warnings are listed in the *Warnings tab*, found below the *Timeline*:



When the [ Show ] button is clicked in the *Warnings tab*, the previous, current and next identification of the cell are shown. It is then possible to see if the warning is appropriate or if it can be ignored:
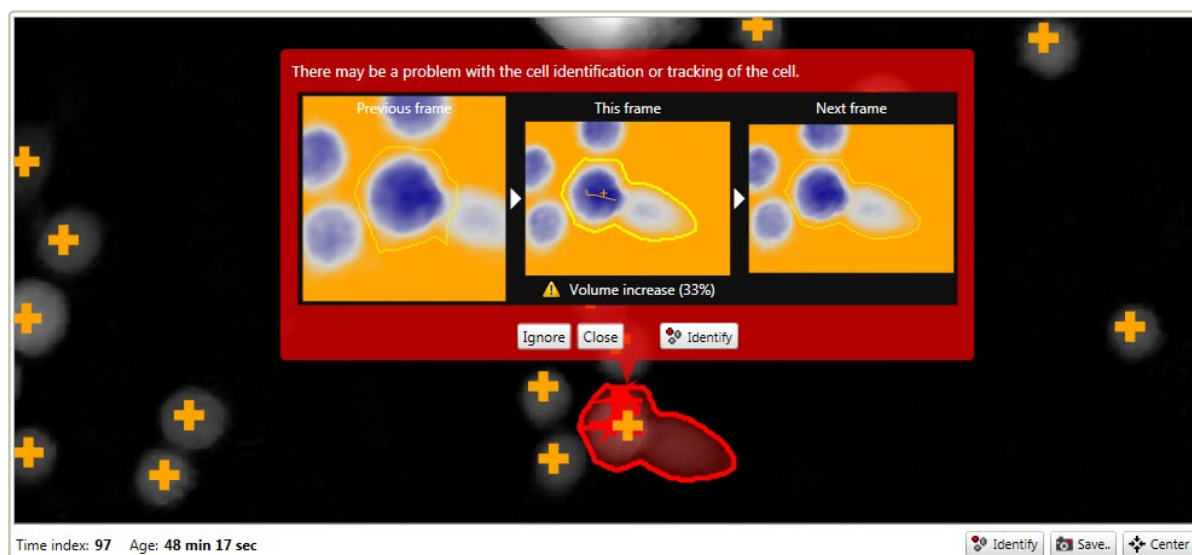


If the warning is caused by a normal cellular event, such as cell division, click [ Ignore ] to ignore the warning.

If the warning is caused by a cell identification error, as in the above example, click [ Identify ] to go to the cell *Identification tab*. After adjusting the identification, click the *Track cells tab* to return.

## DISPLAYING CELL TRACKING

When the *Timeline slider* below the *View area* is moved, tracks showing the cell movements will be displayed.
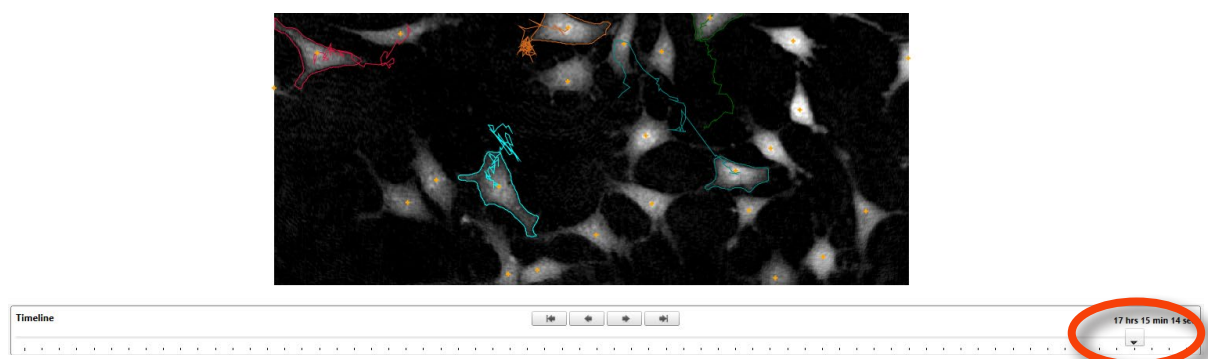


The movements can also be seen in the *Plot movement tab*:
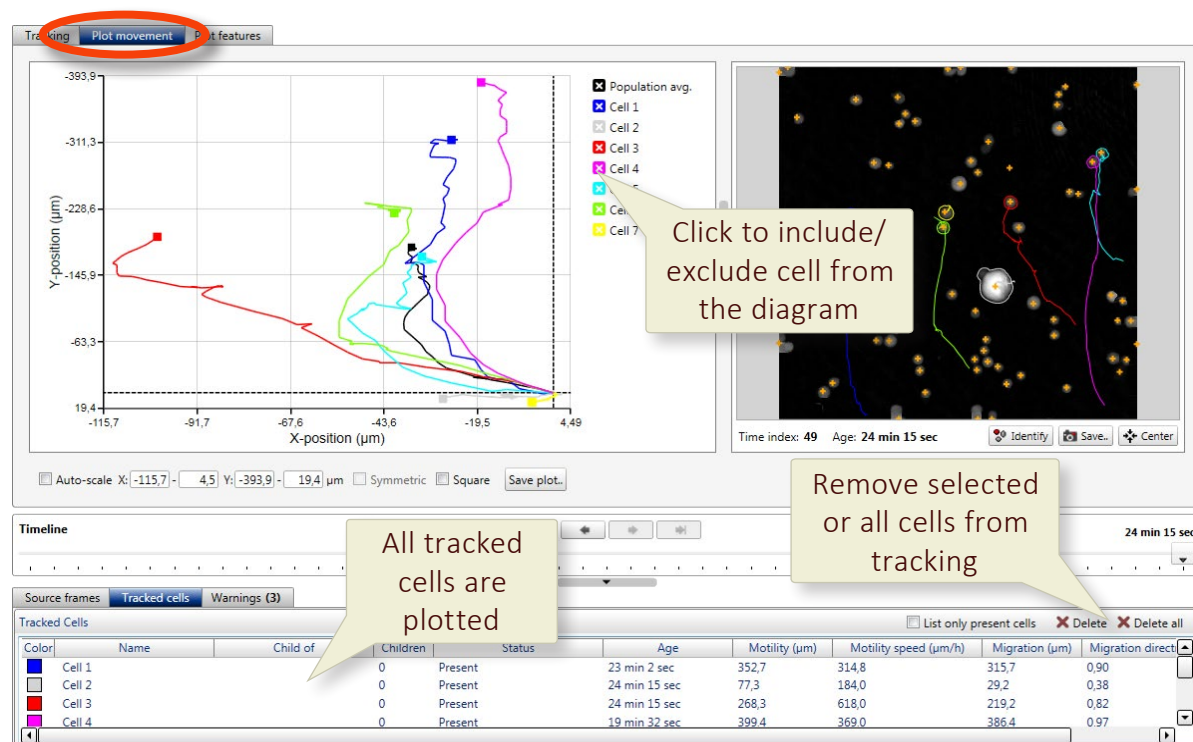


The cell movements are given as x- and y-coordinates over time. Initially, only the average movement is displayed, but by checking the boxes next to the cell names, individual cell tracks can be displayed.

### Set the axis display manually

The plot settings are found below the plot. The values of the x- and y-axis are set automatically to fit all values from all added cells. To set the plot axis values manually, uncheck ☐ *Auto-scale* and then enter the desired x- and y-minimum and maximum values.

## ADJUSTING CELL TRACKING

Sometimes the software will track the wrong cell, e.g. when cells are moving very close to each other and then separate again. This needs to be adjusted manually. Note that the adjustments will be active from the frame of adjustment and forwards through the time-lapse.

**Select the cell to be adjusted**

- Select the *Tracking tab*:



- Activate *Select* in the *Select mode panel*:
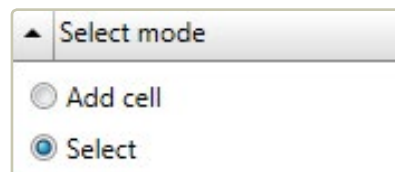


- Click on the tracked cell to be adjusted. A new set of functions will then be available in the *Change tracking panel*:



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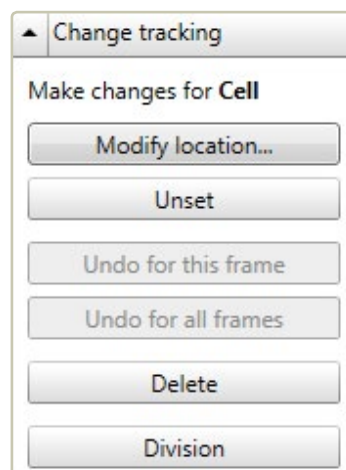
**Transferring tracking from one cell to another**

After selecting the tracked cell, click [ Modify Location ] in the *Change tracking panel*. Then click the cell to which tracking should be transferred. If the tracking was not transferred to the correct cell, repeat the procedure.



**Discontinue cell tracking**

Sometimes a tracking needs to be discontinued, e.g. when a cell moves out of the image area. After selecting the cell to be changed, click [ Unset ] in the *Change tracking panel*. The tracking will be discontinued from the present frame. A new button, [ Set location… ] will appear in the *Change tracking panel*:
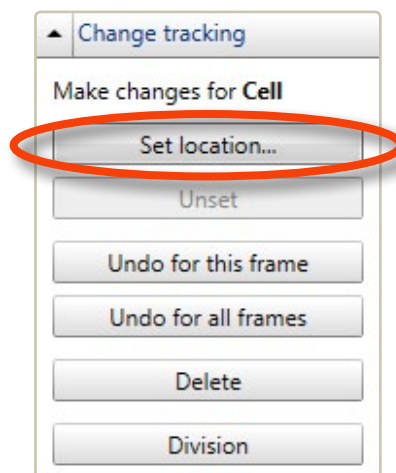


**Continue a discontinued cell tracking**

When a cell has been unset, it will still be present in the *Tracked cells tab*, but noted as not present. In order to resume the tracking in a future frame, select the cell in the *Tracked cells tab* and click [ Set location… ]. Then click the cell in the frame where the tracking should be resumed.

If the manual changes need to be undone, select a tracked cell and click either [ Undo for this frame ] or [ Undo for all frames ] which removes manual changes in the current frame or in all frames, respectively. The tracking will be recalculated accordingly.

## Track cell divisions

When a cell divides, the tracking will follow one of the daughter cells:
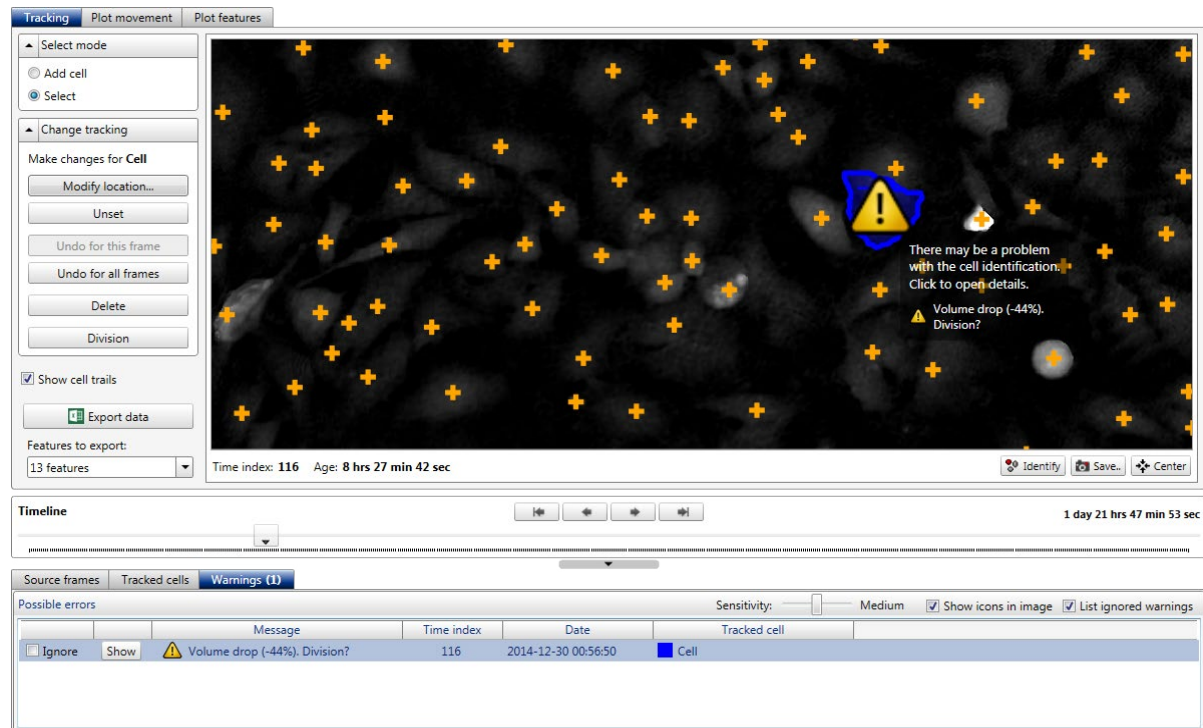


In order to track both daughter cells, select the cell in the frame where it divides, and then click [ Division ]. From then on, both daughter cells will have new names and will be tracked individually. If the mother cell is named e.g. Cell, the daughter cells will be named Cell-1 and Cell-2:
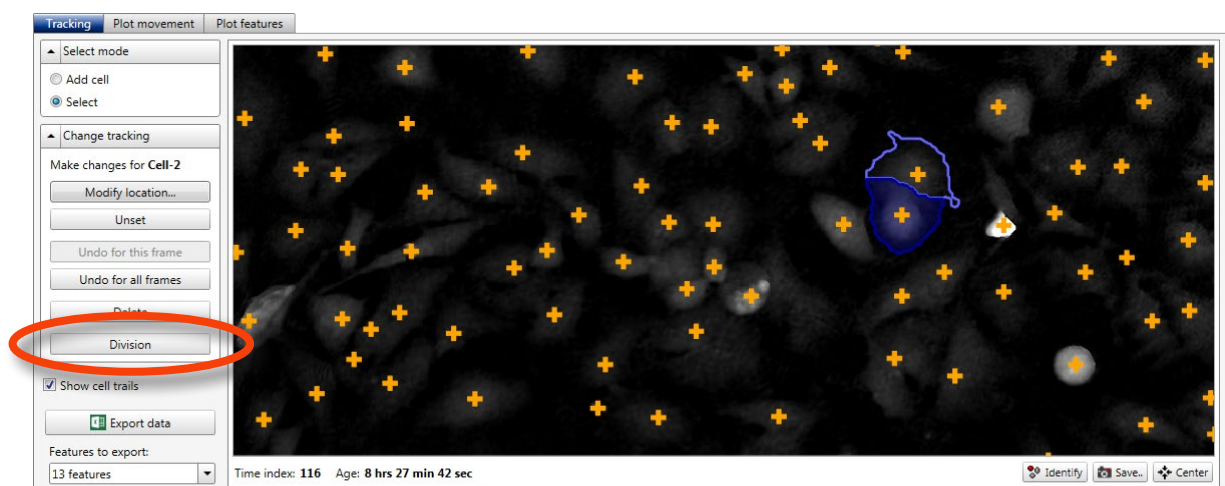
If the semi-automatic daughter cell tracking does not find the daughter cells, they can be added and named manually:

- Add the daughter cell by clicking on the +.

- Go to the *Tracked cells tab*.

- Select the added cell by left-clicking on it.

- Select the mother cell in the *Child of* drop down list. The cell will automatically receive a "daughter" name.


## TRACKING CELL MORPHOLOGY

Once the cell movement has been tracked, cell morphology can be followed over time by using the *Plot features tab*:
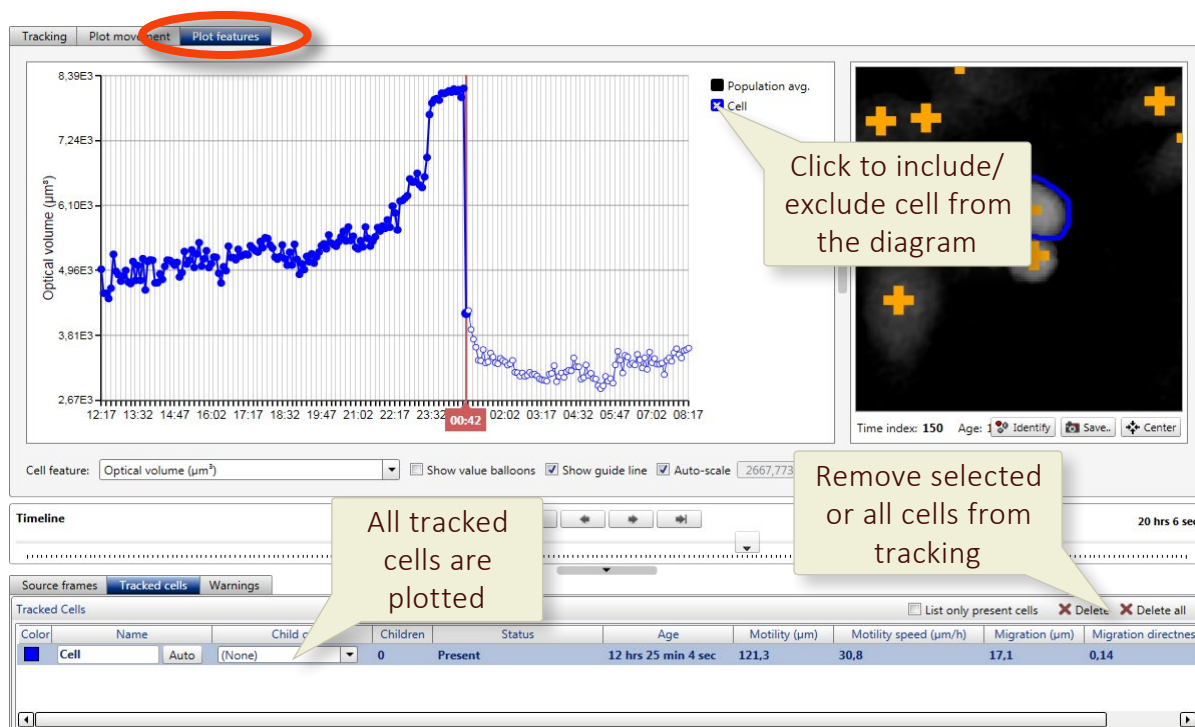
In the above example the optical volume of a cell is tracked over a cell division. By changing the *Cell feature* found below the diagram, a range of different morphological parameters can be tracked:
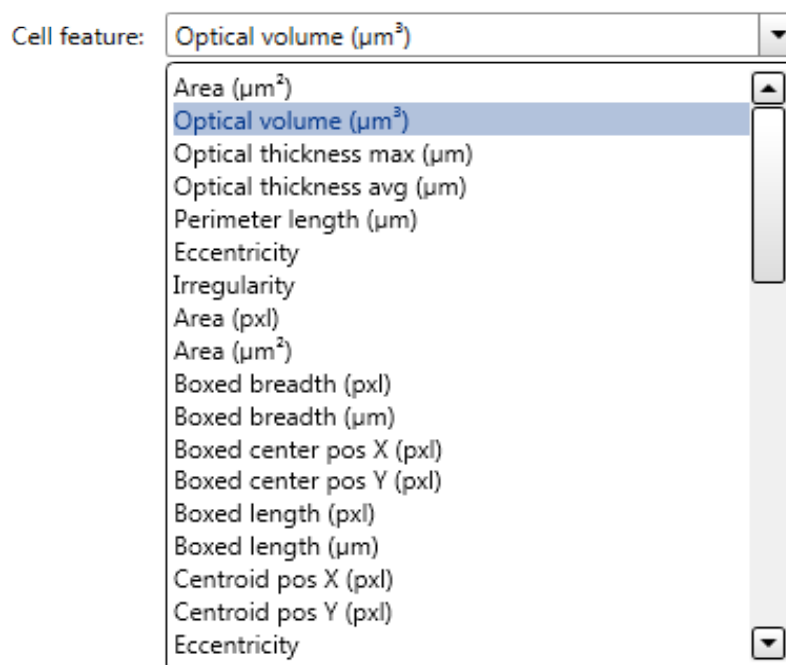


For the full list of parameters and their description, see [Morphological parameters](#) (page 106).

The following diagram options are found below the diagram:

☑ *Show guide line*         displays the current time-point.

☑ *Show value balloons*     displays the value at the current time-point.

☑ *Auto-scale*           sets the y-axis scale automatically. When unchecked, the y-axis scale can be set manually.

Save plot…           saves an image of the tracking diagram.

## MULTIPLE ANALYSES



( 📂 Open... ) opens a saved cell tracking analysis. Analyses are optionally saved when a *Tracking tab* is closed:
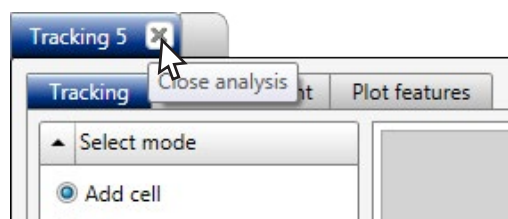
Additional analyses are started by creating additional *Cell tracking tabs*:
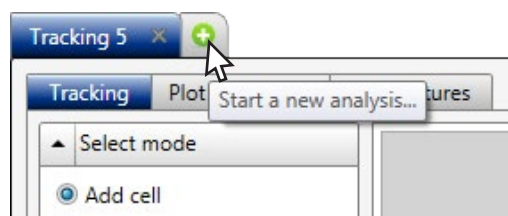


## EXPORT TRACKING DATA

In the *Tracking tab,* selected tracking data may be exported. The data is exported to an XML-file which can be imported to Excel:
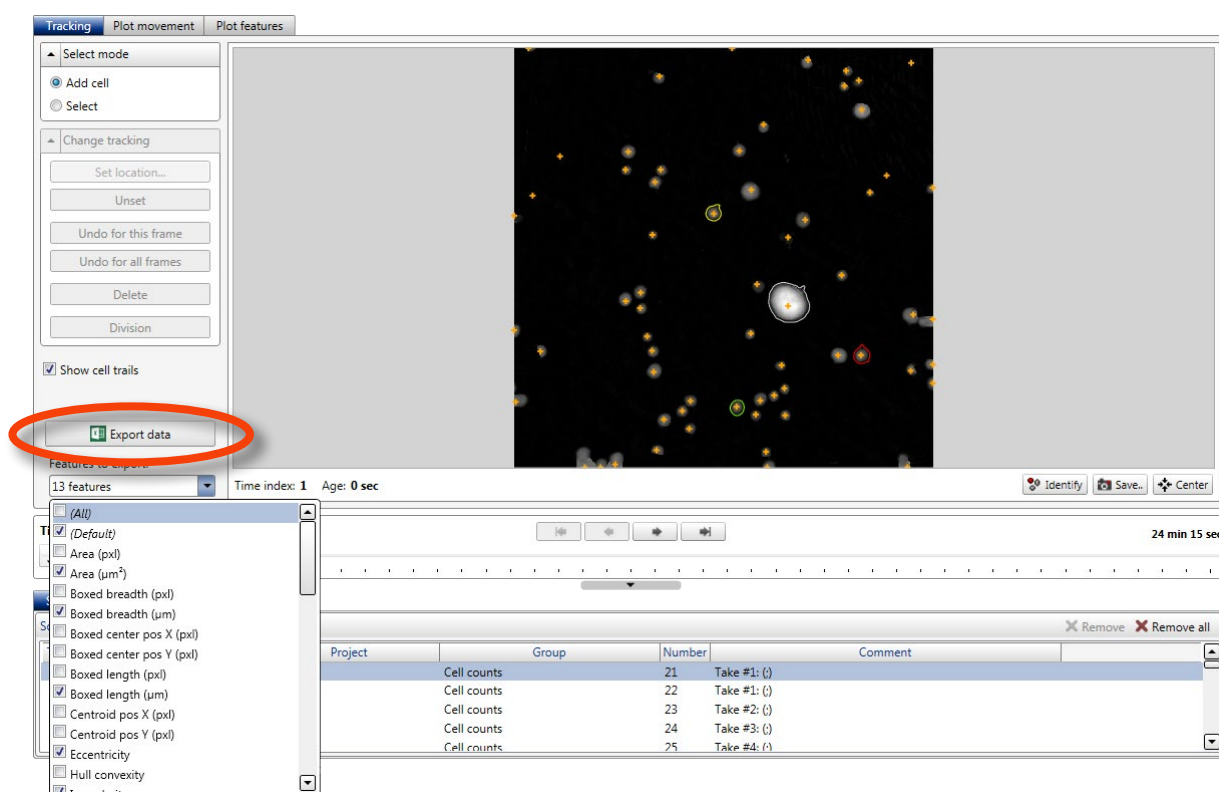
# Analyze and export data

## MEASURE CELLS INTERACTIVELY

A simple tool for interactively measuring distances and cells is found in the *View images tab*:
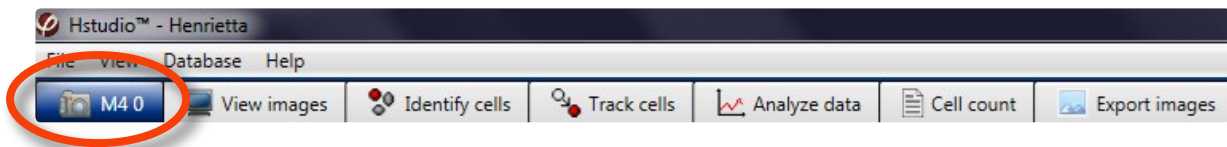


Choose the *View images tab.* Click  Measure  in the *Viewer options panel* to make manual measurements of objects in the viewed image:
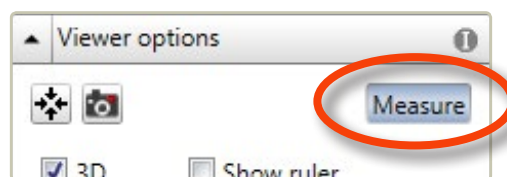


The measurement is started by left-clicking **and releasing** anywhere in the image. The measurement is finished by left-clicking at a new point in the image:
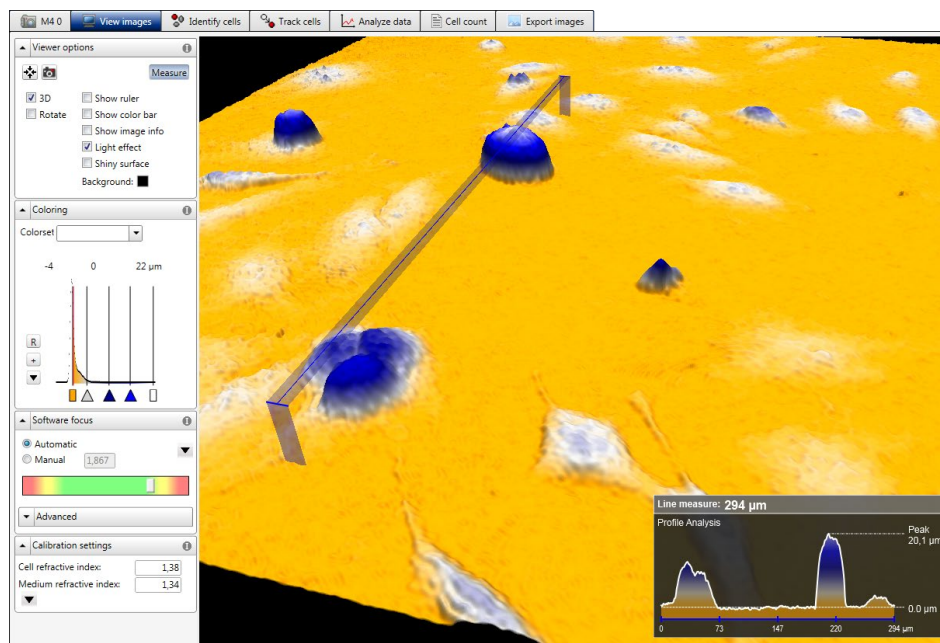


The measured distance is shown with a blue bar. A window displaying the results, including a cell profile image, appears in the *View area*. The data can be saved as an image with the  button in the *Viewer options panel*. It is possible to move the image in the *View area* while the measuring function is activated. The image is moved by clicking and dragging. When  Measure  is clicked again, the measuring function is deactivated.

# ANALYZE RESULTS IN PLOT

Cell population analysis is performed in the *Analyze data tab*:
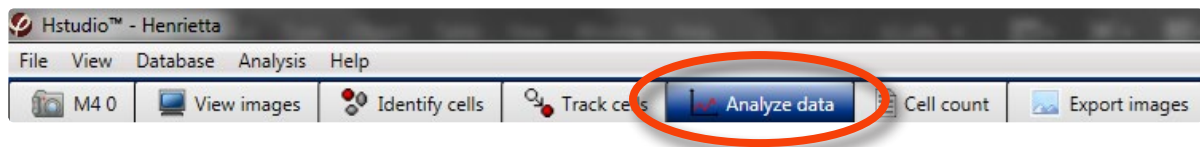


## Start a new analysis

Choose the *Analyze data tab.* If data has not been analyzed previously, a tab will appear with the choices of starting a new analysis or opening a previous:
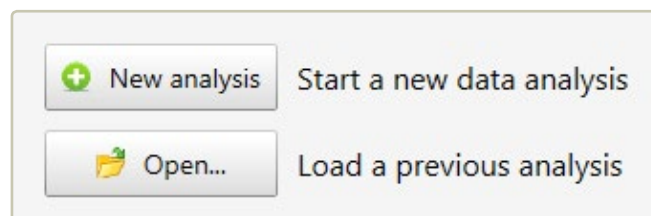


After clicking **New analysis** a message will appear:



Generate the analysis by adding source frames from the frame list to the right.

Select a *Project* and a *Group* in the *Image frame list*, found to the right of the *View area*:
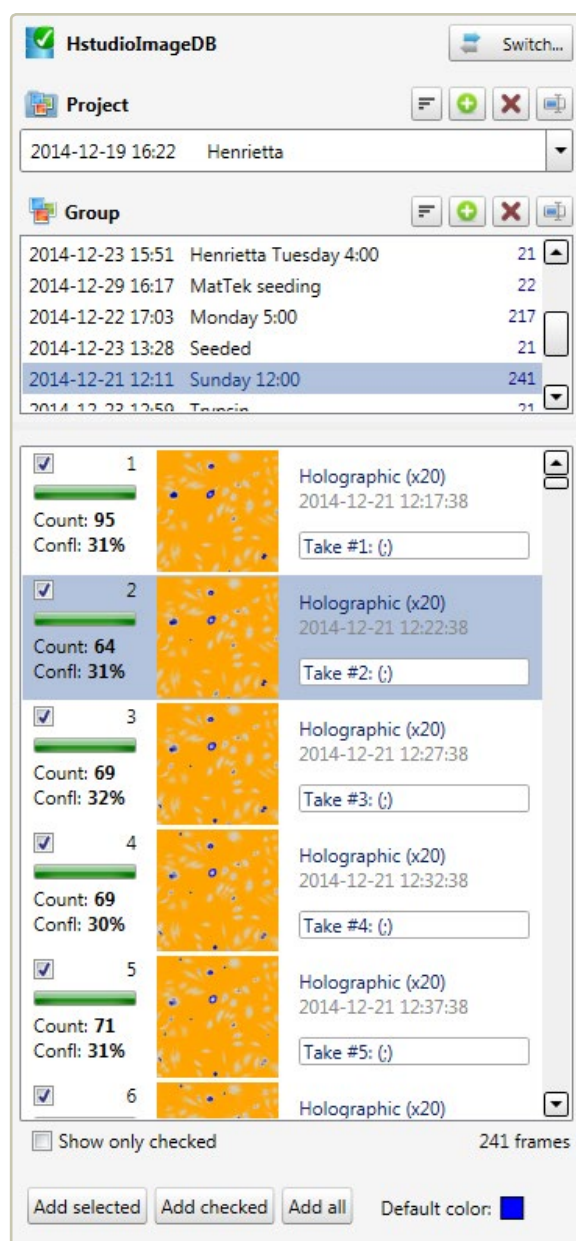


To add data, select image frames and transfer to the plot by using the `Add selected` button, found below the *Image frame list*. Several images can be added simultaneously if they are all selected. The shift key can be used to select several consecutive images and the Ctrl-key to select non consecutive image frames.

The images that are included in the analysis are shown in the *Source frames tab*, found below the *Scatter Plot tab*:
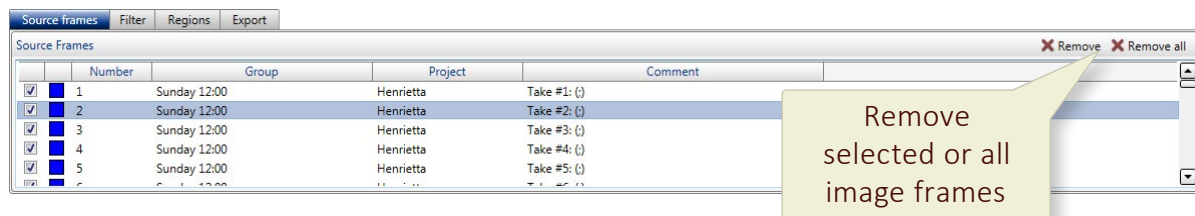


If several images frames are added to the plot, the data from all images will be displayed in the same plot, unless ☐ *Only current frame* is checked:
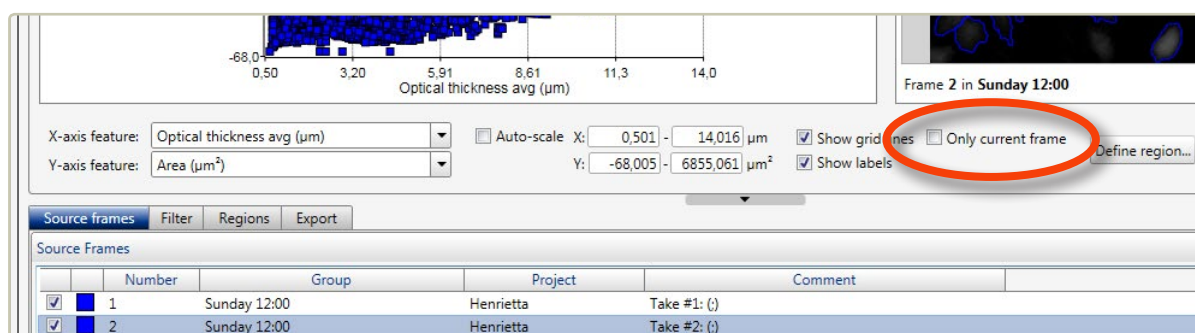


When ☑ *Only current frame* is checked, only the data from the current frame is displayed. Data from the other frames can be displayed when the frame is selected in the *Source frame list*.

## MULTIPLE ANALYSES

Open... will open a saved data analysis. Analyses are optionally saved when a *Data analysis tab* is closed:



Additional analyses are started by creating additional *Data analysis tabs*:
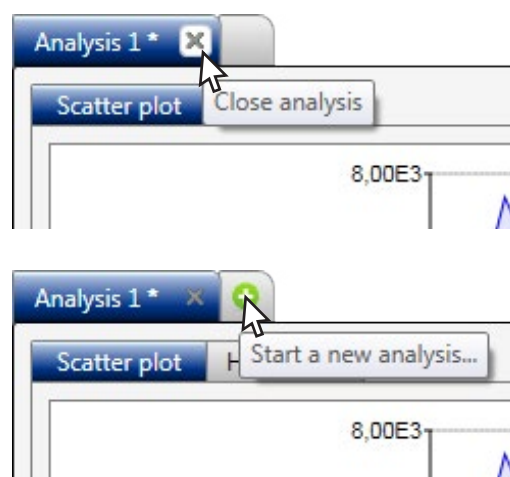
# DISPLAY RESULTS IN SCATTER PLOT

The cell morphology data are first represented as a scatter plot, with the segmented cell images displayed beside the plot. The data point for each cell is represented as a colored box.

If several frames are represented in the plot, the data points from all images will be displayed in the plot simultaneously, while the frame that is selected in the *Image frame list* is displayed in the viewer:
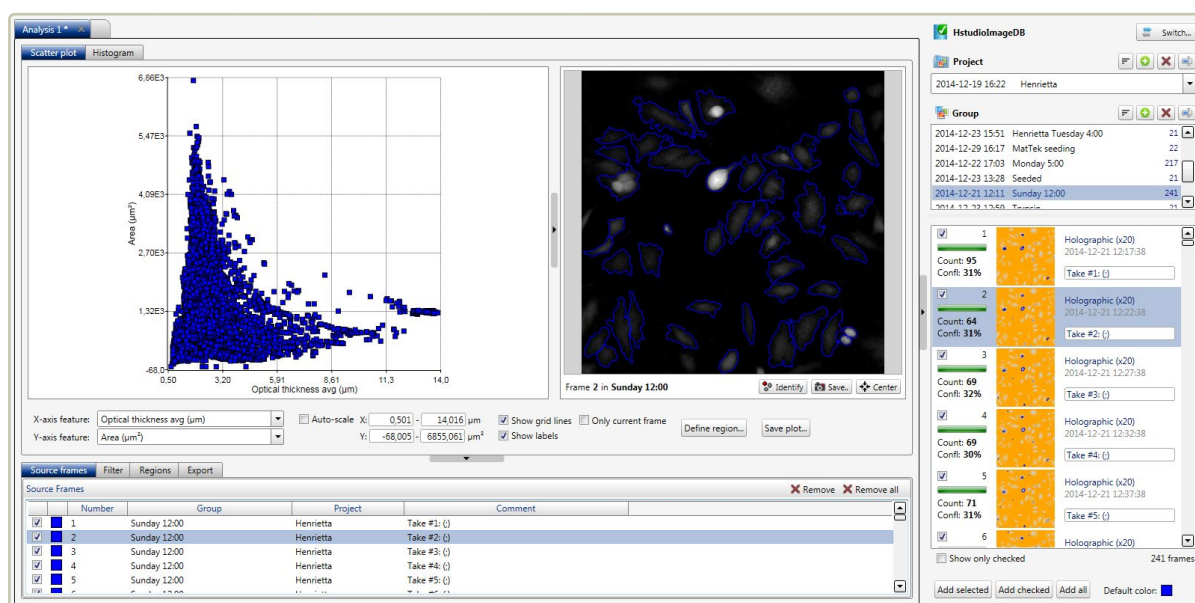


The data points will be displayed in the plot with the color shown in the color box in the *Source frames tab*, found below the *Scatter plot tab*. To change the display color, left-click the color box for the added frame and select a different color:
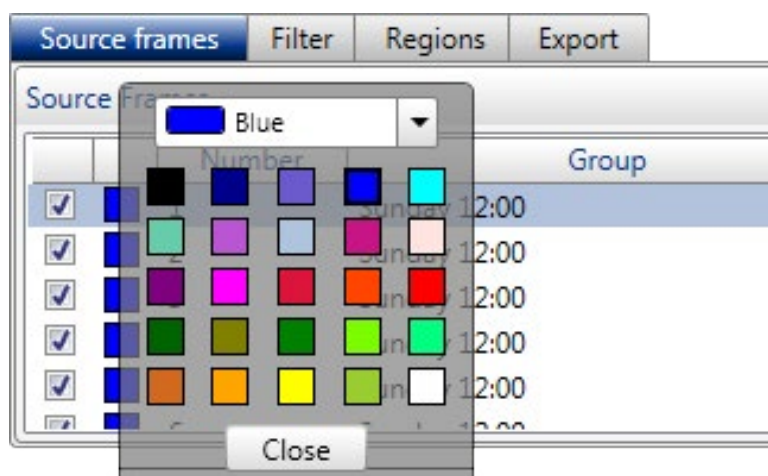
## Identify data points as cells

Hover with the mouse cursor over a data point in the scatter plot. The corresponding cell will then be identified in one of the cell images that have been added to the plot. Data for that cell will be displayed simultaneously:
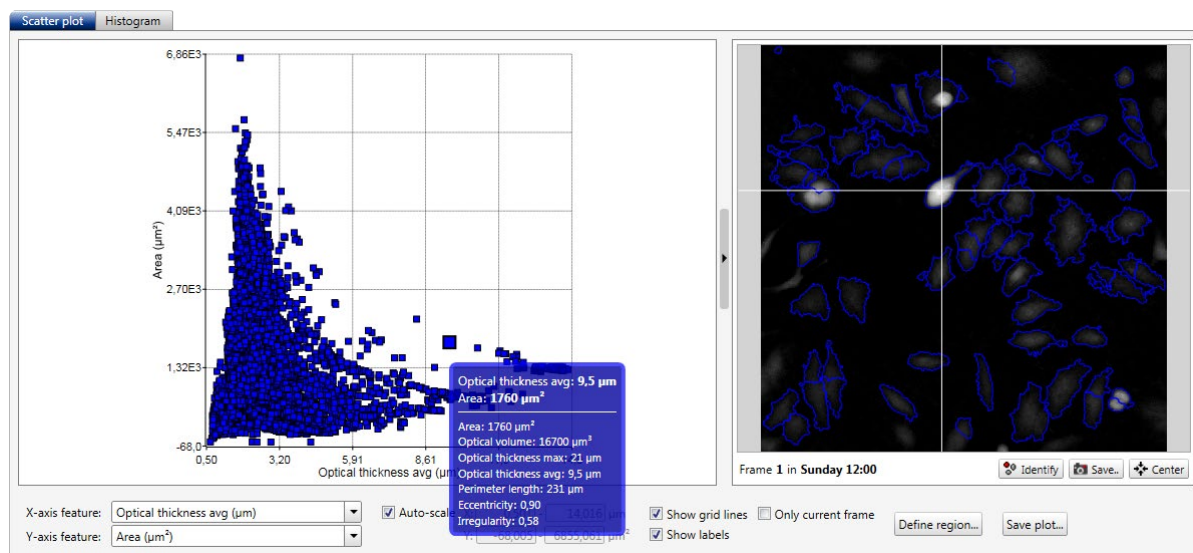


When hovering over a certain cell in an added image frame, the data point representing that cell will be identified in the scatter plot, and data for that cell will be displayed:
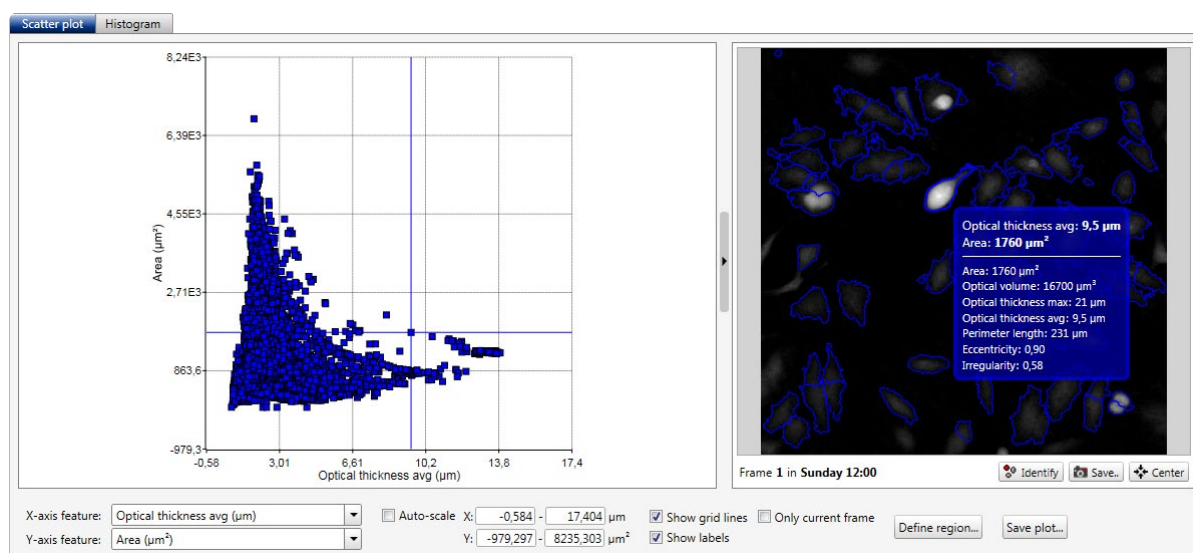
## Change the scatter plot axis units

The axis labels of the scatter plot can be changed by using the listed x- and y-axis features found below the scatter plot. For a full list of axis label parameters, see Morphological parameters (page 106).
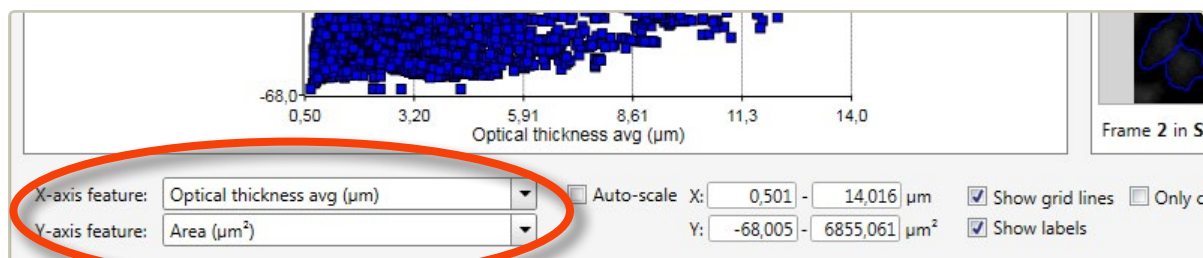


## Set the scatter plot axis range manually

The scatter plot settings are found below the scatter plot. The area of the scatter plot (i.e. the values of the x- and y-axis) is set automatically to fit all values from all added frames. To set the plot area manually, deactivate ☐ *Auto-scale* and enter desired x- and y-minimum and maximum values:
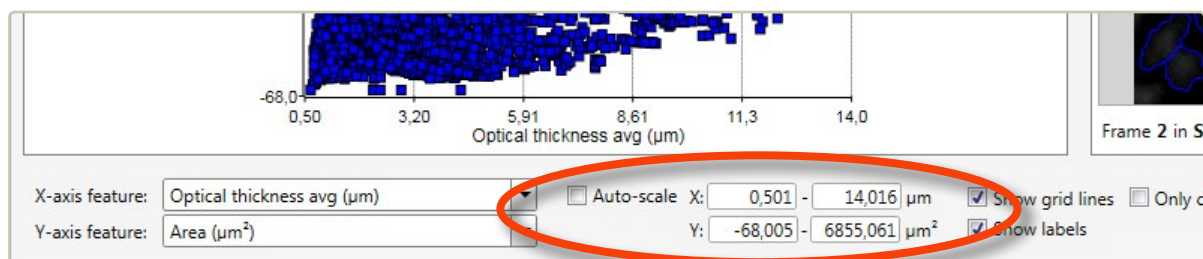


Alternatively, the scatter plot area can be changed with the mouse scroll button. Left-click in the plot and scroll using the mouse scroll button. To move the plot area in the scatter plot, left-click in the plot, hold and drag.

## Create, hide or delete plot regions

The scatter plot diagram can be divided into regions to separate out data from certain cells or cell populations. When right-clicking while hovering over the plot, a menu will open:
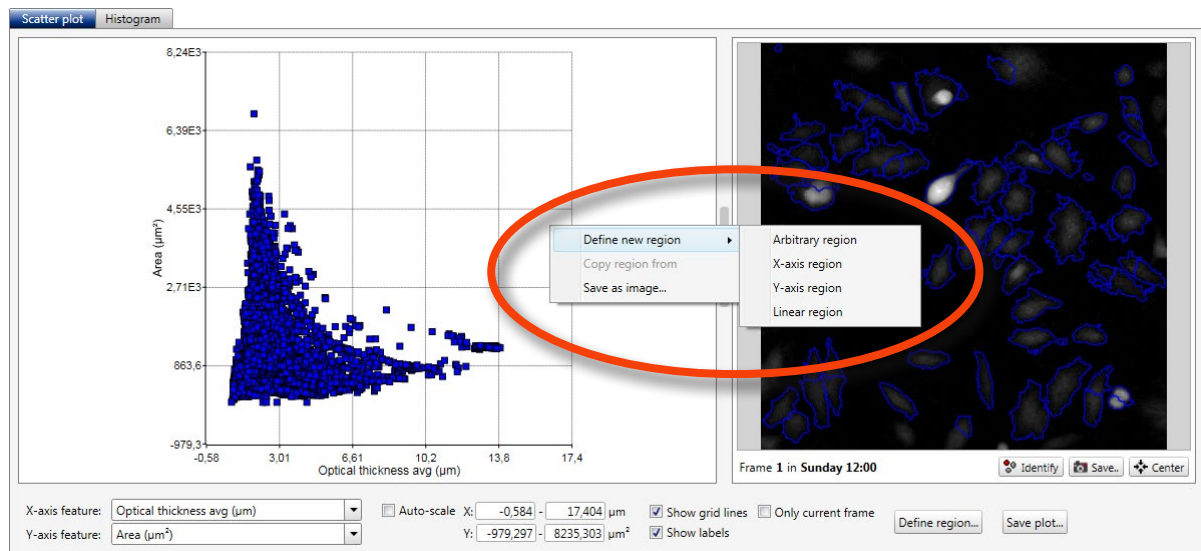


When **Define new region** is selected, a region menu will appear. Alternatively, there is a Define region... button below the **Scatter plot tab**, to the right of **Auto-scale**. There are four region types:

**X-axis region**        The x-axis values are the region cut off values.

**Y-axis region**        The y-axis values are the region cut off values.

**Linear region**        Clicking in the diagram results in straight lines that cut off the region from origin.

**Arbitrary region**     Repeated clicking in the diagram results in an irregular figure that outlines the region. To finish the region, close it off by clicking the starting point.

Select a region type by clicking it. Then click in the plot where the region setting should begin. For the *Linear region* and the *x-* and *y-axis regions* the second click in the plot determines where the region ends. For the *Arbitrary region*, every click in the plot will add to the region. Finish the region by clicking the starting point again. The starting point will expand to indicate that the region is closed. If clicking the region does not make it "stick", try clicking a little slower.
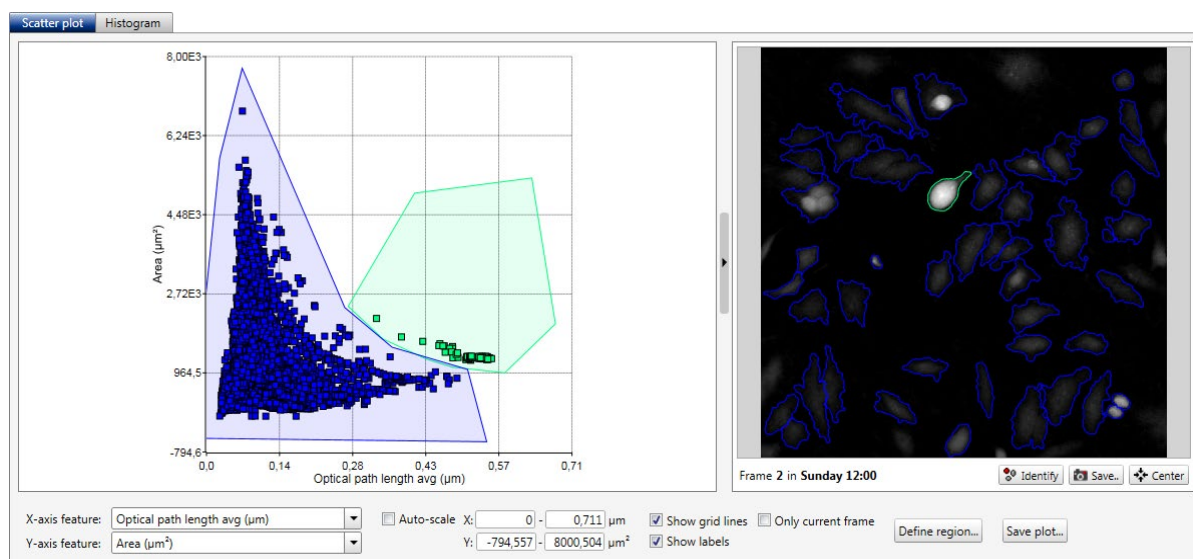


*Scatter plot with two arbitrary regions.*

## The Regions tab

The regions are summarized in the *Regions tab*, which is found below the *Scatter plot tab*. Regions can be hidden by unchecking the region:



Regions can be copied from one analysis to the next by using *Display from other* in the *Regions tab*:

## DISPLAY RESULTS IN HISTOGRAMS

In the *Histogram tab*, the results are displayed as histograms, where one cell parameter is offset against the cell count. If several regions have been added to the scatter plot, they will show up with the appropriate color in the histogram as well.
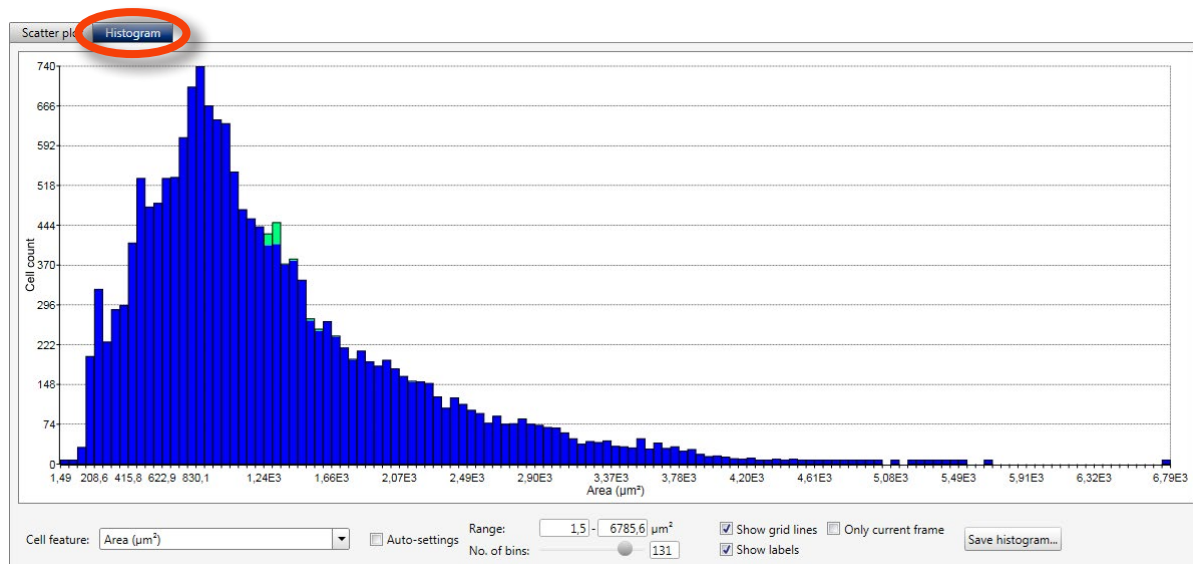


*Histogram with two arbitrary regions.*

### Change the histogram axis units

The x-axis label of the histogram can be changed by using the *Cell feature list* below the histogram. A list of x-axis label parameters are found in Morphological parameters (page 106).
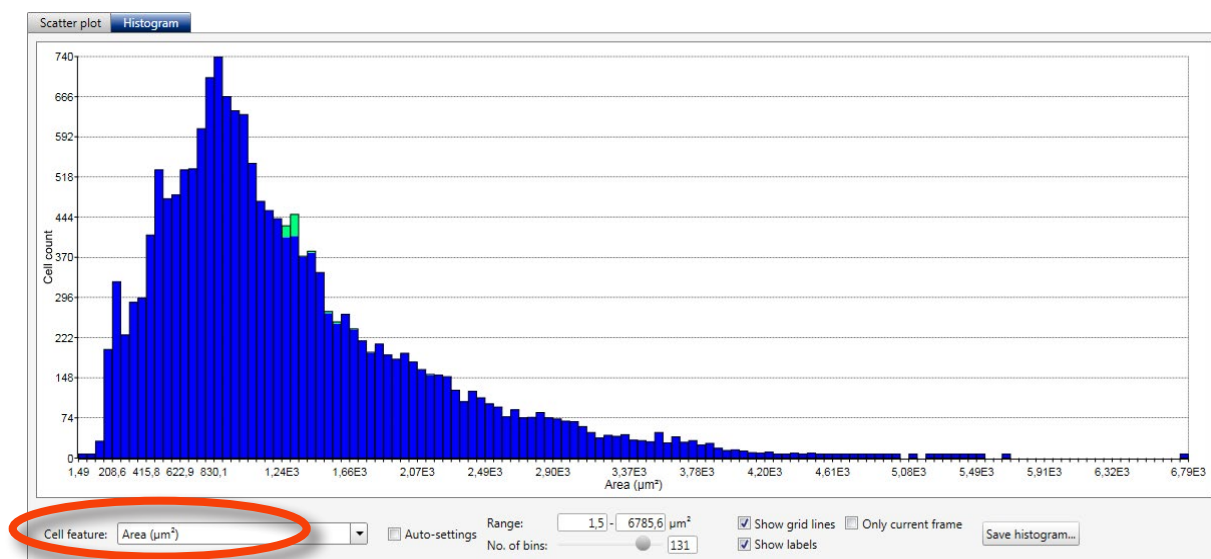
## Change the histogram x-axis intervals and bins manually

The histogram x-axis interval is set automatically to fit in all values from all added frames. To set the histogram x-axis interval manually, ☐ *Auto-settings* must be unchecked. Enter the minimum, maximum values and the number of bins:
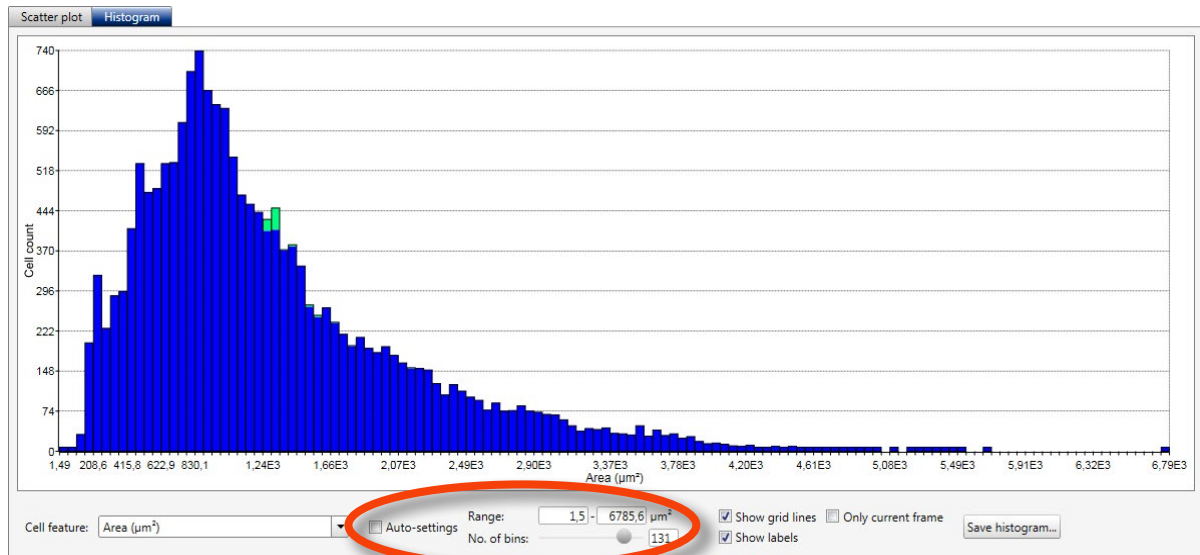


## EXPORT PLOTS AND CELL DATA

In the *Export tab*, cell data from the current plot can be exported to an XML-file, which can be imported into e.g. Excel. Images of the current plot and histogram can be saved as bitmap, GIF, JPEG, PNG or TIFF by using the  Save plot…  or  Save histogram…  buttons.
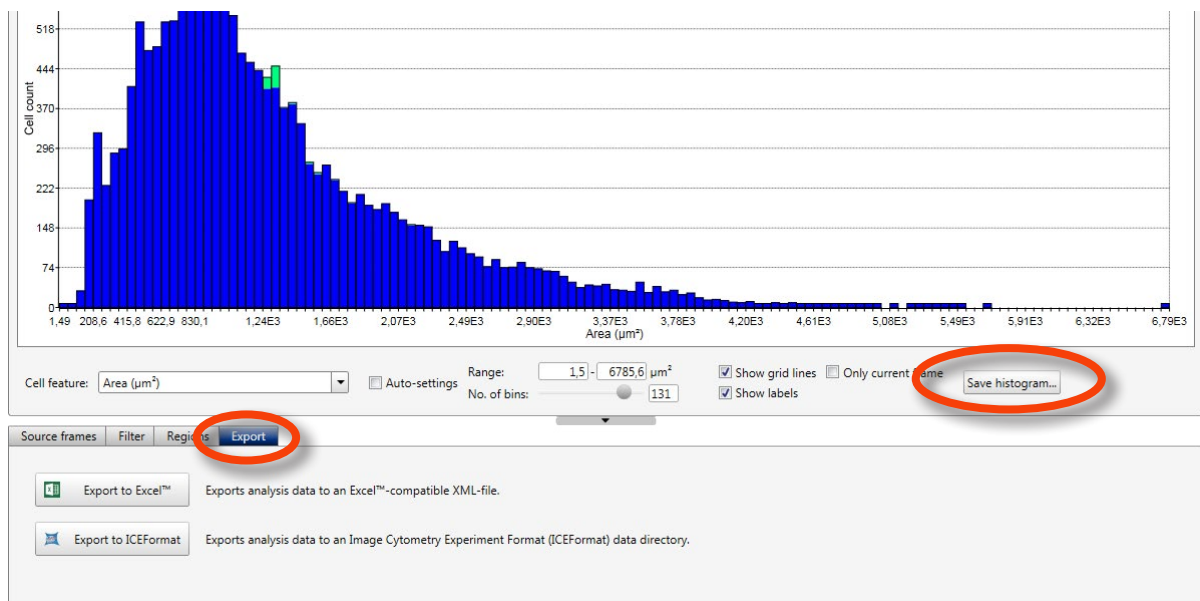
## Export cell data

The *Export tab* is found below the plot area. The Export buttons export cell data to XML or ICE files:
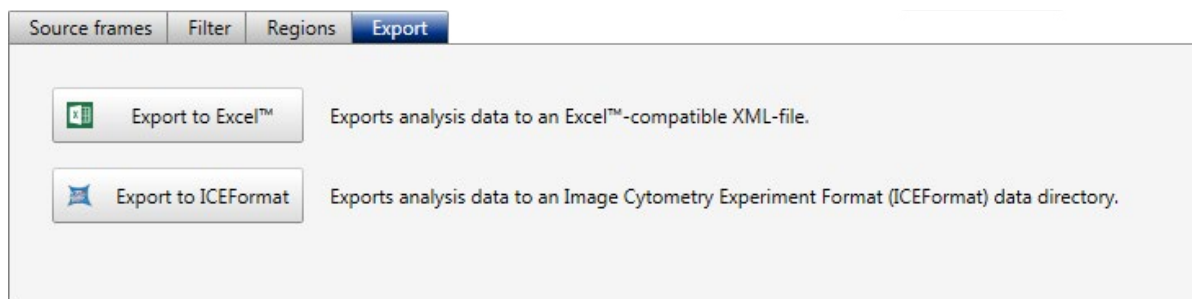


- Click one of the Export buttons to export all cell data. A file browser window will open.

- The file will automatically be named with date and a code, but it is possible to rename the file.

- Save the file to an appropriate place. All data pertaining to the image frames added to the current plot are exported.

## Save plots and histograms

To save a scatter plot or a histogram click the respective Save plot… or Save histogram… button. A file browser window will then open.
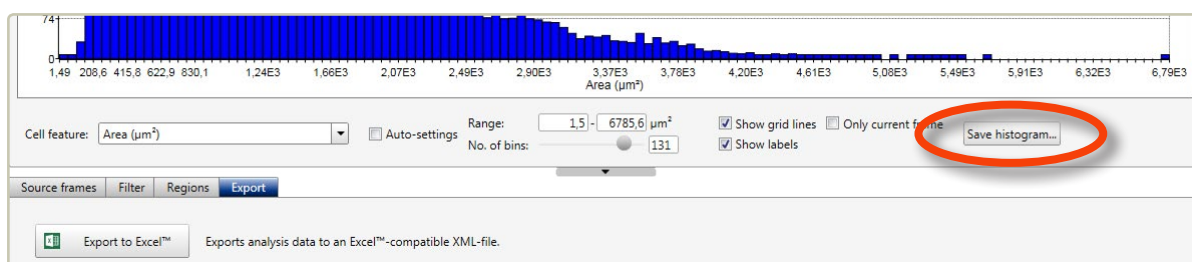


- The file will automatically be named, however it is possible to rename the file.

- Decide which image format you want (bitmap, gif, jpeg, png, tiff).

- Save the file to an appropriate place.

# Cell count

Cells are counted and analyzed for confluence, cell area and volume in the *Cell count tab*:
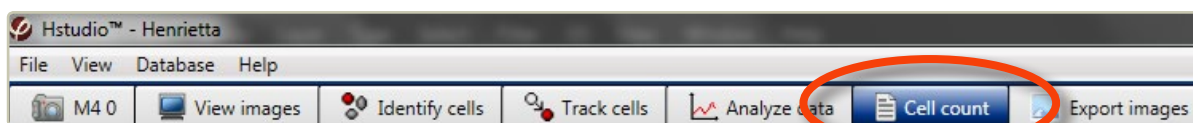


Choose the *Cell count tab*. A text will appear which says that five image frames or more must be added in order to analyze images for cell count and confluence. The frames should be captured at random positions in a cell culture vessel. Select or check the images to be analyzed in the *Image frame list* to the right. Click the appropriate ⬚ Add button, which is found below the *Image frame list*. The added image frames will be shown in the *Source frames panel* below the *Cell count report*.
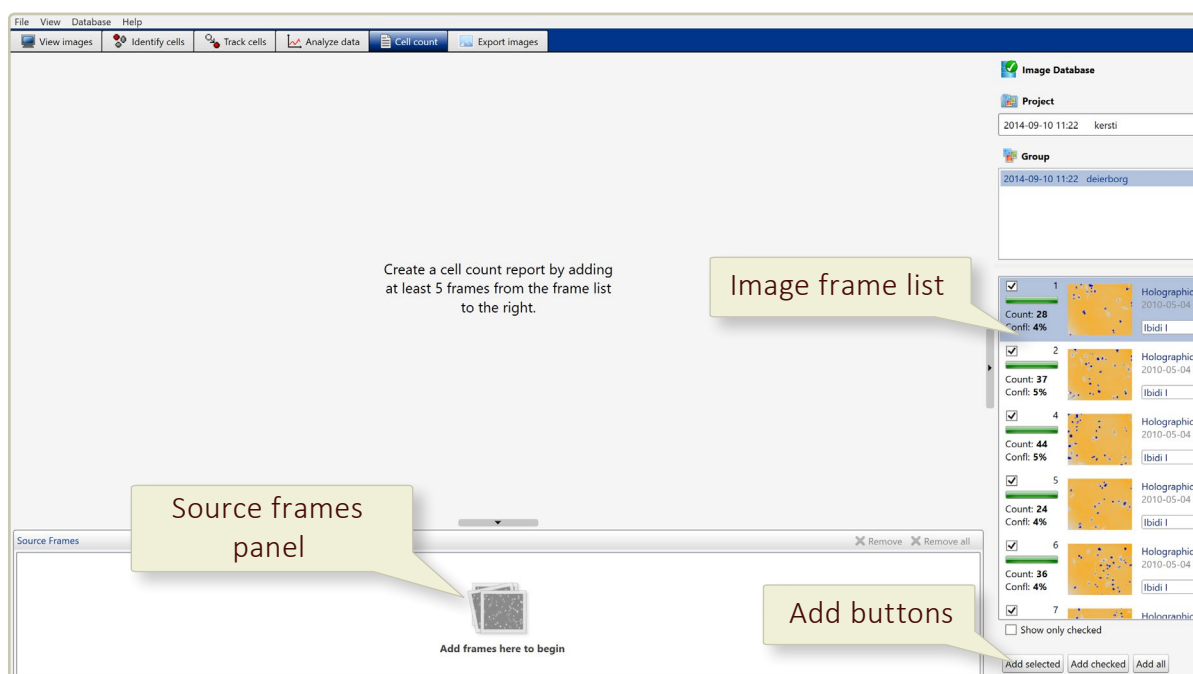


Fill in the correct cell culture vessel growth area and the volume of the cell culture vessel medium content:
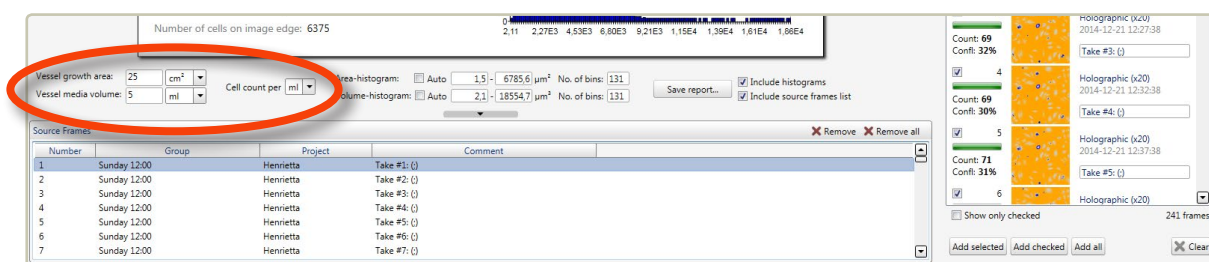
The images are then analyzed, and the results are presented in a *Cell count report*:
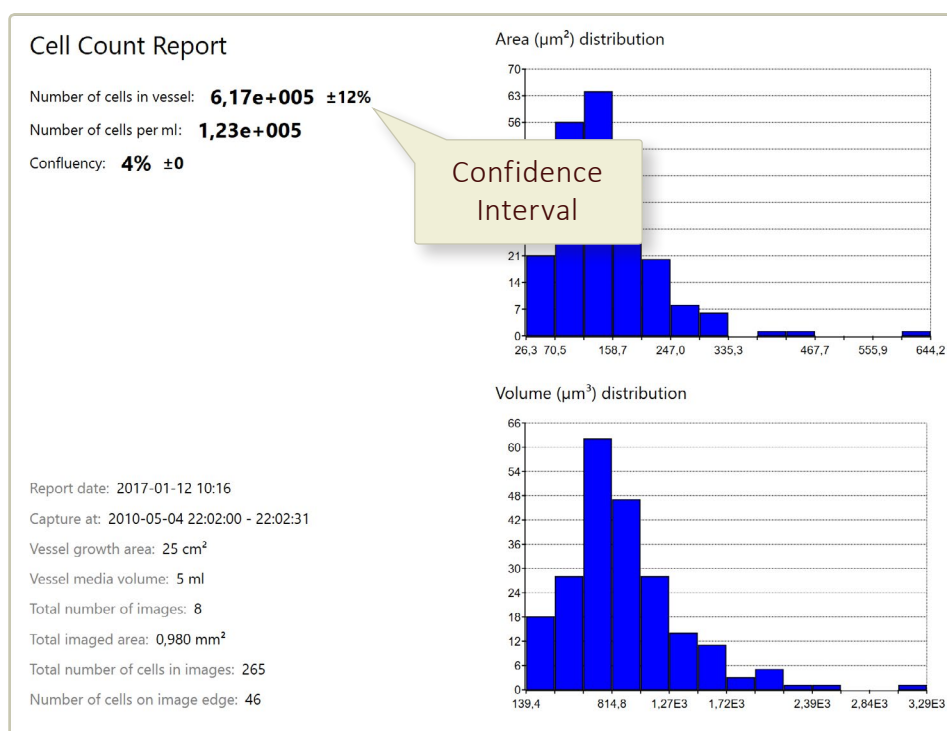


If the confidence interval is too large (larger than 10-15%) more images should be added to the analysis. The more unevenly the cells are distributed in the cell culture vessel, the more images are needed for the cell count.

## ADJUST THE HISTOGRAM PROPORTIONS

The x-axis of the area and volume histograms can be set automatically or manually. For each axis the lowest and the highest value can be set as well as the number of bins that present the data. These adjustments are found below the *Cell count report*:

## REMOVE DATA FROM PLOT

To clear the plot from all data from all image frames, use the [✕ Remove all] button. To remove data belonging to a single frame from a plot, select that frame in the *Source frames panel* and then use the [✕ Remove] button:
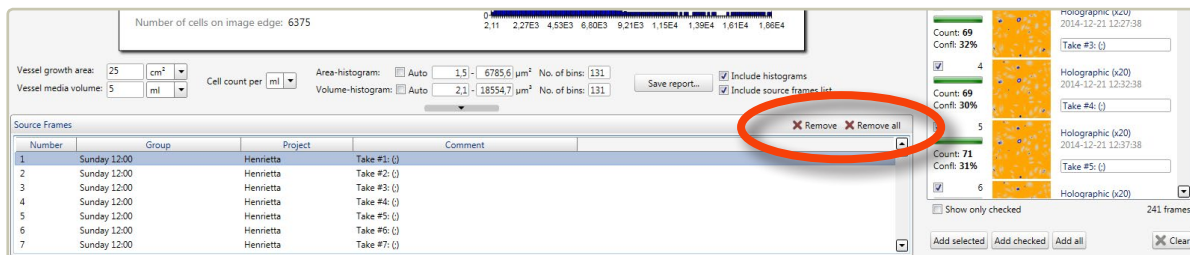


## EXPORT RESULTS

Clicking [Save report…] below the *Cell count report* opens a window where the report can be saved as a pdf or as an xml-compatible file containing all relevant data. Histograms and image frame list may be excluded from the report:
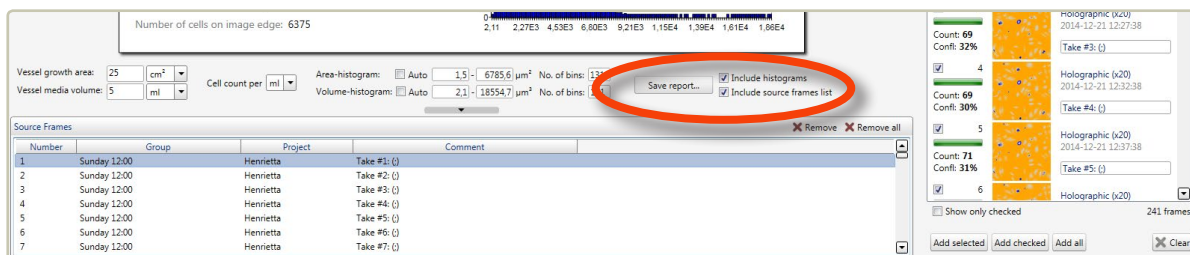
# Export images and movies

## ADD AND REMOVE IMAGE FRAMES

In the *Export images tab*, image frames can be edited and exported either as individual images in several standard formats or as AVI movies.
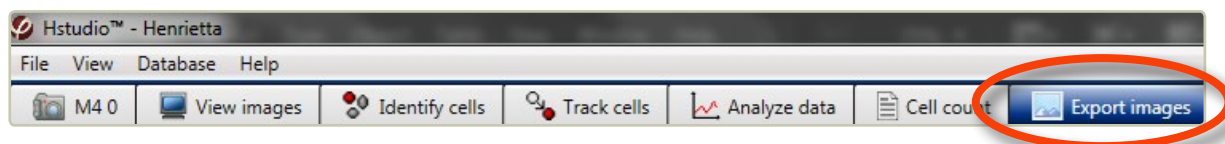


Below the *Image frame list* there are buttons to add image frames to the *View area*. The side panels become active when one or several images have been added.

By clicking the `Add selected` button, found below the *Image frame list*, the data from a single image frame or from several frames can be added when the frames are selected in the *Image frame list*.
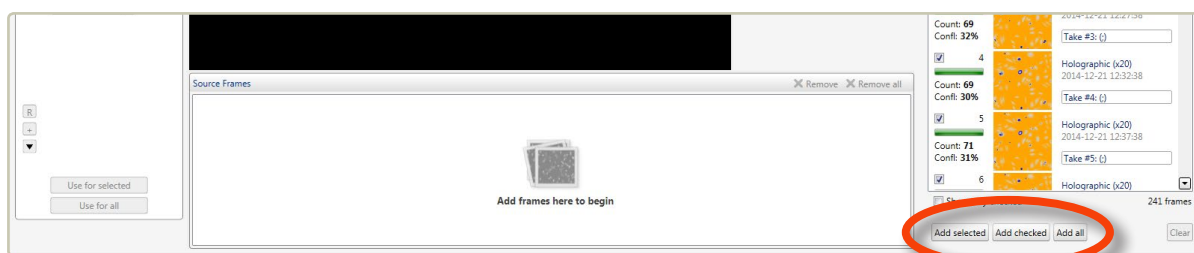


The shift key can be used to select several consecutive images and the Ctrl-key to select non-consecutive images. Images can also be selected by checking the box to the left of each image, and then clicking the `Add checked` button. All image frames can be added by clicking `Add all`. Alternatively, the images can be dragged and dropped into the *Source Frames* window.

The *View area* contains a view of the currently active added image as well as thumb nails of all the added images. By clicking an image thumb nail, it will be displayed in the *View area*:
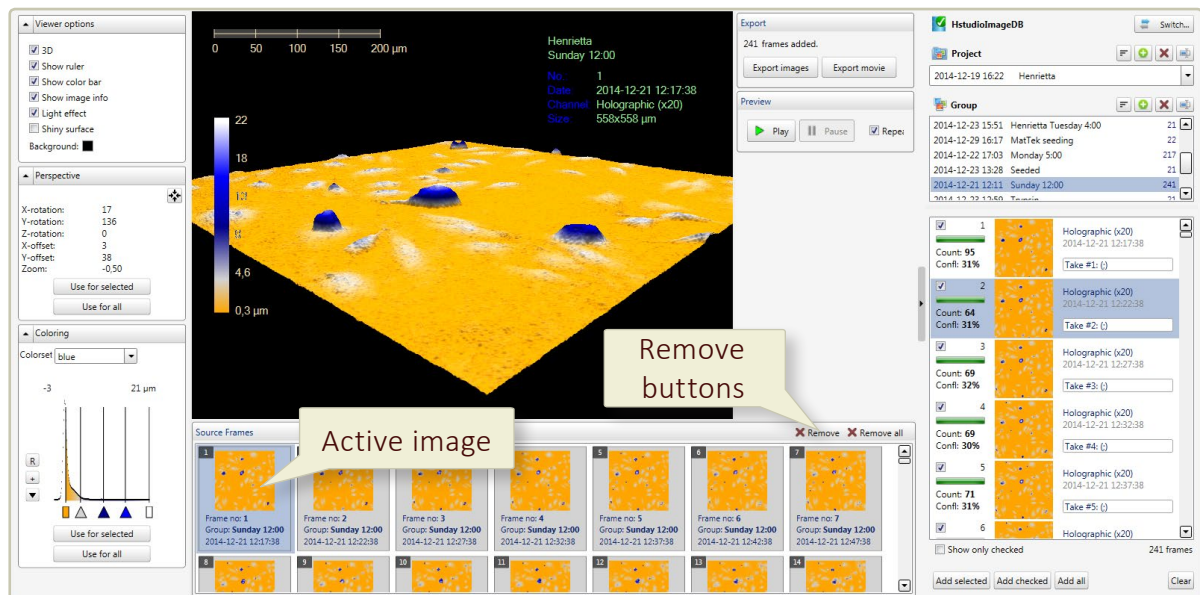


Clicking the ✖ Remove buttons will either remove the selected or all of the added images.

## EDIT THE IMAGES

**Zoom, move or flip the image**

The *Perspective side panel* shows how the image has been moved, flipped or zoomed:
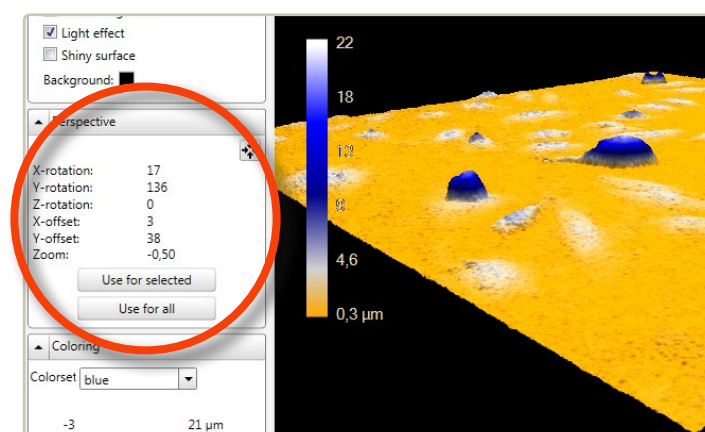


- To zoom, left-click the cell image in the *View area* and then use the mouse scroll button.

- To move the image to a desired location in the *View area*, click, hold and drag using the left mouse button.

- To flip and move the 3D image, click, hold and drag using the right mouse button.

- Click one of the *Use for buttons* to apply the changes to either the added images that are selected or to all added images:
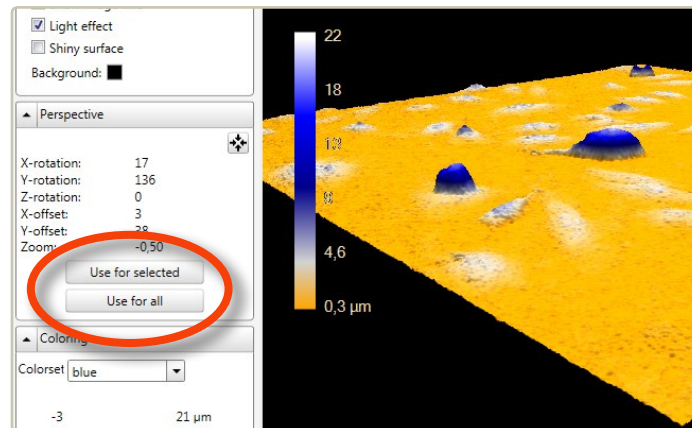


## Adjust the image display

The *Viewer options panel* which is found to the left of the *View area*, is used to adjust the image display:
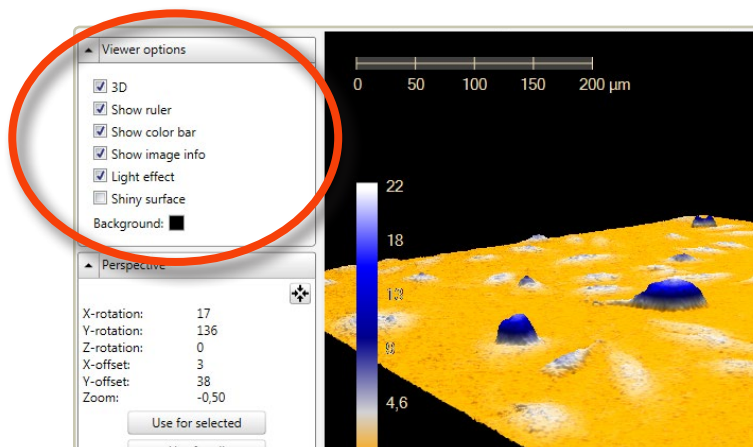


☑ ***3D***      displays the image as a 3D representation.

☑ ***Show Ruler***   displays a horizontal scale bar representative of the distance in X and Y.

☑ ***Show Color Bar***  displays a vertical scale bar representative of the height in Z.

☑ **_Show Image Info_**   displays additional information associated with the image:

          **_First row_**     specifies in which **_Project_** the image is located.

          **_Second row_**   specifies in which **_Group_** the image is located.

          **_Nbr_**       specifies which number the image has in the group.

          **_Date_**      specifies the capture date and time of the image.

          **_Channel_**    specifies if the image is holographic or phase contrast

          **_Size_**      specifies image width and height in μm.

☑ **_Light Effect_**     applies an artificial light source to the image which may sometimes render an improved image.

☑ **_Shiny Surface_**    shows the rendered surface with a reflective visual effect. **_Shiny Surface_** is only active when **_Light Effect_** is checked.

To change the background color, left-click the **_Background color box_** and select a new color.

## Change the image coloring

Use the **_Coloring panel_**, which is found to the left of the **_View area_**, to add or change the pseudo coloring to the images. The colors that are applied will color the image relatively to the thickness of the cells. For additional information on how to set the coloring, see <u>Change image coloring</u> (page 35).

# CREATE AN AVI MOVIE

When image perspective and color looks good, click the [ Use for all ] buttons in both the *Perspective panel* and the *Coloring panel*:
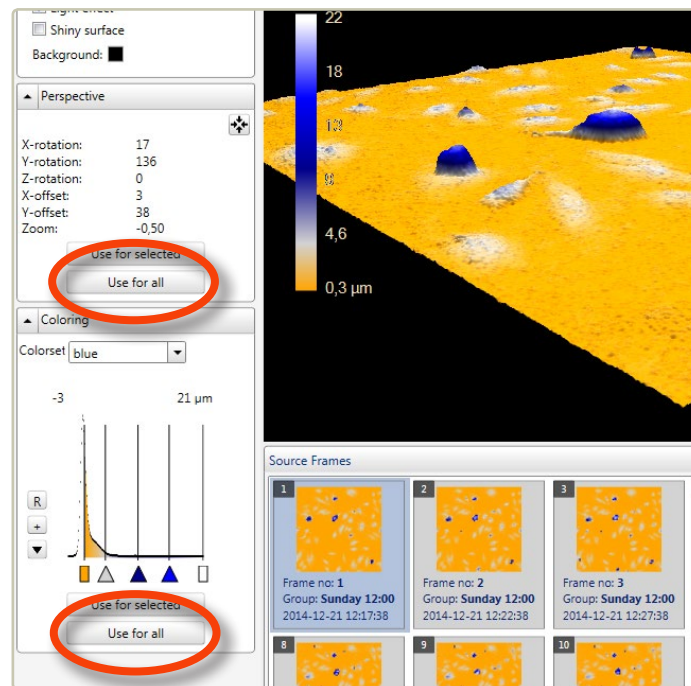


Preview the movie by clicking [ ▶ Play ]:
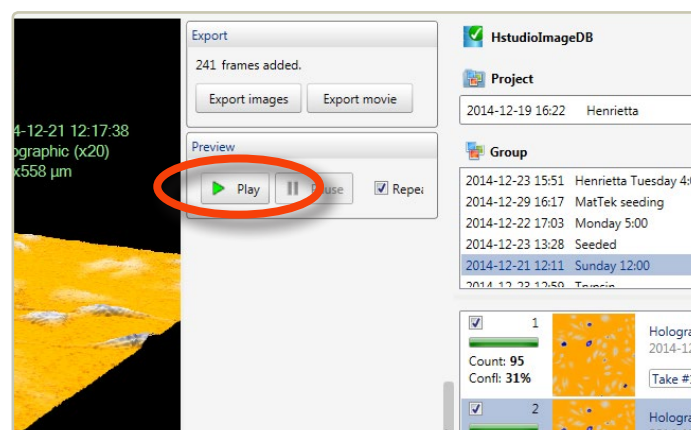
Note that the preview speed of the movie is not the same as for the saved movie, as the movie can be saved at any speed. If the preview looks good, click Export movie in the **Export panel**:
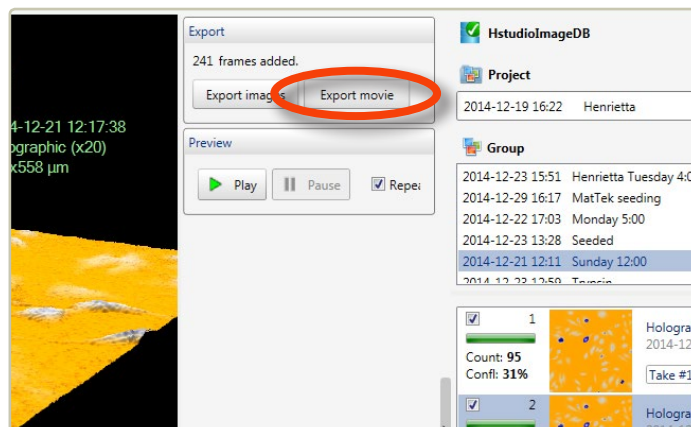


This will open the **Export movie window**:
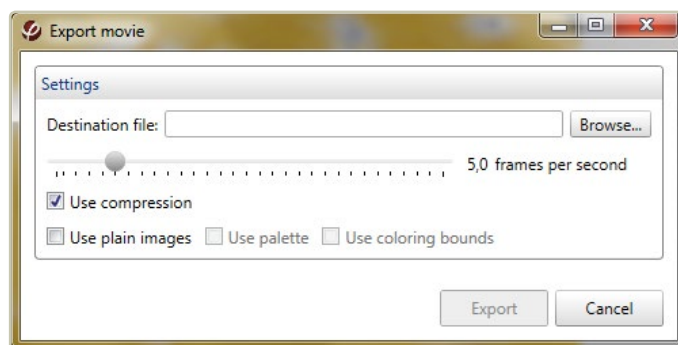


Select the destination folder by browsing. By moving the slide bar, it is possible to set the number of frames per second that will be shown in the AVI movie. When ready, click Export .

# EXPORT IMAGES

When the images have been set up nicely and look good, click Export images :
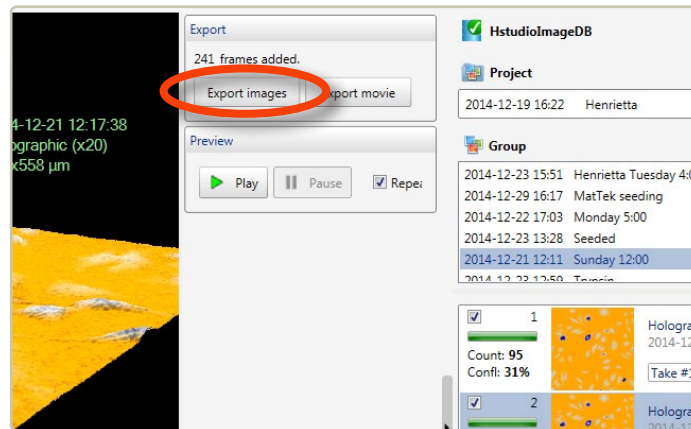


This will open the *Export images window*:
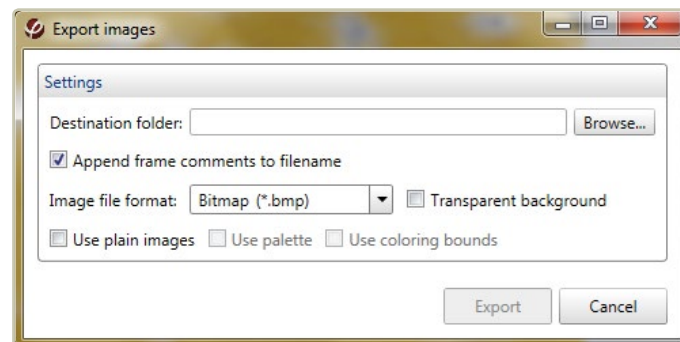


Select the destination folder by browsing. Check the box to add frame comments to the file name. The Image format can be selected in the drop menu. When ☑ *Use plain Images* is checked, no coloring or 3D will be displayed in the exported image. When ☑ *Transparent background* is checked, TIFF-images can be exported with a transparent background. When ready, click Export .

# Database handling and backup

**Note that database data from earlier versions of Hstudio can be used with version 2.7. However, the reverse is not possible. Once a database has been used by version 2.7, it cannot be used by an earlier version.**

To backup, copy or move data, go to the *Image database handler*. It can be reached from *Database menu* or by clicking the [ Switch ] button above the *Project* and *Group:*
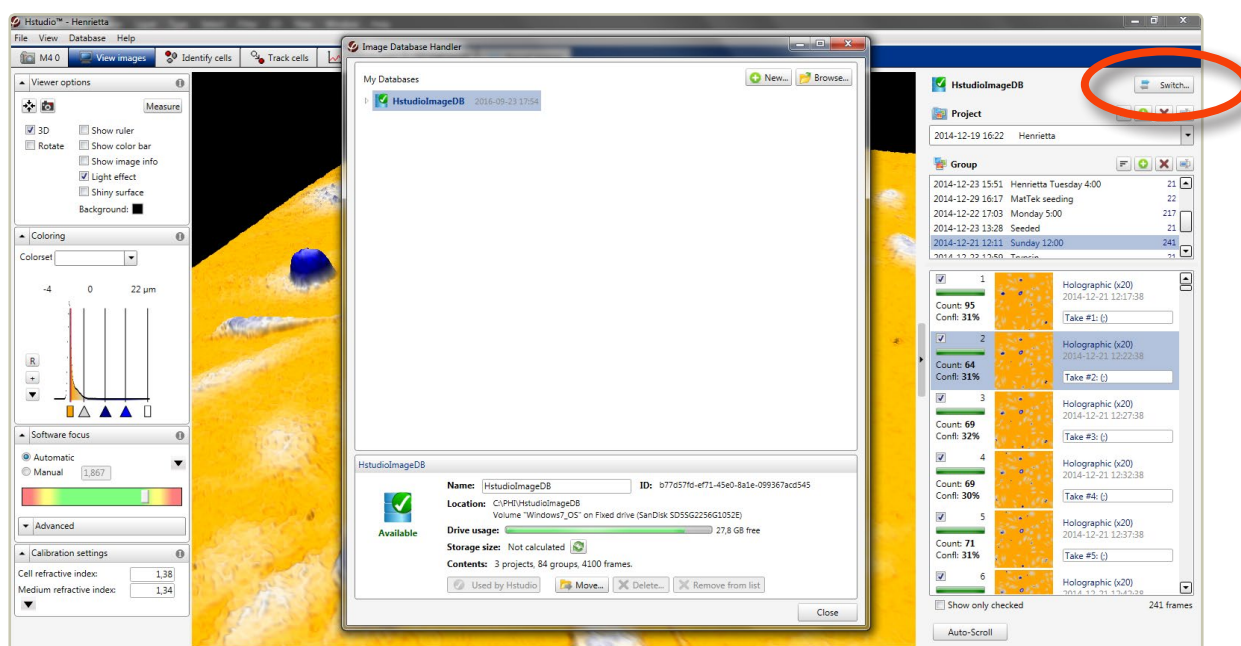


*The Image database handler*

## SWITCH TO EXISTING DATABASE

Databases that are already known to the software are displayed as a list in the *Image database handler* window. In order to add an existing database to the list:

- Click [ Browse… ].

- Locate the desired database, and click OK.

- In the lower part of the database handler window, information on the currently selected database will be displayed. Click [ ✓ Use for Hstudio ] to use the database in Hstudio.
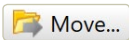
## SWITCH TO A NEW DATABASE

- Create a new database by clicking ⊕ New… . A new window will open.

- Browse to the desired location.

- Name the new database.

- Click Create . The software will now use the new database.
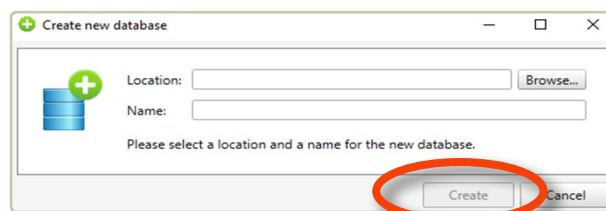


## MOVE A DATABASE

- Select a database in the *Database Handler* list of databases.

- Click 📂 Move… at the bottom of the *Database Handler window*. The *Move database window* will appear.

- Browse to the new location and then click Move . The entire database will be moved to the new location.
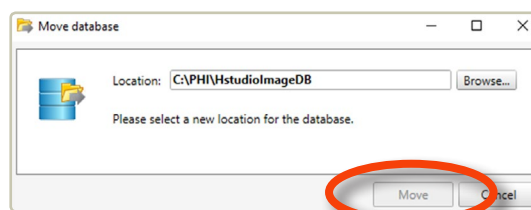
## COPY AND MOVE INDIVIDUAL IMAGES, GROUPS OR PROJECTS

- Click the little arrow ▷ beside the database that contains the data of interest. A list of *Projects* contained in the database will appear. Click the little arrow ▷ beside the *Project* and a list of *Groups* will appear. Click the little arrow ▷ beside the *Group* and a list of images will appear:
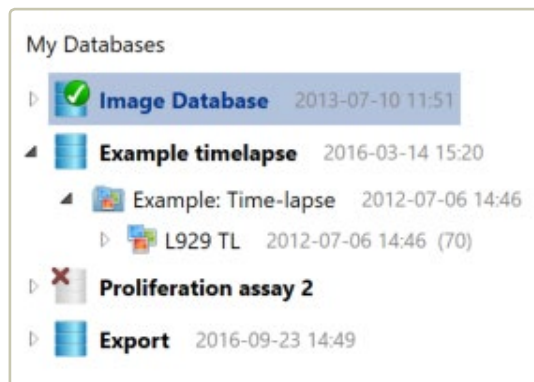


- Right-click on the *Project* or *Group* or image you wish move and select *Copy:*
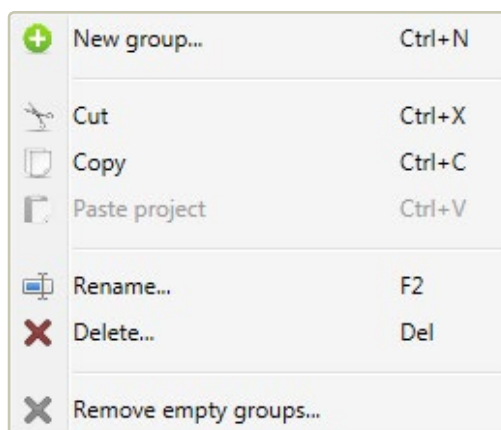


- Go to the desired database and paste the copied *Project* or *Group* or image into the database.


## BACKUP/RESTORE DATA

**To create a database backup**
- Go to the *Image database handler*.

- Click 🟢 New... to create a new database at the backup location.

- Click the little arrow ▷ beside the database that contains the data to backup. A list of *Projects* contained in the database will appear.

- Right-click on the *Project* or *Group* you wish to backup and select *Copy*:
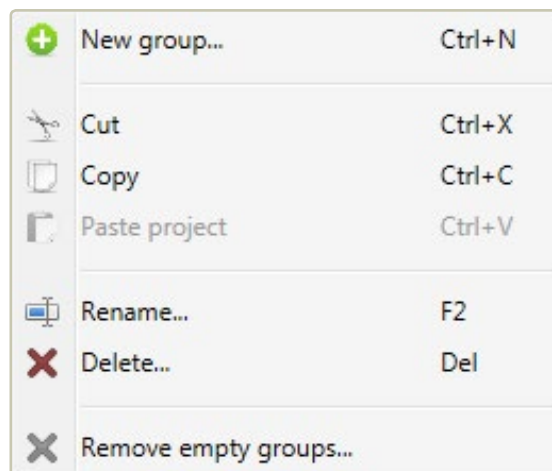


- Go to the backup database and paste the copied *Project* or *Group* into the backup database.

**To restore a database to the computer**
- Go to the *Image database handler.*

- Click ⊕ New... to create a new database on the computer.

- Browse and find the backup database. Copy and paste the backup data into the new folder. The database data are now on the computer and can be accessed by Hstudio.

## TRANSFER DATA FROM ONE COMPUTER TO THE NEXT

- Go to the top menu and click *Database*:
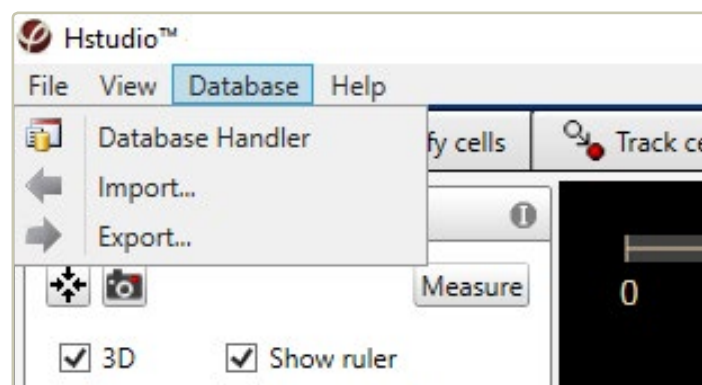
- Select *Export*. The *Export from database* window will appear:
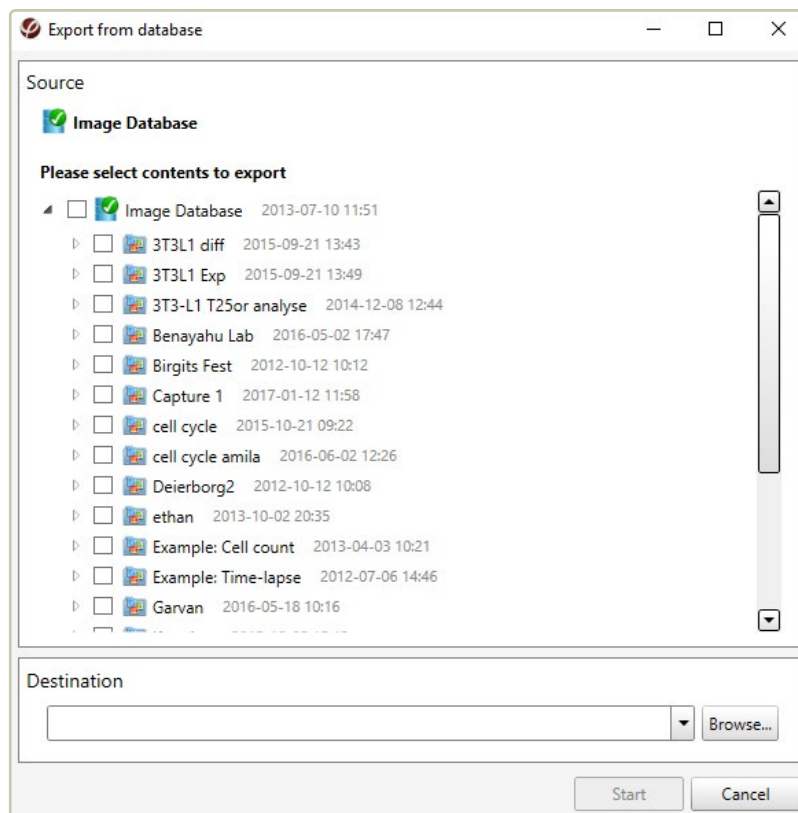


- Check *Projects* and *Groups* that will be moved.

- Browse and create a new folder at *e.g.* a USB hard drive.

- Click Start .

- When the process is finished, connect the USB hard drive to the second computer.

- Start Hstudio.

- Go to the top menu and click *Database*. Select *Import*. The *Import to database* window will appear.

- Browse to find the transfer folder on the USB hard drive.

- Click Start . The database data will be transferred to the database that is currently used by the Hstudio software.

# Troubleshooting

## LIVE CAPTURE
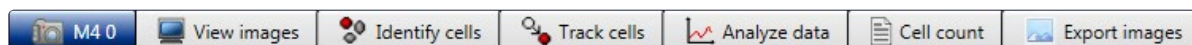


**The Live Capture tab is inactive**

*The software cannot find HoloMonitor*
- Check that the USB and power cables are properly connected.

- Restart Hstudio.

*The drivers are corrupt*
- Start the Windows Device Manager and check for any drivers with exclamation marks. Try disconnecting the USB cable from the instrument and connect it again and wait a couple of minutes. If the problem persists, try to run the installation program again. The driver files are located under C:\PHI\Drivers as sub folders. If a driver displays an exclamation mark in the device manager it may be possible to re-install it by right-clicking in the device manager and selecting Update driver. Then browse to the driver folder under C:\PHI\Drivers.

**It is impossible to focus the live image**

*For HoloMonitor with spacer plates, the wrong distance plate might be used*
- Make sure that the correct spacer plate for the cell culture vessel is used. The bottom of the vessel (where the cells are) should be approximately 1 mm from the objective. Vessels with a thick bottom or a high socket need the thinnest spacer plate. Vessels or slides which are thin should be put on the thickest or medium thick spacer plates. A list of vessels and spacer plates is found at www.phiab.se/support/vessel.

*The cell culture vial is scratched or smudged*
- Even wiping the cell culture vessel clean with ethanol will probably not help much as traces of dirt will still persist. Instead, avoid touching the top and bottom surfaces of the cell culture vessel when handling it, even when wearing gloves. Avoid anything that might scratch the surfaces. Cell culture plastic is very easily scratched.

*There is condensation on the inside of the cell culture vessel*
- The PHI HoloLids (page 22) are designed to handle condensation. It is recommended to use HoloLid whenever possible. If the cell culture vessel is a flask, tighten the cap and gently rinse the condensation away with the cell culture medium by tilting the flask. Avoid getting medium into the cap. If the cell culture vessel is a multi-well plate or a petri dish, allow the vessel to reach room temperature, alternatively switch lids to a new lid or try working without a lid.

- If the cell culture medium is replaced with cold PBS or cold cell culture medium immediately after taking the multi well plates and petri dishes from the incubator, there will not be condensation. Replace the cold liquid with medium again after the analysis.

*The objective and the laser window need cleaning*
- Clean the instrument according to the <u>Cleaning instructions</u> (page 18)**.**

*Auto focus has difficulties to find focus*
- Allow the instrument to warm up in the incubator for at least three hours.

- The cells are very thin or very sparsely distributed in the vessel. Set the software focus manually instead of automatically. Activate manual mode by clicking the `Manual` button in the ***Software focus panel*** in the ***Live capture tab***. Thereafter drag the slider to the green area in the focus bar, and then adjust the mechanical focus. Thin cells have a very narrow focal distance and focusing has to be performed with great care.

- Floating cells disturbed the automatic software focus. Follow the instructions in <u>Re-focus a captured image</u> (page 51).

*Something causes the instrument to vibrate*
- Check that the cables connected to HoloMonitor are slack and not touching anything. Ensure that no vibrations come from sources like centrifuges or hard working air vents.

*The live image focus was OK, but it slowly turned bad and now it can not be set again*
- There is probably a drop of water hanging from the top/lid of the cell culture vessel. As the drop grows larger it will increasingly disturb the image focus. Gently tilt the cell culture vessel and make the drop slide to the side of the vessel.

## The cell image in the view area looks strange

*The cells are very bright and blurry, showing no inner structures*
- The image dynamics are not optimally set. Press the `R` button by the histogram in the ***Coloring panel***.

*The cell image is completely white*
- The image dynamics are not optimally set. Press the `R` button by the histogram in the ***Coloring panel***.

*The cell image is black*
- The laser unit may not be switched on or connected properly. Start by checking that the laser unit is connected properly both to power and to the instrument. If the laser is connected properly and the image still is black, please contact <u>support@phiab.se</u>.

*The images are either zoomed in or out too much*
- Use the mouse scroll to scroll back.

*The image dynamics are not optimally set*
- Press the R button by the histogram in the *Coloring panel*.

## The capture button is inactive

*The Capture button is inactive when no project and group has been selected*
- In that case, the software does not know where to store the captured images. Select or create a *Project* and a *Group*.

## The time-lapse sequence position is unstable

*The time-lapse is drifting in X, Y or Z*
- Sometimes there will be a slight drift the first half hour.

- The sample holder is not properly attached. Ensure that the sample holder is firmly put in place.

- The cell culture vessel is not properly placed in the sample holder. Ensure that the cell culture vessel is firmly put in place.

*The motorized stage needs to be calibrated*
- Contact support@phiab.se.

## The images are not captured at the time points set at the start of the experiment

*There are too many positions too far apart*
- The motorized stage cannot move fast enough to be able to capture the images on time. Instead it works as fast as possible. Set fewer capture points, or allow more time between captures.

## The image looks garbled

### *Wrong spacer plate may be used*

- Ensure that the correct spacer plate is used for the cell culture vessel to place the cells approximately 1 mm from the objective. Vessels with a thick bottom or a high socket should be put on the thinnest spacer plate. Vessels or slides which are thin should be put on the thickest or medium thick spacer plate. When the cells are not positioned within range of the auto focus, the image appear as in the example images below.
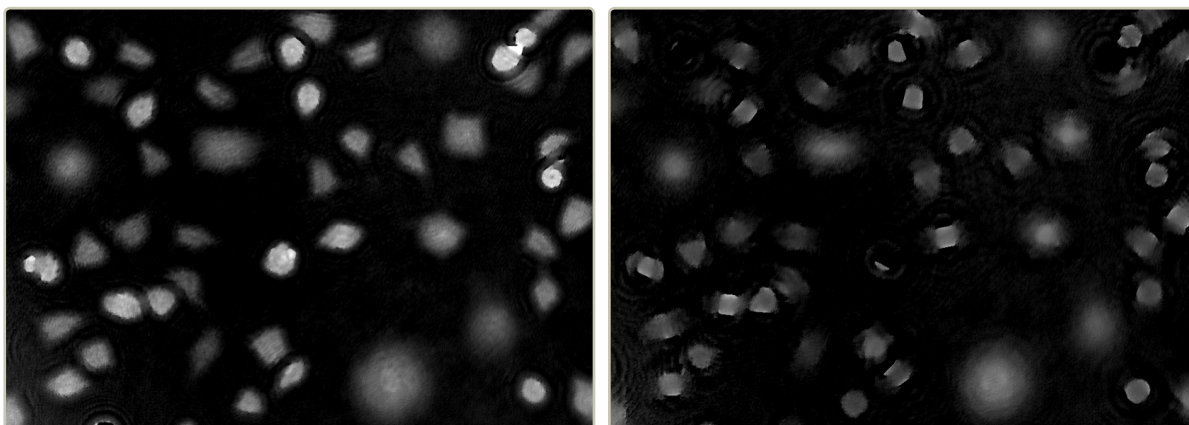


### *Dirt, smudges, scratches, condensation or fingerprints on the vessel, top laser window or objective will affect the image negatively*

- Try moving around the culture vessel to different positions. Make sure that the laser light has a free path through the vessel.

- Clean the instrument as described in the <u>Cleaning instructions</u> (page 18).

- Condensation on the roof of the vessel. Keep vessels at the same temperature and humidity as the surrounding the instrument. Avoid transporting vessels immediately before starting a capture sequence. Use vessels which are condensation free, e.g. IBIDI-micro channel slides, or the <u>PHI HoloLids</u> (page 22).

- Re-calibrate according to the <u>Calibration procedure</u> (page 19).

- Vibrations, check that the cables connected to HoloMonitor are slacking and not touching anything creating tension.

### *The image dynamic display is off range*

- Click the R button in *Coloring panel* to reset the image dynamics display, see <u>Change image coloring</u> (page 35).

*The laser is not optimal*
- Make sure that the laser beam intensity is adequate. When auto-exposure is on (camera properties window in the left side window), the exposure time for an image without a sample should be around 5 ms and never more than 10 ms. If that is the case, contact the local distributor for advice.

*Condensation has formed inside HoloMonitor*
- The HoloMonitor may suffer from internal condensation if the incubator is excessively humid, or if the instrument power cord has been unplugged. Remove the instrument from the incubator and let it dry over-night in room temperature. After drying, calibrate HoloMonitor according to the <u>Calibration procedure</u> (page 19) to ensure that the ***Diagnostic Values*** are in the green before proceeding to incubator acclimatization.
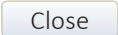
## Only one position in a capture pattern is captured

*Even if several positions have been set in a capture pattern, only one is captured*
- Ensure that the Capture Pattern box in the Capture side panel is checked.

## The images from several parallel time-lapses are not sorted separately

*Each position was supposed to go into a separate group,*
*as selected in the Capture Position window*
- Recreate the ***Capture setup***, and ensure that [ Close ] is clicked. That will close the window and save the set up.

# VIEW IMAGES
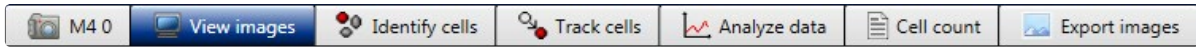
## No image frames are visible in the Image frame list

- No project and group has been selected. Select a *Project* and a *Group*.

- If the ☑ *Only checked* is checked, only checked images will be displayed. If no image frames in the current *Group* have been checked, no frames will be visible. Uncheck ☐ *Only checked*.

## The cell image in the view area is white

- The image dynamics are not optimal. Click the R button in the *Coloring panel.*

## In a series of captured images not all images were good

- There might have been a scratch or a smudge on the vessel where the bad images were captured. Capture extra images and then discard the bad ones.

- A passing disturbance, e.g. floating cell debris, caused the software auto-focus to miscalculate. Follow the instructions how to manually reset an unfocused image.

## IDENTIFY CELLS
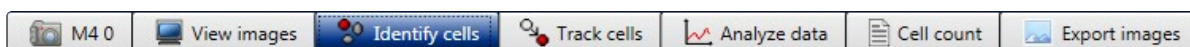
M4 0 | View images | Identify cells | Track cells | Analyze data | Cell count | Export images

### No image frames are visible in the Image frame list

- No project and group has been selected. Select a **Project** and a **Group**.

- If the ☑ **Only checked** is checked, only checked images will be displayed. If no image frames in the current **Group** have been checked, no frames will be visible. Uncheck ☐ **Only checked**.

### The automatic cell identification looks strange

- The **Background threshold method** may not suit the cells. Try a different threshold method.

- If the cells grow at a very high density, the threshold setting methods are not able to identify individual cells. If cells are growing very close to each other they cannot be separated out. Adjust the **Background threshold** in **Adjustment tab**. Make sure that only the background and not any cells are defined as background. Then use the confluency or the cell dry mass parameter to determine the amount of cells instead of the cell number.

### There is background noise in the image that causes the background to be set at an incorrect level

- Make sure that background and laser are calibrated prior to analysis.

### Some cells are incorrectly segmented as two or more

- The minimum object size is set too low. Adjust the Min object size using the slider in the Object definition box under the Adjustment tab so that each cell has one blue marker.

### Two cells are incorrectly segmented as one

- The minimum object size is set too high. Adjust the **Min object size** in the **Adjustment tab** so that each cell has one blue marker.

- The cell confluency is very high and the cells are difficult to separate out. Separate the two cells by using the Add or remove button under the **Manual changes tab** in the **Identify cells tab**.

## TRACK CELLS

| M4 0 | View images | Identify cells | **Track cells** | Analyze data | Cell count | Export images |

### The tracks are very irregular

- The cell segmentation is not properly done. Go to the Identify Cells tab and make sure that the segmentation is properly performed.

### There is a very high number of warnings

- The cell segmentation is not properly done. Go to the Identify Cells tab and make sure that the segmentation is properly performed.

## ANALYZE DATA
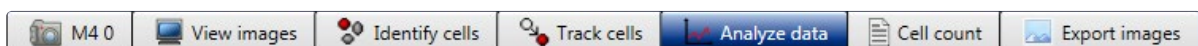
| M4 0 | View images | Identify cells | Track cells | **Analyze data** | Cell count | Export images |

### No image frames are visible in the Image frame list

- No project and group have been selected. Select a *Project* and a *Group*.

- If the ☑ *Only checked* is checked, only checked images will be displayed. If no image frames in the current *Group* have been checked, no frames will be visible. Uncheck ☐ *Only checked*.

### The dots in the scatter plot disappeared

- The changes in settings and display has caused the dots to be outside the range of the scatter plot values. Set the plot area to *Auto-scale*.

## EXPORT IMAGES
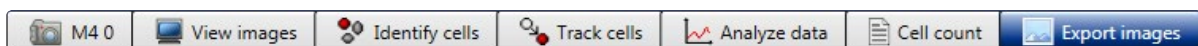
| M4 0 | View images | Identify cells | Track cells | Analyze data | Cell count | **Export images** |

### No image frames are visible in the Image frame list

- No project and group have been selected. Select a *Project* and a *Group*.

- If the ☑ *Only checked* is checked, only checked images will be displayed. If no image frames in the current *Group* have been checked, no frames will be visible. Uncheck ☐ *Only checked*.

### *The cell image in the view area is white*

- The image dynamics is not optimal. Click the R button in the *Coloring panel*.

# Morphological parameters

## HOLOGRAPHIC MICROSCOPY

HoloMonitor creates label-free holographic cell images. Laser light is divided into a reference and an object beam. As the object beam passes through the sample, a phase delay is imprinted on the light beam. By subsequently merging the sample and the reference beam, an interference pattern is created. The interference pattern is the hologram. It is recorded by an image sensor and then used to numerically reconstruct a so-called phase image, which is displayed and analyzed (Mölder et al 2008).
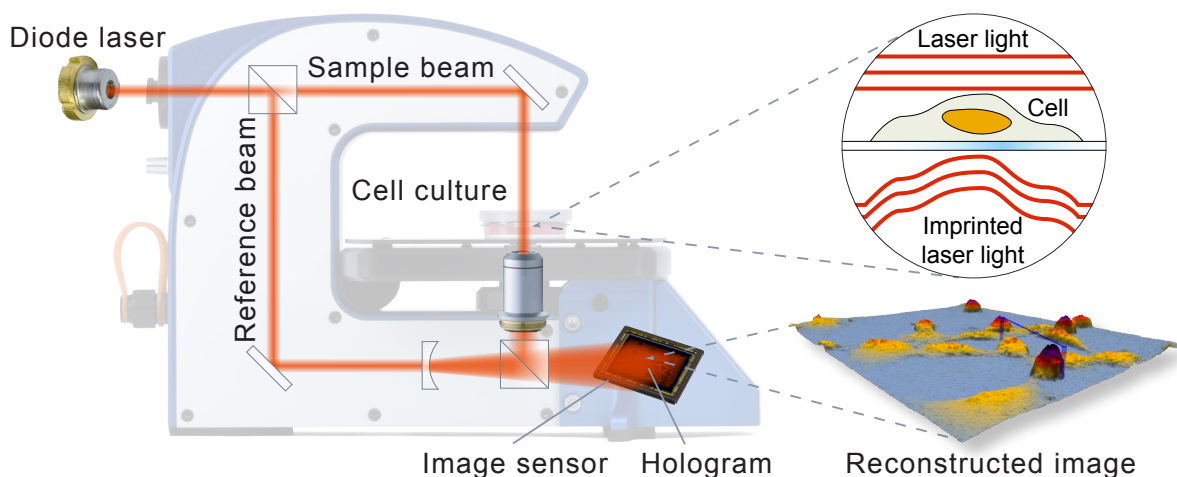


*The holographic setup showing how the laser beam is split. To the right, the laser wave front passes through the cell and the phase delay is imprinted upon the light waves.*

A phase image displays the phase delay, *i.e.* the phase shift of the light, caused by the sample. When the light passes through the cell, it will travel slower. Once through the cell, it will regain its former speed. This delay will cause the phase of the light waves to be offset, or shifted, compared to the reference light. The quantified phase shift is the basic raw image data of holographic microscopy. Cell below described morphology parameters are calculated by the Hstudio software, based on the phase image.

The estimated cell thickness *(T)* of a cell in pixel *i* is obtained from the phase shift *(φ)*, the wavelength of the laser light *(λ)*, the refractive index of the cells *(n_c)* and the refractive index of the surrounding medium *(n_m)*:

$$T_i = \frac{\lambda}{n_c - n_m} \cdot \varphi_i$$

The default values used for $n_c$ and $n_m$ (1.38 and 1.34 respectively) are average refractive index values based on publications worldwide (Dunn & Richards-Kortum 1996, Farina & Verkman 1996, Rappaz et al 2005 and Kemper et al 2010). The default refractive indexes may be changed, as described in Change default cell and medium refractive index (page 36). Small variations in the refractive index do not cause any significant changes in the calculations.

The refractive index of the surrounding medium should not deviate too much (+/- 0.08) from the refractive index of the cells. It should also not be identical with the refractive index of the cells.

It is important to remember that the phase delay in each pixel in a phase shift image is the sum of the phase shift created by variations in refractive index along the optical path. It is therefore not possible to determine whether a variation in phase shift is caused by a change in cellular thickness or by a refractive index change in the cellular area imaged by a pixel.

## CELL MORPHOLOGY PARAMETERS

### Area
The cell area *(A)* is calculated in the same way as when using conventional microscopy:

$$A = N \cdot s_{xy}^2$$

where $s_{xy}^2$ is the calibrated size of the area imaged by a single pixel and where $N$ is the number of pixels which image the cell area.

### Optical Volume
Independent of its shape, the estimated volume of a cell *(V)* is calculated from the phase shift (Kemper et al. 2010):

$$V = \frac{\lambda \cdot s_{xy}^2}{n_c - n_m} \cdot \varphi_{sum}$$

where $\varphi_{sum} = \sum_{i=0}^{N} \varphi_i$ .

### Optical thickness
The average cell thickness $(T_{avg})$ is obtained from the cell volume and the cell area:

$$T_{avg} = \frac{V}{A} = \frac{\lambda}{n_c - n_m} \cdot \frac{\varphi_{sum}}{N}$$

### Optical thickness max
The maximum cell thickness $(T_{max})$ is obtained from maximum phase shift:

$$T_{max} = \frac{\lambda}{n_c - n_m} \cdot \max_{i=1\ldots N} \varphi_i$$

## Optical path length

Optical path length or optical path difference *(OPD)* is the extra optical distance the illuminating laser light travels when it passes through the more optically dense cell:

$$OPD_{avg} = T_{avg} \cdot (n_c - n_m) = \lambda \cdot \frac{\varphi_{sum}}{N}$$

$$OPD_{max} = T_{max} \cdot (n_c - n_m) = \lambda \cdot \max_{i=1...N} \varphi_i$$

*OPD* is expressed in µm. Notice that contrary to cell thickness, *OPD* is independent of the refractive index of the cells *($n_c$)* and the cell culture medium *($n_m$)*.

## Boxed length and breadth

In order to measure the length and the width of the cell, a frame is fitted around the cell. The frame that fits the cell while covering the smallest area is selected for measurements. The parameter depends on the threshold setting that distinguishes background from cell. The boxed length *(L)* and boxed breadth *(B)* are the inside length and breadth of the rectangle that precisely encloses the whole cell.
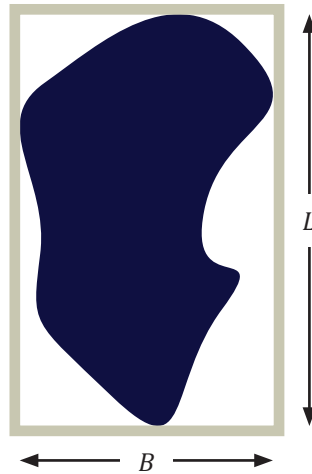


## Boxed center position x and y

The geometric center position of an area-minimized (aligned) bounding box that encloses the cell.

## Centroid position x and y

The center of mass is the arithmetic mean of all points weighted by the thickness of the cell. If a physical object has uniform density, then its center of mass is the same as the centroid of its shape. Usually, but not always, the mass center corresponds to the cell nucleus.

### Eccentricity

How elongated the cell is, or how much the cell deviates from being a circle. A value of 0 corresponds to a circle and the more elongated the cell is the higher the eccentricity value becomes, approaching 1. The calculations are based on a threshold setting that distinguishes background from cell.

### Hull convexity

A measure on how much the 3D cell shape deviates from the perfect convex shape. A cell is usually rather smooth in 3D. A higher value means less dips in cell thickness i.e. a more perfect shape.

### Irregularity

A measure of how much the circumference of the cell deviates from the circumference of a perfect circle. A value of 0 means the cell is circular and higher values mean a longer, more irregular outline. The calculations are based on a threshold setting that distinguishes background from cell.

### Migration distance

The migration distance is the shortest distance between the starting point and the end point of the cell path.

### Motility distance and speed

The motility distance is the actual distance traveled by the cell between the starting point and the end point of the cell path. The motility speed is the motility distance divided by the time it took for the cell to travel from the starting point to the end point.

### Migration directness

How direct the cell movement towards its end-point is (migration distance/motility distance). A number close to one represents a direct movement. A number below 0.5 represents a non-direct random movement.

### Peak position

The position of the pixel with the highest measured thickness in the cell. For healthy normal cells this usually corresponds to nucleus and nucleoli.

### Perimeter length

The length of the perimeter around the cell. The calculations are based on a threshold setting that distinguishes background from cell.

## Phase shift

The shift of the light caused by the cells when the light passes through. Phase shift is the original parameter that is measured with digital holography. The different ways to look at the phase shift may reveal different effects of cell treatments.

**Phase shift avg.**    The average measured phase shift in each pixel within the cell area

**Phase shift max.**    The maximum measured phase shift in a pixel within the cell area.

**Phase shift min.**    The minimum measured phase shift in a pixel within the cell area.

**Phase shift std. dev.**    Standard deviation of the measured phase shift within the cell area.

**Phase shift sum**    A different way of calculating the texture correlation. The phase shift sum of a cell *($\varphi_{sum}$)* is the sum of the phase shift in all pixels of the cell:

$$\varphi_{sum} = \sum_{i=1}^{N} \varphi_i$$

where *N* is the number of object pixels. *$\varphi_i$* is the average phase shift in radians created by the area imaged by object pixel *i* divided by *2π*.

## Roughness

Roughness is calculated by subtracting a mathematically smoothed image from the actual image in each pixel, and gives an indication of the smoothness/roughness of the cell. The calculations are based on the phase shift in each pixel of the thresholded cell. A healthy cell usually has a low degree of roughness, while a dying or dead cell usually has more roughness. The roughness of cells may be differently distributed in the cell after different treatments. The different ways to look at the roughness may reveal different effects of cell treatments.

**Roughness avg.**    The average of the roughness distribution.

**Roughness kurtosis**    How peaked the value distribution is. A positive kurtosis distribution has a sharper peak and longer, fatter tails (e.g. a Student´s t distribution) while a negative kurtosis distribution has a more rounded peak and shorter, thinner tails (e.g. a Bernoulli distribution). A neutral kurtosis distribution (=0) corresponds to a normal distribution.

**Roughness RMS**    The root-mean-square of the surface roughness. This value is also known as the quadratic mean. All the values are squared, then averaged and thereafter the square root of the quadratic mean is presented.

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| *Roughness skewness* | How symmetrically the roughness values are distributed around the mean. A negative skew indicates that there are more values that are higher than the mean and a positive skew indicates that there are more values that are lower than the mean. Zero skew indicates that the values are symmetrically distributed around the mean. |

## Shape convexity

A measure on how convex the outline of the cell is. The area of a cell is usually more or less irregular and uneven. This is a measure of how uneven the shape of the area is.

## Texture

Texture is a measure of the variation of the cell structure. It describes smoothness, coarseness and regularity. The texture of an imaged cell may differ depending on where the cell is in its life cycle or depending on different treatments. The provided texture parameters are all different ways of presenting the texture. The different ways to look at the texture may reveal different effects of cell treatments. These texture features are reported in Haralick et al. 1973.

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| *Texture clustershade* | Clustering is the process by which the natural groupings are determined, such that the objects in each group exhibit more similarity to one another than to objects in other groups. Cluster shade is a measure of skewness of the gray levels. When the cluster shade is high, the image is not symmetrical. |
| *Texture contrast* | Measures the thickness contrast between a pixel and its neighbor. |
| *Texture correlation* | Measures the joint probability occurrence of specified pixel pairs, i.e. how likely it is that pixels with certain phase shifts occur together. |
| *Texture correlation info1* | A different way of calculating the texture correlation. |
| *Texture correlation info2* | A different way of calculating the texture correlation. |
| *Texture energy* | Provides the sum of squared elements in the gray-level co-occurrence matrix. Also known as uniformity or the angular second moment. |
| *Texture entropy* | Entropy is a statistical measure of randomness that can be used to characterize the texture of the cell image. |
| *Texture homogeneity* | Measures how many different gray levels the image holds. |

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| *Texture maxprob* | High values occur if one combination of pixels dominates the pixel pairs in the window. |

## REFERENCES

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Farina and Verkman (1996) Cell Volume and Plasma Membrane Osmotic Water Permeability in Epithelial Cell Layers Measured by Interferometry, Biophysical J., Vol. 71: 3511-3522

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Mölder et al (2008) Non-invasive, label-free cell counting and quantitative analysis of adherent cells using digital holography, J. of Microscopy 232: 240–247

Rappaz et al (2005) Measurement of the integral refractive index and dynamic cell morphometry of living cells with digital holographic microscopy, Optics Express, Vol. 13: 9

Kemper et al (2010) Label-free quantitative cell division monitoring of endothelial cells by digital holographic microscopy, Journal of Biomedical Optics 153

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