



# holo monitor<sup>®</sup>

## App Suite

## App Suite 3.4

# Setup and Operation Manual

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Revision 3

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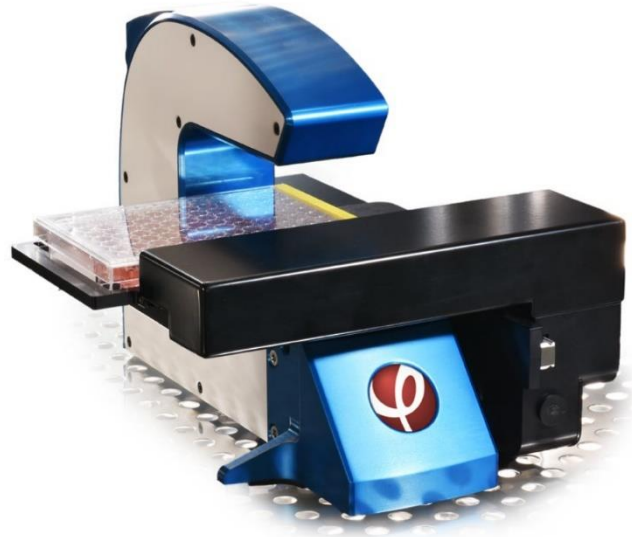
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## INTRODUCTION TO HOLOMONITOR® M4

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
- Wound healing
- Cell division/mitosis
- Toxicology/dose-response



HoloMonitor M4 is based on digital holographic microscopy. This technique measures how the phase of light shifts when it passes through the cells and does not require any kind of labeling or staining. All images are holographic reconstructions, based on the hologram captured by the HoloMonitor. The technology is described more in depth in the Analysis Parameters section of this manual.

HoloMonitor M4 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. HoloMonitor is equipped with a computer-controlled motorized XYZ-stage.

Applications are described in detail in separate protocols. All protocols can be found on <https://phiab.com/resources/manuals-protocols/>.

## THIS MANUAL

The first part of this manual includes information on instrument safety and handling.

The second part includes guides on how to use the different parts of App Suite software.

The third part is a troubleshooting guide.

Applications are described in detail in separate protocols. All protocols can be found on <https://phiab.com/resources/manuals-protocols/>.

## INSTRUMENT SAFETY

HoloMonitor M4 is CE -labeled. The CE label is subject to the general principles set out in Article 30 of Regulation (EC) No 765/2008, in accordance with

- Electromagnetic Compatibility (EMC) **2014/30/EU** (*former 2004/108/EC*)
- Low Voltage Directive (LVD) **2014/35/EU** (*former 2006/95/EC*)

and referring to the harmonized standards

- **IEC 61010-1:2010** (Safety requirements for electrical equipment for measurement, control, and laboratory use.)
- **IEC 60825-1:2014** (Safety of 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 to 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 manufacturer's 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.



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

### WARRANTY.

From the date the Product is sold to an end-user for the first time, as evidenced by the original proof of purchase, Manufacturer warrants the Product is free from defects in materials and workmanship ("Defects") as follows:

Twelve (12) months for the main device for accessories sold separately or included in the sales pack of the main device.

During the warranty period, Manufacturer will, in a reasonable time, remedy the Defect free of charge by either repairing or replacing the defective Product or the defective part of it at its option provided that you have informed Manufacturer of the Defect before the warranty period expires. When repairing or replacing your Product, Manufacturer may use new or re-conditioned parts or products.

### Limited Warranty.

Manufacturer does not provide any warranty for the following: Errors or damage caused by: (i) misuse or not using your Product in accordance with the user guide, (ii) using your Product with, or connecting it to, any product, accessory, software, or service not manufactured or supplied by Manufacturer, (iii) any products combined with your Product by a third party, (iv) damage or errors caused by hacking, cracking, viruses, or other malware, or by unauthorized access to services, accounts, computer systems or networks; or (v) other acts beyond Manufacturer's reasonable control.

## COMPUTER REQUIREMENTS

HoloMonitor is controlled by the proprietary HoloMonitor App Suite software (App Suite) running on an external computer. App Suite needs to be installed prior to installation of the HoloMonitor base unit. These are the recommended computer specifications:

Operating system:	Windows 10, 64-bit
Processor:	Intel Core i7 or AMD Ryzen 7
Memory:	16 GB RAM (8 GB minimum)
Hard drive:	512 GB SSD (256 GB minimum) and external USB3 hard drive for backup and transfer
Display:	Full HD (1920x1080) or higher
Other:	2 USB ports

## INCUBATOR REQUIREMENTS

The HoloMonitor is designed to work in an incubator with 95% humidity and 5% CO<sub>2</sub> at 37°C but will work from 10-40°C and at ambient humidity and any combination of the ambient gases (CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub> etc.).

The incubator needs to have sufficient space for the HoloMonitor stage to move during the experiments.



**Note that when sterilizing the incubator with heat or chemicals other than ethanol, HoloMonitor and all cables need to be removed from the incubator.**

Operating temperature	10-40° C
Operating humidity	Max 95 %
Space required in incubator	40×27×19 cm (w×d×h)

## SOFTWARE

### Software installation

App Suite is installed through a single installer executable that guides through the installation process. The same installer is used regardless of purchased applications within the software. The installer can be downloaded from the PHI website (<https://phiab.com/product/holomonitor-app-suite-software/>)

### Software activation

After installation, App Suite needs to be activated by entering the License ID and activation password which were provided during system installation. The activation step requires internet access. Once App Suite is activated, the purchased applications become available. A request for activation will appear automatically the first time someone uses the software.

One software license is valid for two computers.

### License Status

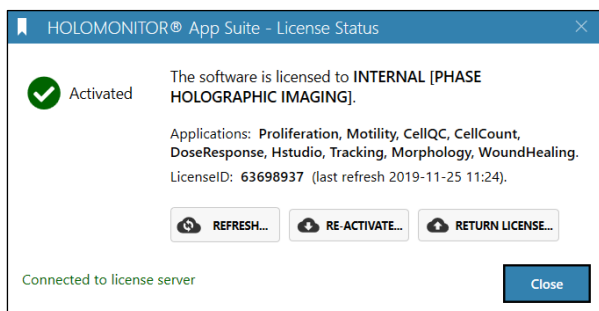
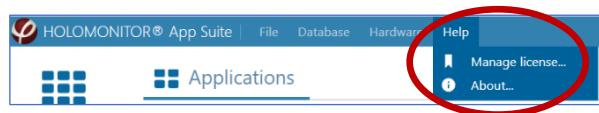
The current license status can always be accessed via the Help tab in the top menu.

Here it is possible to refresh, re-activate or return the current license.

*Refresh* will update the license. This may be necessary for newer versions of the software or if new applications have been purchased.

*Re-Activate* makes it possible to switch to another License ID, e.g. switching from a time-limited to a standard license, without reinstalling the software.

*Return License* is used to inactivate the software on the current computer. Each license can be active on two computers. A trial license can only be activated once on one computer. For issues with returns, please contact [support@phiab.se](mailto:support@phiab.se).



### Internet Access

During software activation or for service purposes it is convenient if the computer can access internet when needed.



Note that Windows Update may restart the computer, thus disrupting any ongoing experiments. We therefore recommend scheduling updates to a time when the computer is not used or simply disabling internet connection before an experiment so that no updates are downloaded and installed when the experiment is running.

## Computer Screen and Sleep mode settings



Set the computer to never turn off the screen and/or go to sleep, otherwise the computer may shut down and stop an experiment.

## HARDWARE INSTALLATION

The HoloMonitor should be delivered and assembled by trained personnel. They will ensure that the stage is correctly mounted and calibrated.

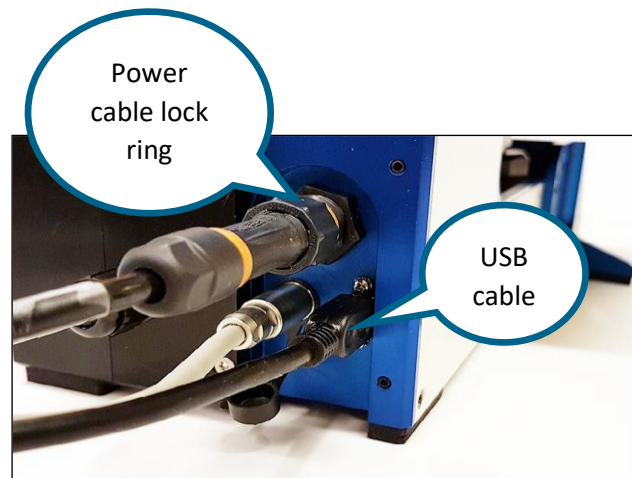
### Instrument Assembly inside incubator

1. Use a paper towel dampened with 70 % (v/v) ethanol to wipe down the cables and optical fiber that will be inside the incubator. If there is a cable port on the incubator, insert the cables through that port.

2. Connect the USB-cable to the computer.

3. Connect the power cable to the wall outlet.

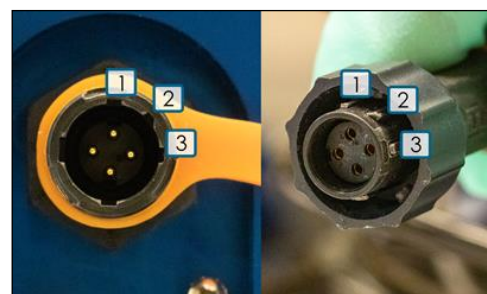
4. Clean the instrument exterior. The instrument can be wiped down with a paper towel dampened with 70 % ethanol. Cleaning must be performed so that no liquid enters the interior of the instrument.



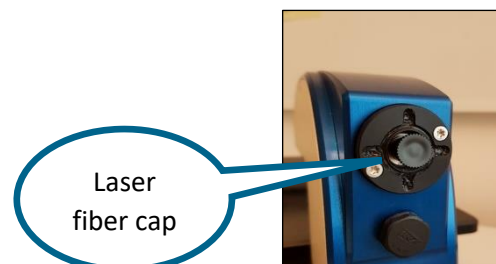
5. Place HoloMonitor M4 inside the cell culture incubator. Make sure that there is space for the motorized stage to move.

6. Wipe down the USB-cable with ethanol and connect it to the instrument.

7. Wipe down the power cable with ethanol and connect it to the instrument. Ensure that it is properly aligned before tightening the lock ring. The contact has ridges in a pattern that will fit slots in the connector. When properly aligned, the lock ring will be guided into place when tightened.

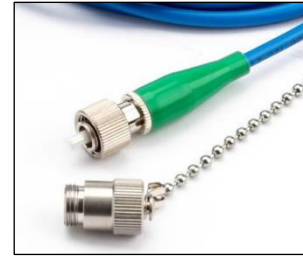


8. Remove the black cap covering the laser fiber connection point.

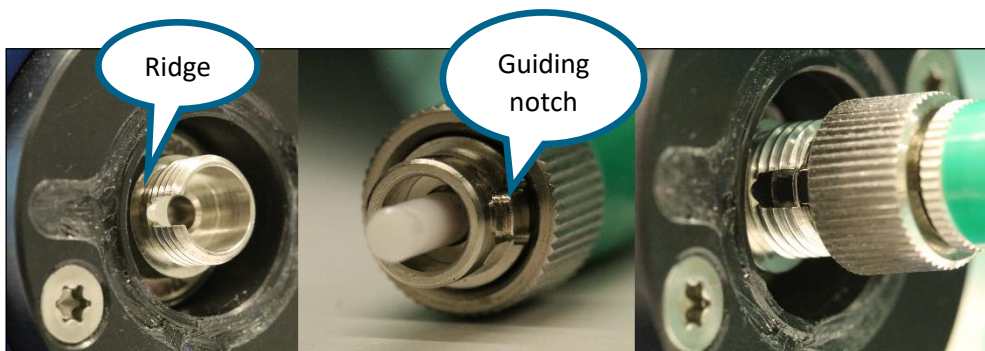




9. Carefully remove the protective cap from the Optical fiber tip. Save the protective cap for future use.



10. Gently insert the optical fiber tip into the fiber connector. Make sure that the metal ridge on the fiber tip is aligned with the guiding notch in the connector socket before tightening the lock ring (see image below).

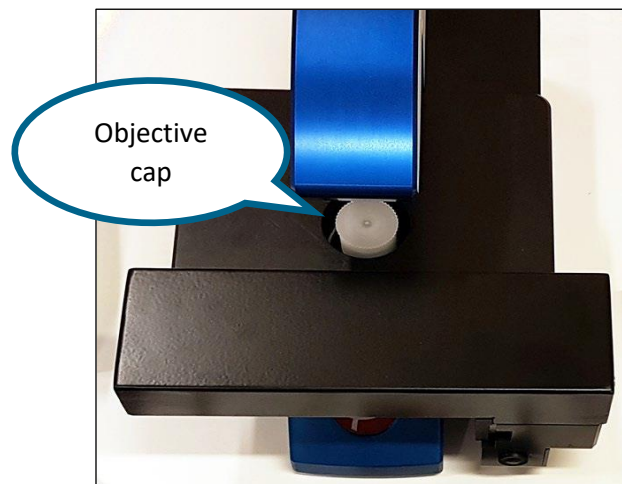


11. Connect the Laser unit power supply to the Laser unit and a wall outlet.

12. Close the incubator and wait at least 3 hours for the instrument to acclimatize to the incubator heat and humidity.

13. When the HoloMonitor is inside the incubator and acclimatized, the objective protective white cap can be removed.

14. Run the Auto-calibration procedure.





To avoid build-up of internal condensation, the instrument power must always be switched on while HoloMonitor is inside the incubator.



Do not lift HoloMonitor by holding the motorized stage. Only hold the blue and white body of the instrument when lifting.



The optical fiber must be handled gently. Do not forcefully stretch or bend the fiber. Do not touch the fiber tip.

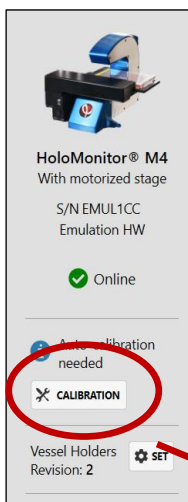


The laser needs approximately 30 seconds to warm up before the laser light is visible. An additional 5 minutes is required before it is fully stabilized.



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.

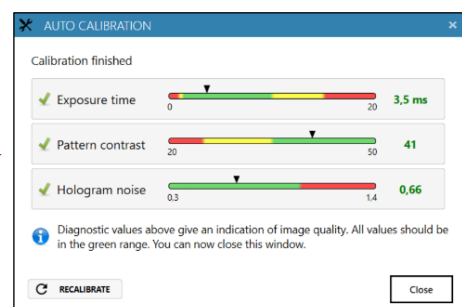
## Image Calibration



Re-calibrate the HoloMonitor before each use, especially when setting up an experiment. Auto-calibration gives a background image that is used by App Suite to lower background noise and increase image quality. The calibration procedure takes approximately one minute.

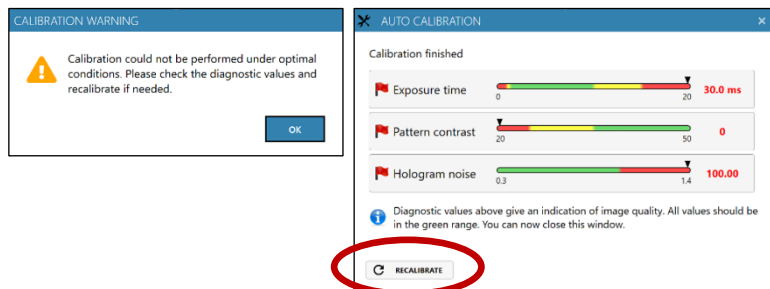
Start App Suite and wait for the HoloMonitor M4 to initialize. When HoloMonitor is ready, click the Calibration button below the instrument information.

Follow the instructions and ensure that there is no sample on the stage. Click continue, and the calibration is performed automatically.



When the calibration is finished, the calibration wizard will display three diagnostic values measuring the image quality. All values (indicated by black arrow heads) should be in the green range.

If App Suite fails to perform a calibration, a warning will appear. After verifying that HoloMonitor is properly installed and cleaned, click Recalibrate.



## Auto-calibration trouble shooting

*All calibrations values are in the red*

If you cannot get the Calibration wizard to pass a calibration step, and all values (Exposure time, Pattern contrast and Hologram noise) are in the red ranges, this might indicate that the laser is not able to produce enough light.

If all values are in the red, this could be caused by:

- Laser not properly attached to HoloMonitor M4
- Electricity cable is not connected to the laser hub
- Dirty optics
- Dirty laser tip or seating
- Laser requires normal periodic recalibration
- Dirt/condensation inside HoloMonitor

### Check the laser

- Is the red light shining on the objective?
- If not, is there power to the laser and the green light on the laser module is blinking?
- Does the image in the App Suite live capture tab look normal?
- Is the optical fiber cable damaged or crushed?
- Is the laser connection to the back of the HoloMonitor M4 positioned correctly and tightened?
- Inspect the Laser tip. Unscrew the Laser connection from the back of HoloMonitor and see if the tip is scratched or dirty.
- When the laser connection is detached from the HoloMonitor M4, check if the laser light shines in a strong and perfect red circle when shining on a white paper. If not, it is either dirty, or the tip could be damaged or cracked.

### If the laser tip needs cleaning

- Wipe the tip with a dry cotton swab in **one** direction.
- Take a new cotton tip and clean the hole where the laser tip is seated.
- Carefully reconnect the laser, paying attention that it is **keyed**. The guiding metal ridge on the laser connection must slip into the guiding notch in the connector socket at the back of the HoloMonitor. Connections must be finger tight.

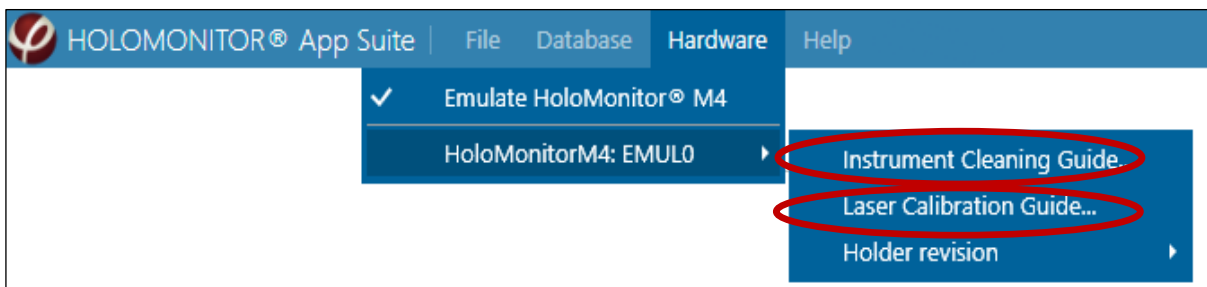
After checking the laser, recalibrate

After testing all above, re-run the Calibration wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range.

*Pattern contrast is in the red range or unstable*

An unstable Pattern contrast value in the green range or a value in the yellow/red range could indicate that the laser needs periodic recalibration. Go to the top menu, select Hardware and then the current HoloMonitor. Activate the Laser Calibration Guide and follow the instructions.

Afterwards, re-run the Auto-calibration wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range.



*Hologram Noise is in the red range*

A Hologram noise value in the red range could indicate that there is dirt on the optics or dirt inside HoloMonitor. Go to the top menu, select Hardware and then the current HoloMonitor M4. Activate the Instrument Cleaning Guide and follow the instructions. You will probably have to repeat the cleaning 2-4 times depending on how dirty the optics are. Afterwards, re-run the Image Calibration wizard. After successful auto-calibration all values (black arrow heads) should be in the green range.

*If nothing works*

If any of the Diagnostic values remain outside the green range after several cleaning attempts and/or laser calibration, contact the technical support at [support@phiab.se](mailto:support@phiab.se) for further assistance.

## HOLODRY™

The HoloDry™ canister is designed to keep the interior of HoloMonitor® M4 dry and free from internal condensation while operating inside a cell incubator. For optimal HoloMonitor M4 performance, and to qualify for warranty service when needed, HoloDry™ canisters need to be replaced at least once a month.

To mount a HoloDry canister, the HoloMonitor M4 microscope needs to be equipped with a protruding spout. Recent systems are prepared for HoloDry, but older microscopes need a simple upgrade, where the spout replaces the previous filter plug.

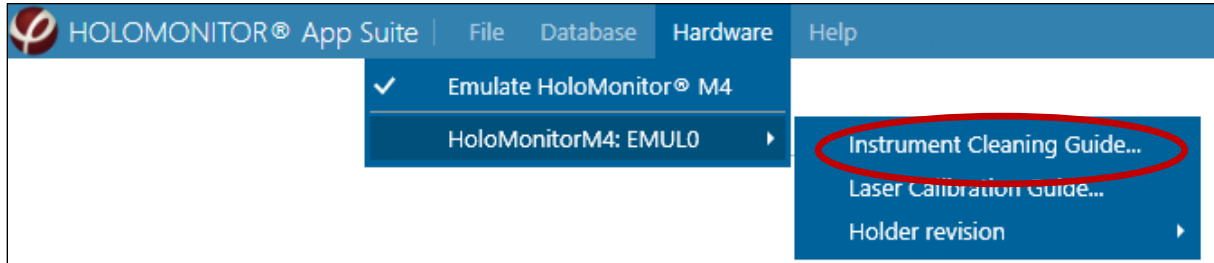
For further information, see the HoloDry protocol (<https://phiab.com/resources/manuals-protocols/>).



## CLEANING INSTRUCTIONS

### *Cleaning optical surfaces*

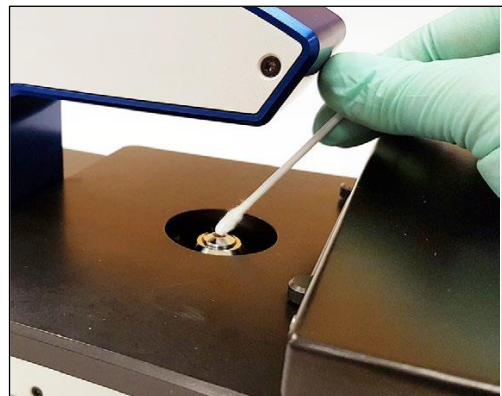
Go to the top menu, select Hardware and then the current HoloMonitor. Activate the Instrument Cleaning Guide and follow the instructions.



You might have to repeat the cleaning several times depending on how dirty the optics are. Afterwards, re-run the Auto-calibration wizard as described above. After successful auto-calibration all values after Auto-calibration (black arrow heads) should be in the green range.

In short, the cleaning is performed as follows:

Clean the top of the objective with a new cotton swab dipped in 70 % ethanol. Use a dry cotton swab to remove ethanol residues before it dries. The top of the objective is concave, and it is important to dig into the concave surface. Cleaning needs to be performed with some vigor.



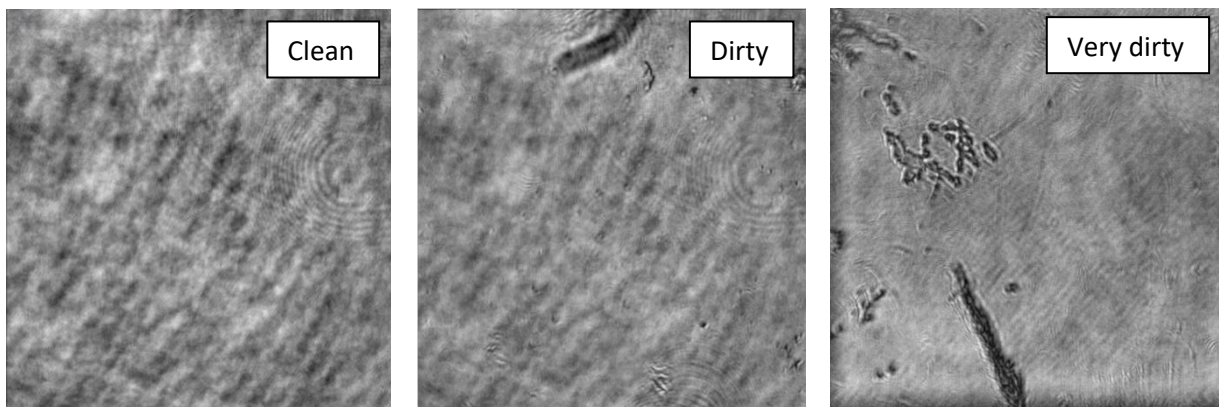
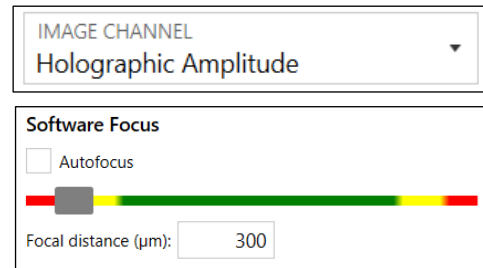
Clean the top window with a new cotton swab dipped in 70 % ethanol. Use a dry cotton swab to remove ethanol residues before it dries.



To validate that the optical surfaces are clean, use the Auto-calibration procedure. Repeat cleaning if the Diagnostic Values are not in the green ranges.

To assist the cleaning, look at the image in the Live View, and look if you see any small specks, flecks or dots that could be dirt. Confirm this by moving the stage to another position. If the specks are still in the field of view, then they are on the optics. Inspect after cleaning that they are gone, or that there are less of them.

Set the image channel in the Live View to Amplitude. Then uncheck autofocus and set the focus to 300. Any specks or other dirt now in focus are positioned on the objective tip.



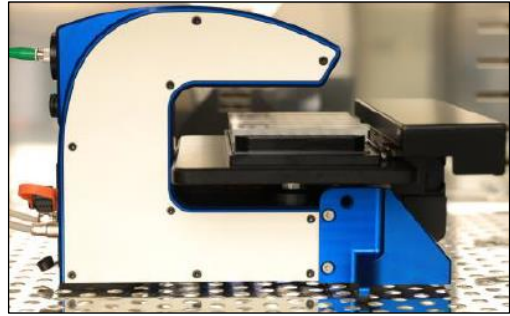
If any of the Diagnostic values remain outside the green range after several cleaning attempts, contact the technical support at [support@phiab.se](mailto:support@phiab.se) for further assistance.

*Cleaning instrument exterior*

All outside surfaces of the base unit and the motorized stage can be **wiped down with 70% ethanol**.



Avoid having any liquid entering the interior of the instrument.

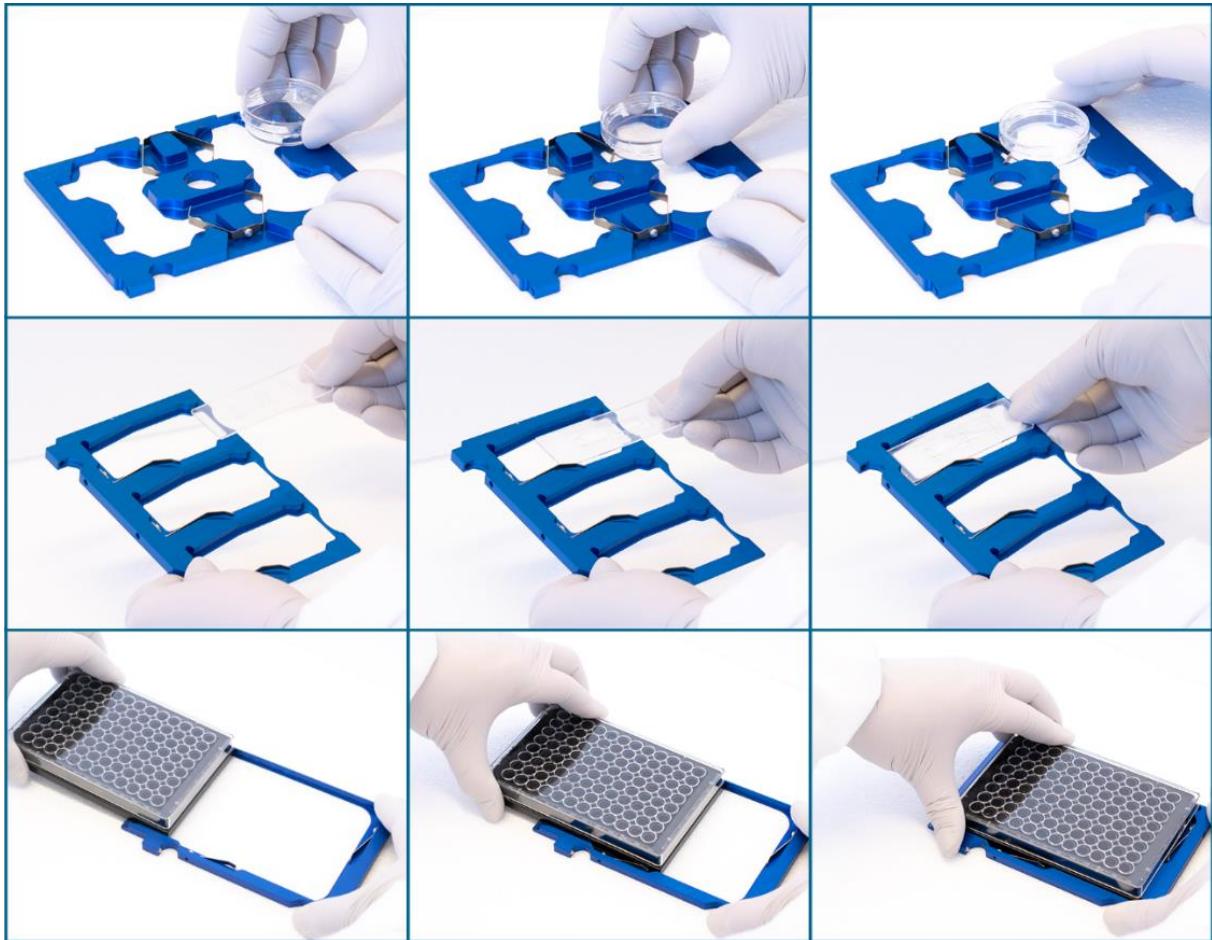


On a regular basis, wipe down the cables connecting the instrument using 70% ethanol to minimize any microbial build-up.



## USING THE SAMPLE HOLDER

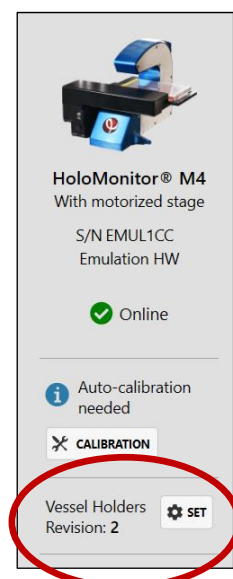
HoloMonitor M4 with motorized stage is delivered with a set of blue cell culture vessel holders called *revision 2*. There is a standard  $\varnothing$  35 mm Petri dish holder, a microscope slide holder, and a standard multi-well plate holder. In App Suite, there are vessel maps to match the holders.



There is a previous, black version of holders (*revision 1*) with a microscope slide holder, a standard multi-well plate holder and a petri dish holder. In order to



use the older holders, the Revision number must be reset in the software, otherwise the Vessel Maps will not be accurate.



## Loading the sample holder

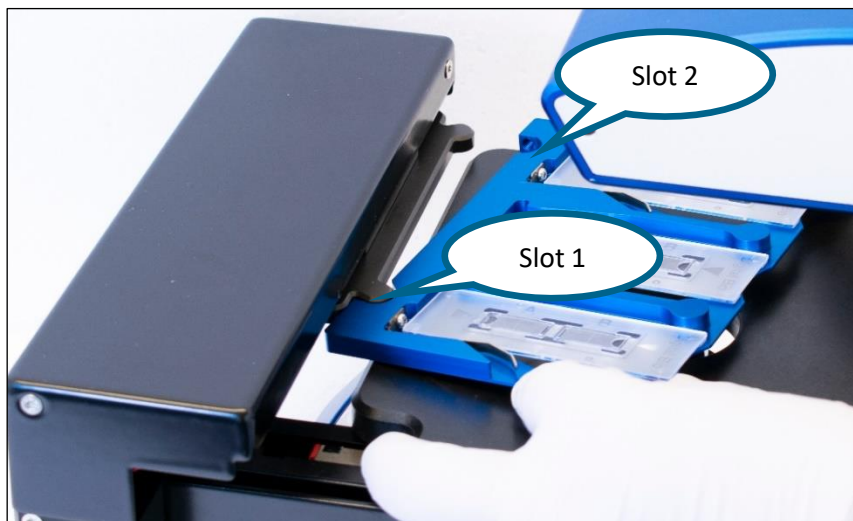
Before use, wipe the sample holder with 70% ethanol and put it in the LAF-bench, with the two slots facing towards the user.

1. Place the cell samples on to the holder. Place the sample as shown above and ensure that the vessels are correctly fitted on to the holder. For multiwell plates, slide them sideways along the tracks on to the Vessel holder with the bevel/s to the left. There is a spring that holds the vessel in place.



Note that multiwell plates are not symmetrical, and if the plates are placed the wrong way the Vessel Maps will not be accurate.

1. Thereafter, replace the standard lids with the appropriate HoloLid, following the HoloLid protocol (<https://phiab.com/resources/manuals-protocols/>). There are several types of HoloLids suitable for different cell culture vessels.
2. Rest the loaded sample holder on the HoloMonitor stage. Then click slot 1 in place, and thereafter slot 2.



## LONG-TERM STORAGE

If the HoloMonitor M4 is not going to be used for two weeks or more, remove it from the incubator and store it at room temperature with a dust cover. Protect the objective and the laser fiber connector with their respective covers.

The HoloMonitor should not repeatedly be taken out of and placed back into the incubator as temperature fluctuations can cause disturbances. Once the HoloMonitor is removed for long term storage, leave at room temperature at least two days.

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



**Turn off the laser unit before disconnecting the laser fiber from HoloMonitor.**



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

## SAMPLE REQUIREMENTS

### Cells

The HoloMonitor is intended for use with living, non-labeled, eukaryotic cells.

- Adherent cells growing as monolayers can be analyzed in all the vessels mentioned below.
- Suspension cells need to be mounted on a microscope slide or placed in a Countess® counting chamber, or another single use hemocytometer without grid, or a cell culture vessel similar to IBIDI slides. For the Cell Count App, we recommend Countess cell count chamber which can be ordered from [www.thermofisher.com](http://www.thermofisher.com).

### Cell culture vessels

For the best cell care and imaging quality, use

- ibidi® plastic ware (channel slides), chemotaxis slides, 35 mm culture dishes or 24-well plates with wound healing inserts

- Sarstedt 6-well plates with HoloLid™

- Sarstedt Lumox® 24 or 96 well plates with HoloLid™

Glass-bottom Petri dishes are highly recommended.

Vessel	Vendor cat. number	Growth area, cm <sup>2</sup> /well
Sarstedt TC-dish 35	83.3900	8.00
Sarstedt TC 6-well plate	83.3920.005	8.80
Sarstedt lumox® 24-multiwell plate	94.6000.014	1.90
Sarstedt lumox® 96-multiwell plate	94.6000.024	0.34
ibidi® µ-dish 35 mm, high	81156	3.50
ibidi® µ-plate 24 Well Black	80241	1.90
ibidi® chemotaxis slides	80326	-
ibidi® µ-slide	80106	-
Eppendorf CCCadvanced® FN1 - 6 well	0038110010	9.40

### Keep the cell culture vessels clean and unscratched

It is very important to keep the cell culture vessels perfectly clean and unscratched. Even wiping the cell culture vessel clean with ethanol will probably not help much as traces of dirt and tissue fiber will 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.



Note that all smudges and scratches will affect image quality.

## HOLOLIDS™

HoloLids™ have been specifically designed to eliminate image disturbances caused by surface vibrations and condensation inside the cell culture vessel. HoloLids™ are available in several different formats. They are made of poly-methyl-methacrylate (PMMA, acrylic glass), a non-toxic material often used in medical surgery implants, dentures, etc. For more information see <https://phiab.com/resources/manuals-protocols/>.

HoloLids have an optically neutral window which dips into the cell culture medium. This eliminates image disturbances caused by vibrating medium surfaces or by the transient condensation which often occur inside cell culture vessels. In order for the lids to fulfill their function, an appropriate amount of cell culture medium needs to be added to each well.

Vessel	HoloLid™ PHI cat. #	Final volume mL/well	Growth area cm <sup>2</sup> /well
Sarstedt TC-dish 35 (cat. # 83.3900)	71110	3.0	8.0
Sarstedt TC 6-well plate (cat. # 83.3920.005)	71120	3.0	8.8
Sarstedt lumox® 24-multiwell plate (cat. # 94.6000.014)	71130	1.9	1.9
Sarstedt lumox® 96-multiwell plate (cat. # 94.6000.024)	71140	0.17	0.2
ibidi® µ-dish 35 mm, high (cat. # 81156)	71111	2.5	3.5
ibidi® µ-plate 24 Well Black (cat. # 81156)	71131	2.5	1.9
Eppendorf 6-well plate (cat. # 0038110010)	71150	3.0	9.4



Optically neutral  
window in contact  
with cell culture  
medium

# HOLOMONITOR® APP SUITE SOFTWARE

## Introduction

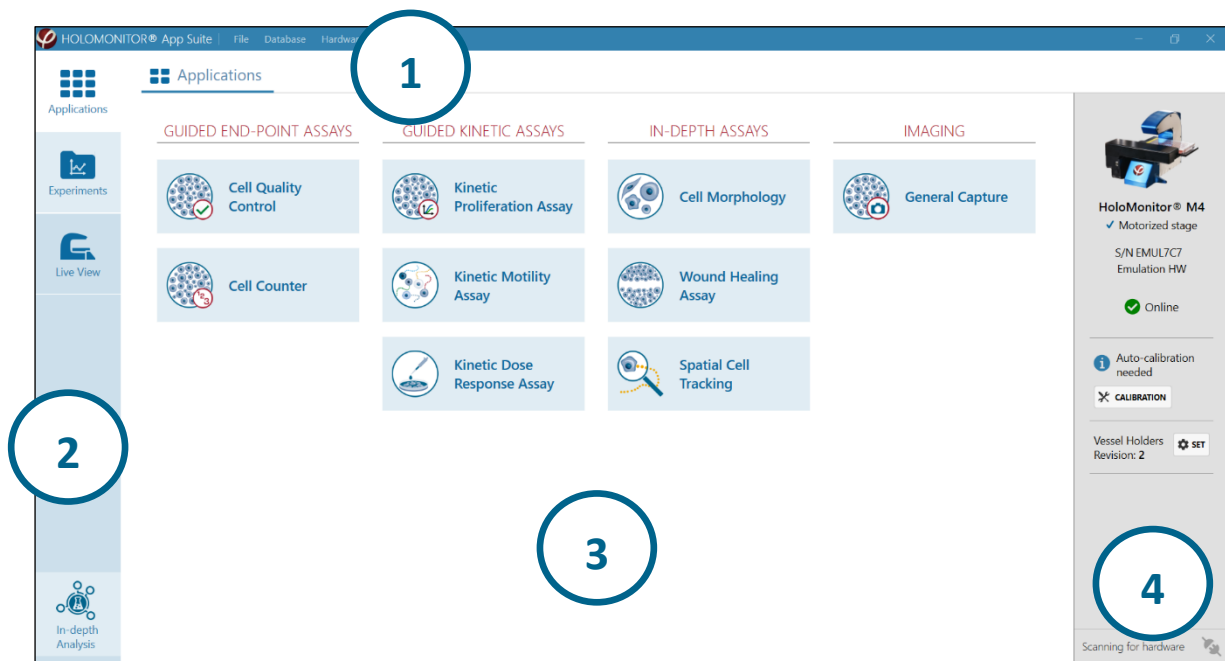
HoloMonitor App Suite™ software is specially designed for capture and analysis of holographic microscopy images using HoloMonitor M4. It enables the user to capture both single images and time-lapses. Based on images captured at regular intervals, time-lapses can be used to study slow cell processes. The user captures images of the sample using HoloMonitor M4, which are then analyzed in the App Suite software.

The imaging procedure is simple and 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 .xlsx or .xml-files for further work in Excel or similar software. 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.

HoloMonitor App Suite software comprises applications that perform assays such as Kinetic Proliferation, Kinetic Motility, Kinetic Dose Response, Cell Counting, Cell Quality Control, Cell Morphology, Wound Healing, and Single Cell Tracking using the HoloMonitor M4. Each assay has a specific software application as well as a protocol. This manual will describe the software functions common for all assays, while assay-specific details are found in each protocol.

In order to activate applications, specific licenses are needed. For more information, please visit [www.phiab.com](http://www.phiab.com), contact your distributor or [support@phiab.se](mailto:support@phiab.se).

## Overview



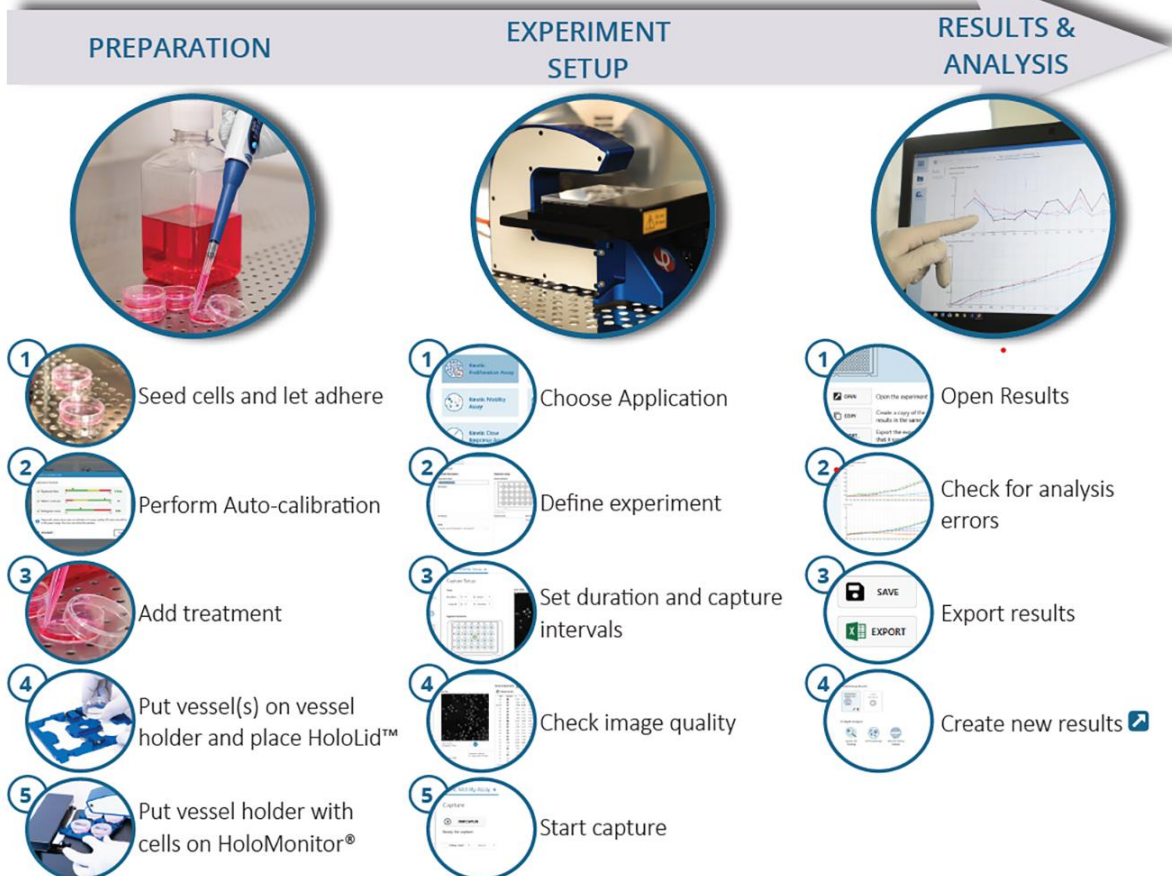
1. **In the top menu**, the Database Handler, the Instrument Cleaning Guide, the Laser Calibration Guide and the HoloMonitor M4 Emulator can be accessed. The Database Handler allows for copying, editing, transferring, and deleting data. The HoloMonitor Emulator is a virtual HoloMonitor M4. When activated it allows access to all live functions of the App Suite software.
  
2. **In the left-side panel**, the user can navigate between the main components of the software: Applications, Results, Live View and In-Depth Analysis. Applications are shown by default. Selecting one of the components will display the corresponding details in the center panel.
  - **Applications** contains the Application library. Clicking the Application name gives access to a short description, as well as a button to start the assay. Only applications unlocked by the license key are active. The procedure for each assay is described in the corresponding protocol. The protocols can be found at <https://phiab.com/resources/manuals-protocols/>.
  - **Experiments** contains a list of all accessible previous experiments with their analyses. The experiments can be re-analyzed at any time.
  - **Live View** allows the user to study cell samples without setting up an experiment.
  - **In-depth analysis** accesses software for detailed studies of individual cells and for the wound healing assay analysis.
  
3. **In the center panel** all applications of the software are displayed. Active (licensed) applications are highlighted in blue.
  
4. **The right-side panel** presents connected instruments, calibration status and vessel holder specification. Two instruments can be connected simultaneously.



## Workflow for guided applications

All applications have a similar workflow that guides the user through setup to image acquisition. The guided assays provide results immediately during the experiment while the results for the In-Depth applications can be extracted after analyses. The exact procedure for each application is described in a separate protocol. The protocols can be found at <https://phiab.com/resources/manuals-protocols/>.

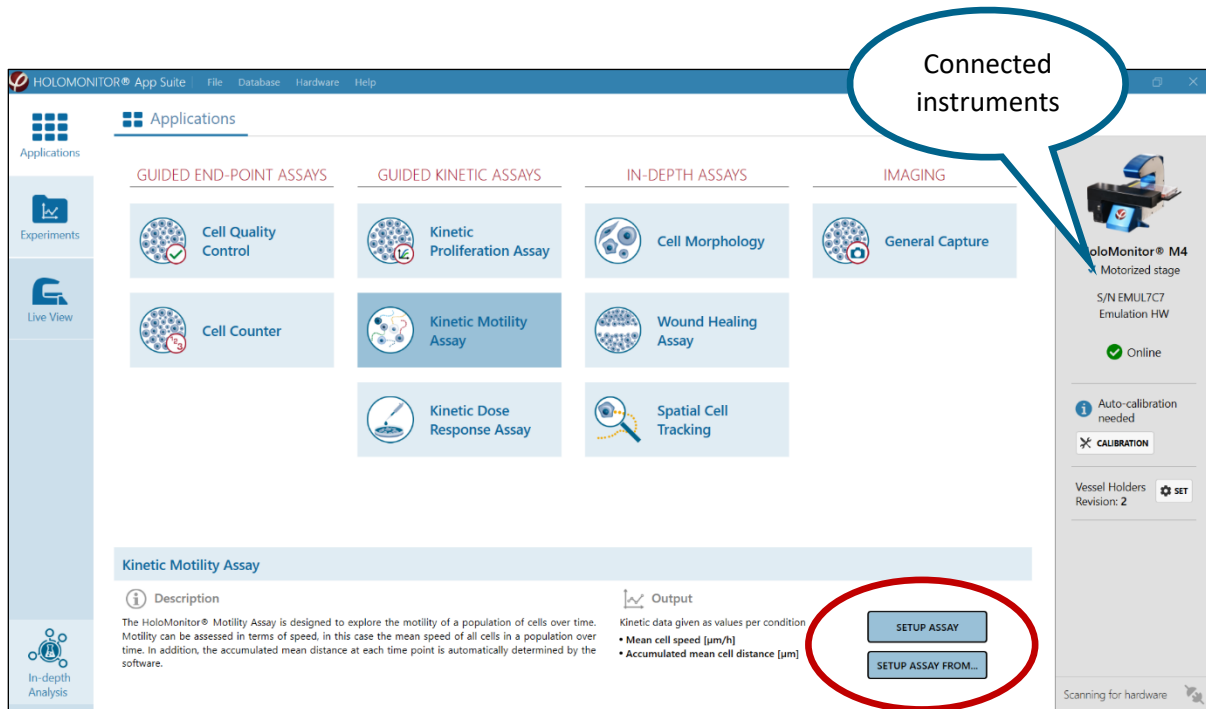
### WORK FLOW:





## Applications

The Applications library is open by default when the software starts up. It contains the tools for setting up and capturing experiments. Before starting an application, a HoloMonitor M4 must be connected.



## Assays in short

### Cell Quality Control

The Cell Quality Control assay can be used to ensure that the cell count and/or confluence for adherent cells is appropriate at the start of an experiment. It can also be used as a tool to detect undesired changes in the cell culture as compared to previous experiment(s). The output is cell count, cell confluence, mean cell area, mean cell volume and mean cell diameter.

### Cell counter

The Cell Counter assay is designed to automatically count suspension cells using a non-grid Countess counting chamber. Cell count is provided as number of cells per volume unit, i.e. the same principle as with hemocytometer cell counting. A volume converter tool will automatically provide the stock suspension volume needed for seeding the cells in upcoming experiments or the next passage. The output is cell count, mean cell area and mean cell volume.

### Kinetic Proliferation Assay

The Kinetic Proliferation assay is designed for a continuous automated and detailed proliferation analysis of adherent cells. At each timepoint individual cells are automatically identified and counted by the software. The output is cell count and cell confluence over time.

### Kinetic Motility Assay

The Kinetic Motility assay is designed to explore the motility of a population of adherent cells over time. At each timepoint, individual cells are automatically identified and their position is compared to the previous timepoint. The output is the mean cell speed of the population and the accumulated mean distance.

### [Kinetic Dose Response Assay](#)

The Kinetic Dose Response assay is designed for automated and detailed analysis of drug responses in adherent cells. The cells are automatically identified and counted by the software. Results are presented as an interactive dose-response curve where results for each condition can be displayed for any selected time point. The output is cell count, cell confluence and mean cell volume.

### [Cell Morphology Assay](#)

The Cell Morphology assay enables quantification of a wide range of features for individual adherent cells as well as cell populations. Scatterplots visualize correlations between controls and treated cells and make it possible to distinguish between cell populations. The output consists of more than 30 different cell features. The results are also accessible in excel spread sheets.

### [Wound Healing Assay](#)

The Wound Healing assay enables the study of cell migration and/or cell proliferation over time using adherent cells. The kinetic gap closure data as well as cell front velocity are provided semi-automatically. Output data include gap width, cell covered area and cell free area over time.

### [Single Cell Tracking](#)

Using the Single Cell Tracking application, adherent cells can be monitored and analyzed for cell motility, migration, and morphology of individually selected cells. The cells can be tracked over time even throughout mitosis. The results can be used to distinguish between random cell movement and cell migration, and to follow morphological changes over time. The output consists of movement diagrams, cell speed and direction of movement, cell family trees and diagrams showing cell features over time with more than 30 different cell features.

### [General Capture](#)

The General Capture module can be used for flexible setup of an experiment. The created image databases can be used for analysis by licensed applications. The output consists of time-lapse images and videos.

### [Output Variables](#)

A more detailed description of the output variables is found in the Analysis Features section in the back of this manual.

## Setting up an experiment

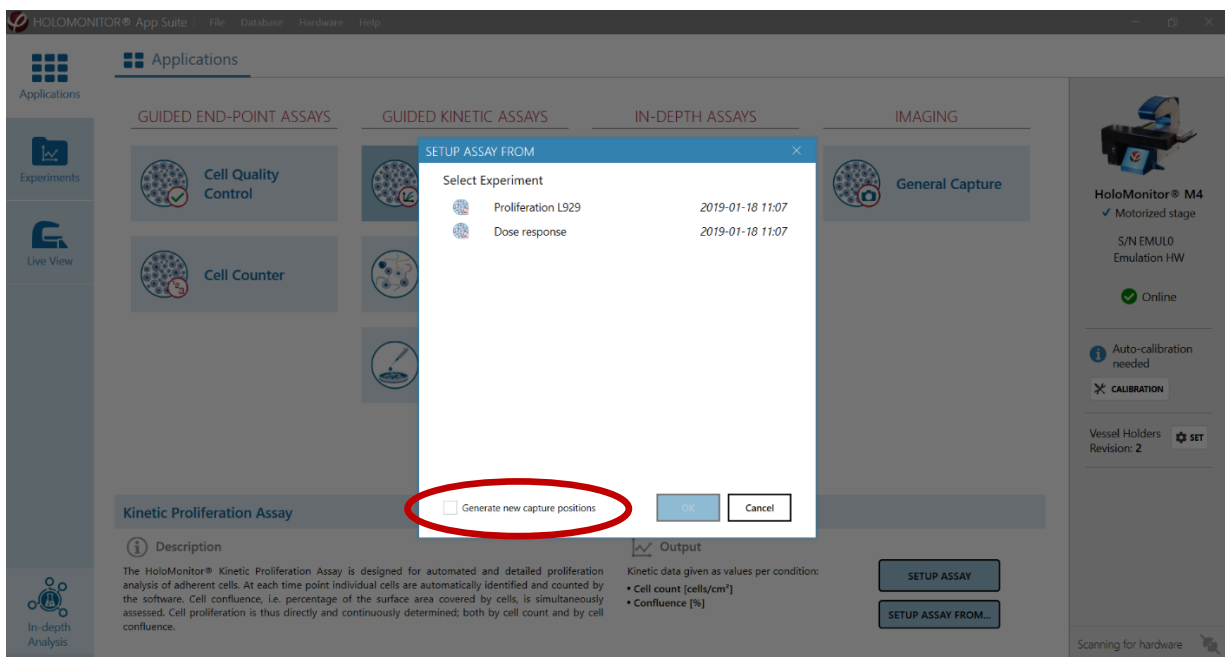
### Setup Assay

Selecting an application gives access to a short description of the application as well as the output parameters, and a button to Setup assay or Setup Assay From.

The exact procedure for each assay is described in the corresponding protocol. The protocols can be found at <https://phiab.com/resources/manuals-protocols/>.

### Setup Assay From

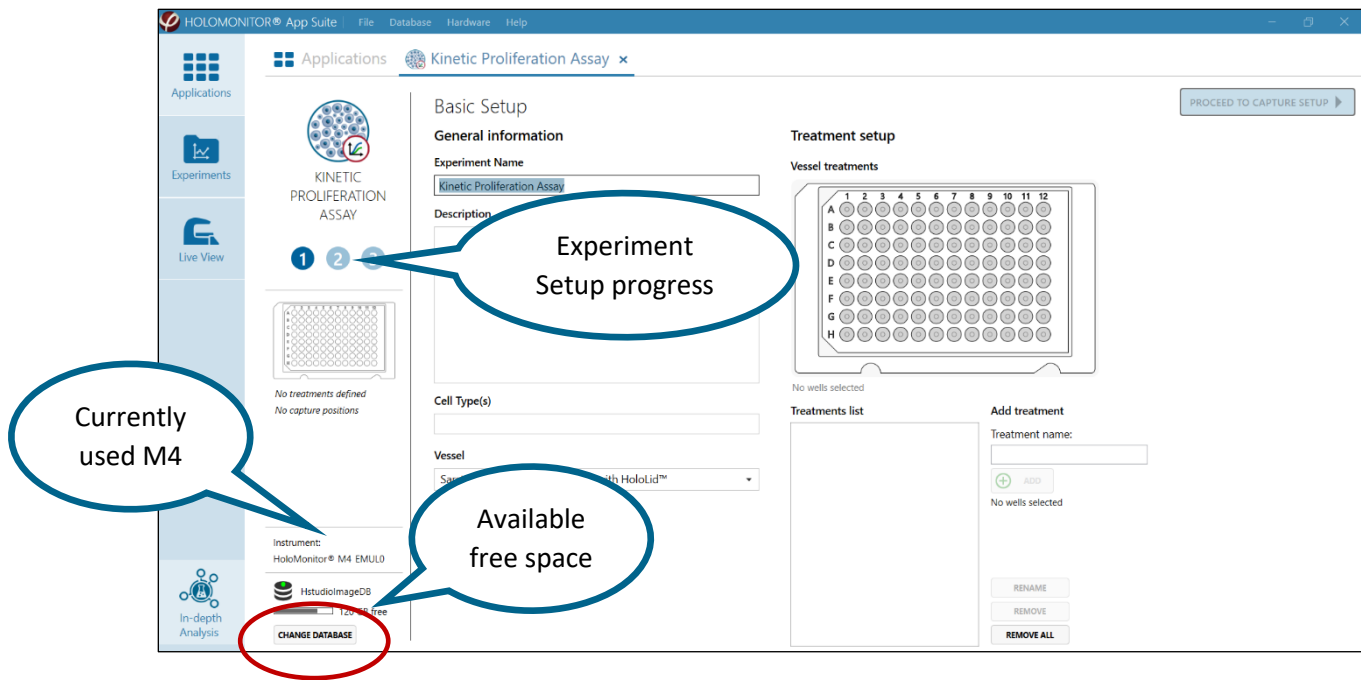
The Setup Assay From... button is used to set up a new experiment based on a previous experiment. It is possible to set up an assay from previous experiments of the same type (see image below). All properties from the selected experiment will be applied by default for the new experimental setup, including the capturing positions. To create new capturing positions for the new experiment, check Generate New Capture Positions.



### Experiment Progress

Once Setup Assay has been initiated, the progress of experiment setup and capture can be followed to the left on the Applications page, where setup steps 1, 2 and 3 are indicated by blue circles.

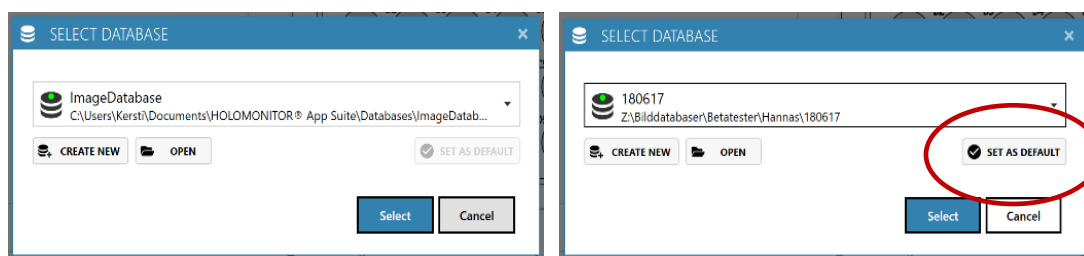
As the experiment setup proceeds, more details will be displayed below the setup progress indicators, such as cell culture vessels used, number of treatments added, the duration of the experiment and the storage space required.



### Switching Database

In the lower left corner, the available free storage space on the computer and the name of the currently used database are given, together with the possibility to switch to another database or create a new database (see image above). The data base used for an experiment can be changed while the experiment is set up, and the data can be moved afterwards, but not while the experiment is running.

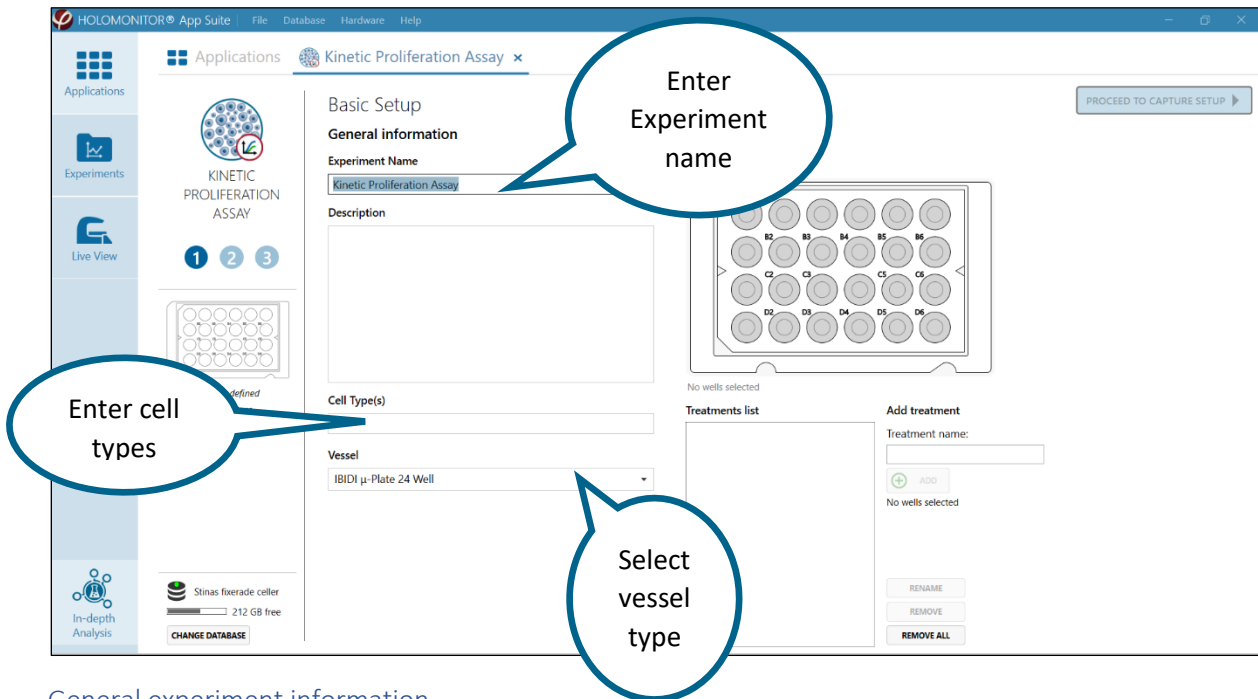
The currently used database is shown in the Select Database dialog box. Use the scroll list to select another database and then set it as default (see images below). If the database is not found in the scroll list, click the Open button, and browse to find another database to set as default. There is also the option to create a new database to set as default.



Note that when running an experiment, data needs to be stored on the computer running App Suite. Storing data onto an external hard drive, server or cloud storage during an experiment may cause all data to be lost due to erratic USB connections or weak internet connection.

## Basic Setup

Enter experiment parameters in this page, including selecting which vessel to use and which treatments that are added to each well. Other comments regarding the experiment can be entered here as well. The information will be saved with the experiment and are searchable.



### General experiment information

#### Experiment Name and Description

Enter an experiment name in the Experiment Name textbox and add details on the experiment in the Description and Cell Type(s) text boxes (both optional).

#### Vessel

Select the appropriate cell culture vessel map in the Vessel drop-down menu. The following vessels are supported with black and blue vessel holder configurations:

#### Black vessel holder: revision 1

- Countess Cell Counting Chamber Slide x 3
- Generic Microslide Std 1mm x 3
- IBIDI μ-Slide 1 x 3
- IBIDI μ-Plate 24 Well
- IBIDI μ-Plate 24 Well - with HoloLid™
- IBIDI μ-Plate 24 Well, Culture-Insert 2 well
- Sarstedt Lumox® Multiwell 24
- Sarstedt Lumox® Multiwell 24 - with HoloLid™
- Sarstedt Lumox® Multiwell 96
- Sarstedt Lumox® Multiwell 96 - with HoloLid™
- Sarstedt TC Plate 6 Well Standard.F
- Sarstedt TC Plate 6 Well Standard.F - with HoloLid™

#### Blue vessel holder: revision 2

- Eppendorf CCCadvanced® FN1 - 6 Well
- Eppendorf CCCadvanced® FN1 - 6 Well - with HoloLid™
- Generic Microslide Std 1mm x 3
- Generic Petri dish x 4
- Generic Petri dish - with HoloLid™ x 4
- IBIDI μ-Dish 35 mm high x 4
- IBIDI μ-Dish 35 mm high - with HoloLid™ x 4
- IBIDI μ-Dish 35mm high, Culture-Insert 2 Well x 4
- IBIDI μ-Slide 1 x 3
- IBIDI μ-Plate 24 Well**
- IBIDI μ-Plate 24 Well - with HoloLid™
- IBIDI μ-Plate 24 Well, Culture-Insert 2 well
- Sarstedt Lumox® Multiwell 24
- Sarstedt Lumox® Multiwell 24 - with HoloLid™
- Sarstedt Lumox® Multiwell 96
- Sarstedt Lumox® Multiwell 96 - with HoloLid™
- Sarstedt TC Plate 6 Well Standard.F
- Sarstedt TC Plate 6 Well Standard.F - with HoloLid™
- Sarstedt TC-Dish 35 mm x 4
- Sarstedt TC-Dish 35 mm - with HoloLid™ x 4

### Treatments or Conditions Setup

Here, treatments or conditions that have been added to which wells is defined. Wells that are selected together constitute a treatment or a condition, and their results will be pooled and presented together. The treatments and conditions can be redefined after the experiment (see Edit Treatments, Conditions and Doses in the Results section), thus allowing for a different presentation of results.

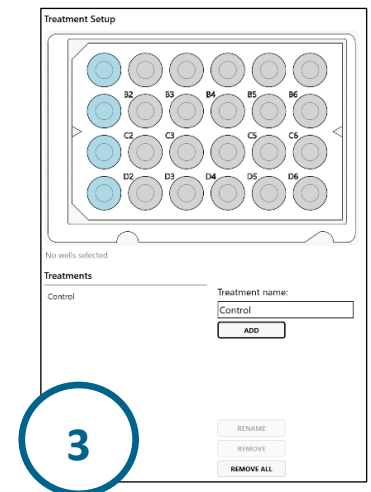
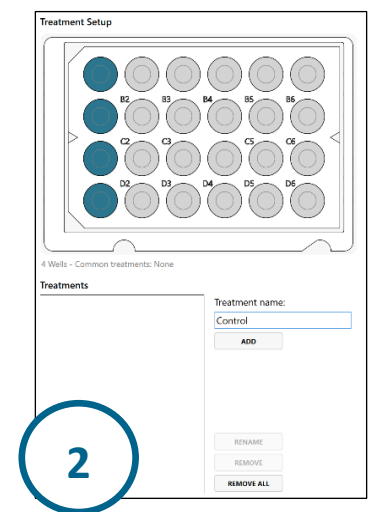
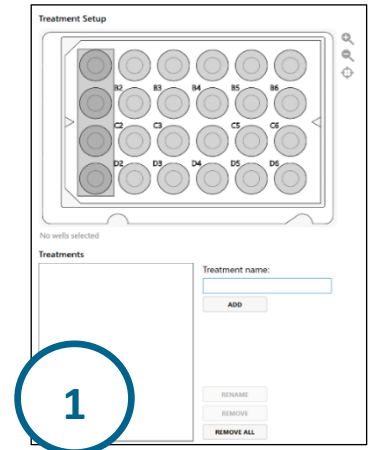
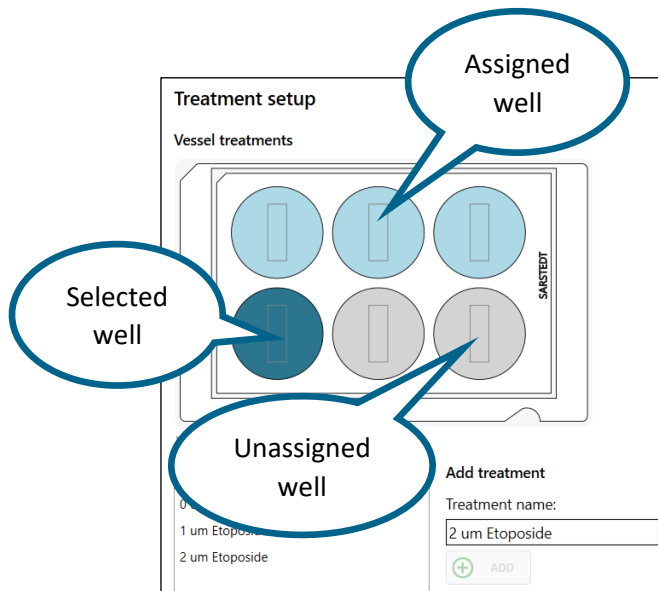
#### Add treatments

This page is slightly different for different applications.

1. To define the first treatment, start by selecting wells in the vessel map. Wells can be selected by left clicking or by marking them with a rectangle using the mouse cursor.
2. The selected wells will be dark blue.
3. Enter a treatment name in the text box and then click Add New Treatment to save the selection with the treatment name. The wells will then turn light blue.

To define another treatment, select the next set of wells, type the treatment name and click Add. Repeat until all wells that are included in the experiment have been labeled.

All wells may also be added as a single treatment.



#### Set dose-response conditions

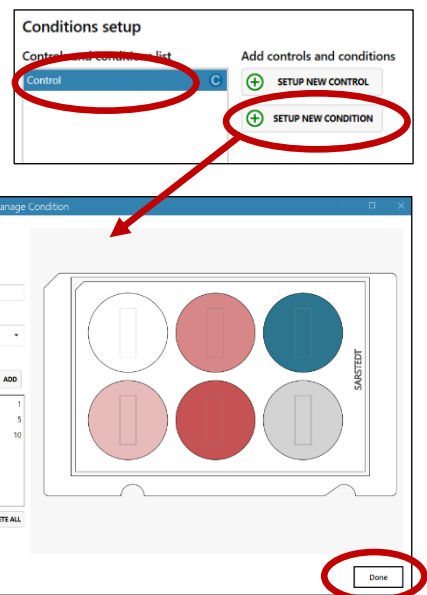
For dose-response experiments, a control must be set before other experiment conditions can be added. Clicking Setup New Control will open Manage Control (see image below). Select the well/wells that comprise a control, enter the control name and click done.

Note that one experiment can have more than one control, e.g. if there are two different cell lines. Use Setup New Control to add more controls. Note that the controls must have unique names, otherwise it will not be possible to save them.



Select one of the controls and then click Setup New Conditions in order to add the associated conditions. Note that the conditions must have unique names, otherwise it will not be possible to save them.

Click Done to add the changes and close the window.



#### Rename or remove treatments

To rename or remove treatments, select the items to be changed and use the buttons next to the treatments list.

#### Proceed to Capture

When the treatment or conditions setup is done, click the Proceed to Capture button in the upper right corner. This button will be inactive until treatment or conditions information has been added to at least one set of wells.

## Capture setup

In this window, the frequency of capture and duration of the experiment are set. The capture positions can be validated to ensure good capture quality. For each application there are suitable default settings.

Setup progress

Storage requirement

Capture duration and interval

Vessel map

Live View

Positions list

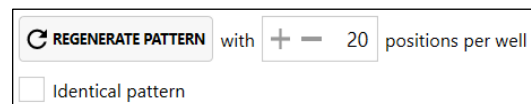
Image of current position

### Set capture duration and interval

Change the duration of the experiment and the interval between capture timepoints by clicking the “+” and “-”, or by entering numbers in the text boxes.

### Set capture positions automatically

Change the number of capture positions either by clicking the “+” and “-”, or by entering numbers in the text box below the vessel map. Then click Regenerate Pattern to generate a new random capture pattern which is unique for each well.



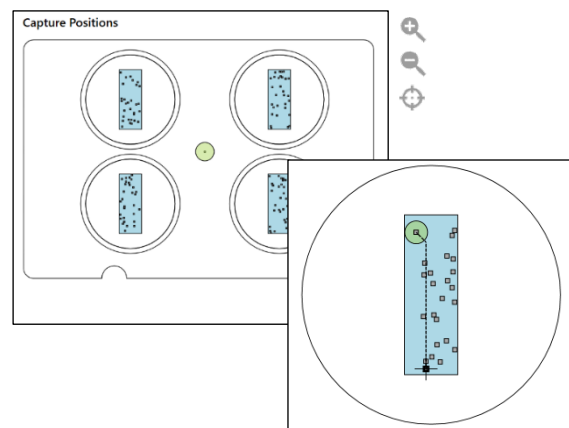
To generate an identical capture pattern in each well, check Identical Pattern before clicking Regenerate Pattern.

### Moving the stage to the capture positions

The capture positions are marked with little grey boxes in the vessel map in the Capture Positions window.

The green circle shows the current objective position. When clicking one of the positions, the motorized stage will move to that position.

The current field of view is shown in the Live View window.





## Pan and zoom the Vessel map

To better see the Vessel map, it can be moved by click-and-drag, and it can be zoomed in or out by click-and-scroll. Using the buttons to the right of the vessel map is another way to zoom in and out as well as to reset to original size (see buttons to the right). The vessel map can also be reset by double-clicking the left mouse button.



Positions List (96)

WELL	PREVIEW	X	Y	FOCUS
B1	N/A	0,629	-0,200	-1,350
B2	N/A	0,393	0,386	-1,350
B3	N/A	-0,517	0,262	-1,350
B4	N/A	-0,476	0,074	-1,350
B5	N/A	-0,611	-0,150	-1,350
B6	N/A	-0,364	-0,391	-1,350
B7	N/A	-0,683	0,039	-1,350
B8	N/A	0,613	0,116	-1,350
B9	N/A	-0,524	0,353	-1,350
B10	N/A	0,181	-0,606	-1,350
B11	N/A	0,547	0,320	-1,350
B12	N/A	0,423	-0,148	-1,350

DELETE SELECT ALL

VISIT POSITION Go to the selected position

APPLY CURRENT FOCUS Set the current focus to all selected positions

ADD CURRENT LOCATION Create a capture position at the current location

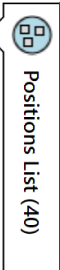
REGENERATE POSITION Move the selected position to a new random location in the same well

REPOSITION Replace the selected position to the current stage position

COPY TO ALL WELLS Copy the selected position to all wells

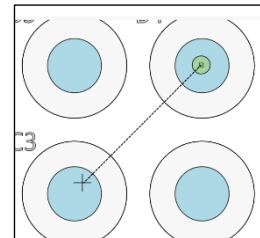
## Positions list

Each capture position that is marked in the Vessel map is also listed in the Positions list. Access the list by clicking the list button in the upper right corner of the page (see button to the right). The list shows the X and Y coordinates and the focus settings for each position. It is possible to set positions manually, and to edit existing positions.



## Set capture positions manually

To set capture positions manually, start by opening the Positions list. Then move the sample by clicking the desired position in a blue field in the vessel map. The stage will move the sample to the selected position (see image to the right, green marker corresponds to objective, "+" corresponds to new position).



The current field of view, *i.e.* the image showing the current sample position, is shown in the Live View window. If the image quality is good, click the Add Current Location button at the bottom of the Positions list to set the current position.

## Edit capture positions

To visit, reposition or delete a capture position, select the corresponding position in the Positions List and use the appropriate buttons.

*Delete* will delete all selected positions.

*Select All* will select all positions in the list.

*Visit Position* moves the motorized stage to the selected position, and the image will be displayed in the Live View window.

*Apply Current Focus* will apply the current focus setting to all selected image positions.

*Add Current Location* will add the current position to the positions list, and it will be included in the capture.

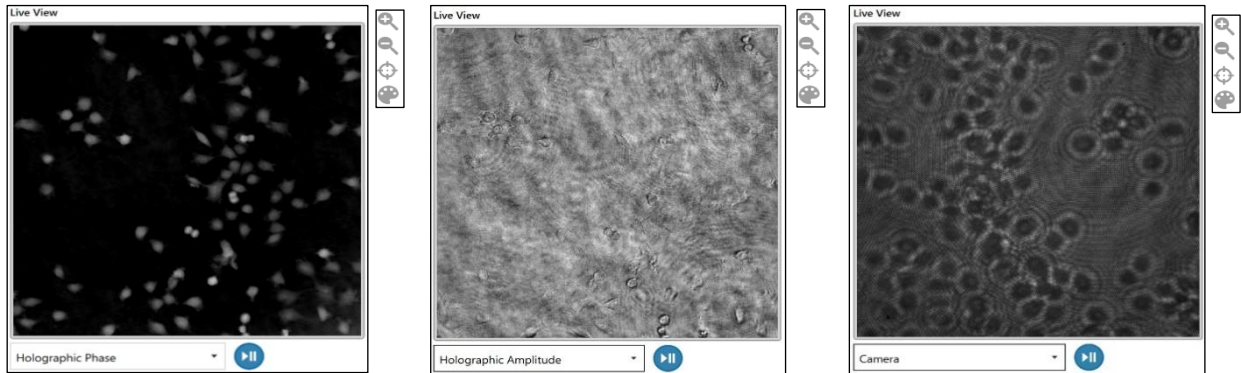
*Regenerate Position* will automatically replace the selected position with a random new position.

*Reposition* will replace the selected capture position with the current stage position.

*Copy to All Wells* will copy selected positions to all wells.

### The Live View Window

The Live View window shows the field of view of the current sample position. Three different versions of the cell image can be shown:




*Holographic Phase* shows the reconstructed holographic image based on the phase information. This is the commonly used image type in the M4 system.

*Holographic Amplitude* shows the reconstructed image based on the amplitude information.

*Camera* shows the interference pattern, i.e. the hologram, captured by the HoloMonitor camera. The hologram is the basis for reconstructing the holographic images.

### *Paus live imaging*

Use the blue button  to paus the showing of live images.


### *Pan and zoom*

To better see the image, move it with a mouse by click-and-drag, and zoom in or out by click-and-scroll. Use the buttons to the right of the Live View window as another way to zoom in and out as well as to reset to original image size (see image to the right). The image can be also reset by double-clicking left mouse button.



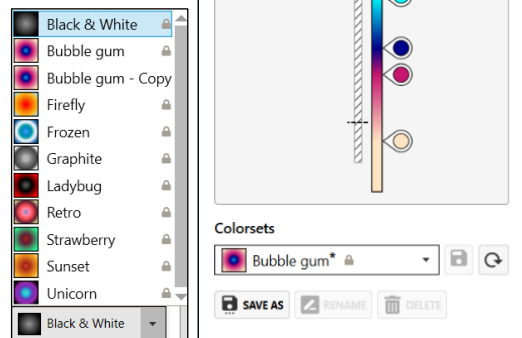
Note that these actions will only pan and zoom the image. The sample and the motorized stage will not be moved or zoomed.

### Image coloring

Click the color button  to the right of the Live View window to open the Coloring window. There, the color balance of the window can be adjusted and the colors corresponding to cell thickness can be added.

The default colorset for any image is Black & White. There are several pre-set colorsets to choose from and it is easy to create new, personal colorsets. Once a colorset has been selected, it will be applied to all captured images in the experiment.

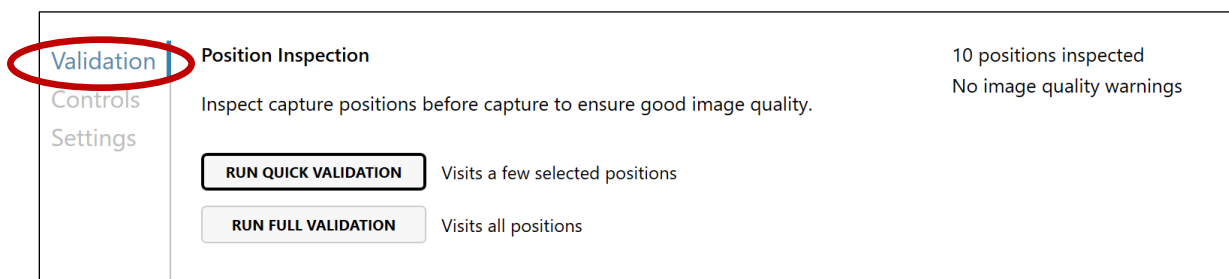
- Switch to a pre-existing colorset by using the Colorsets dropdown list.
- Create new color sets by right clicking in the Coloring window and then click Add Color.
- Click-and-drag to move the color pins to a suitable position in the color set.
- Save any changes.



### Validating Capture Positions

To ensure that the experiment turns out well, the capture positions should be validated by using the Position Inspection tool. The tool is found in the Validation tab. The validation is performed by automatically capturing and evaluating images of the positions selected for the experiment.

Note that this function is not available for End-Point Assays.

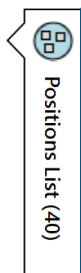


### Run Quick Validation or Run Full Validation

The Position Inspection tool has two options: Run Quick Validation or Run Full Validation. The Quick Validation investigates 10 positions, selected randomly among the capture positions, while Full Validation investigates all the Capture Positions.

After validation, the software will either indicate that no image quality warnings were found, or that there were warnings.

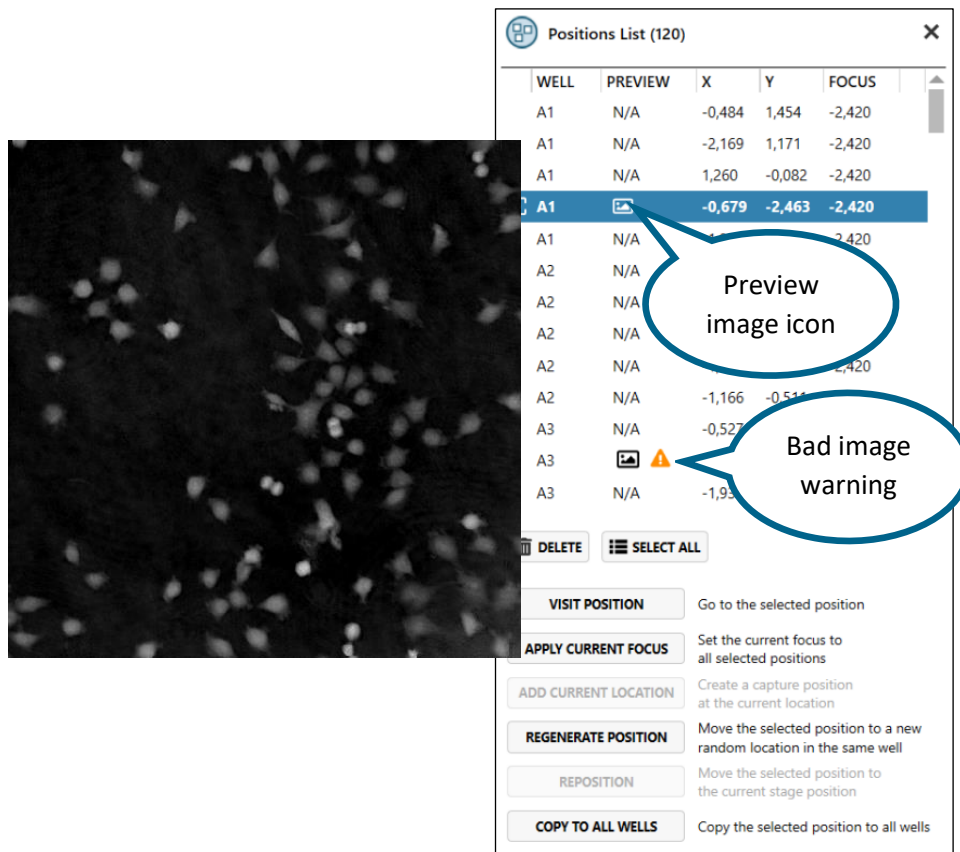
There are Previews of the validated positions in the Positions list. Display Positions list by clicking the list button in the upper right corner (see list button to the right).



### Viewing validated positions

When a position has been validated, a preview image has been captured. Hovering the cursor on top of a preview icon in the Positions list will display the preview image temporarily.

Display the live image in the Live View window by double clicking an image icon or highlighting an image position and then click the Visit Position button.



### Editing capture positions with warnings

If the software has determined that the image quality is insufficient, a warning sign appears beside the icon. Select the position in the Positions List to delete the position or reposition it using the buttons below the Positions List.

*Delete* will delete all selected positions.

*Select All* will select all positions in the list.

*Visit Position* moves the motorized stage to the selected position, and the image will be displayed in the Live View window.

*Apply Current Focus* will apply the current focus setting to all selected image positions.

*Add Current Location* will add the current position to the positions list, and it will be included in the capture.

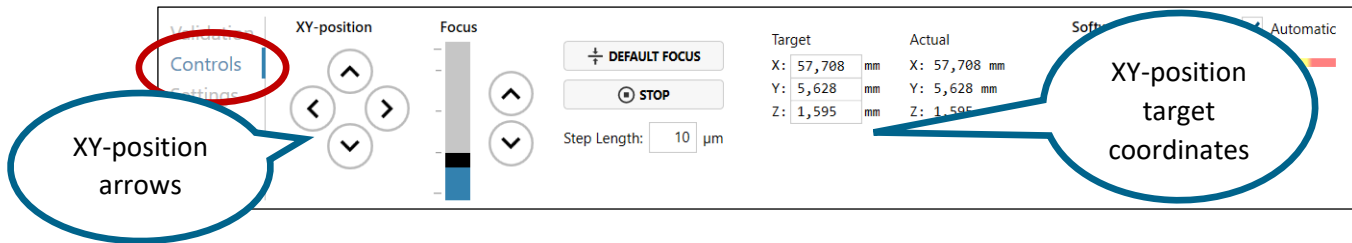
*Regenerate position* will replace the selected position with a random new position in the same well.

*Reposition* will replace the selected position with the current stage position.

*Copy to All Wells* will copy selected positions to all wells.

### Motorized stage controls

The motorized stage will move automatically, following the capture pattern that was set during Capture setup. Sometimes manual positioning or focusing is necessary to achieve a higher precision. Click the Control tab to find the motorized stage controls. Stage movements in XY directions can be controlled, and both hardware and software focus can be adjusted. The current field of view will be displayed in the Live View window.

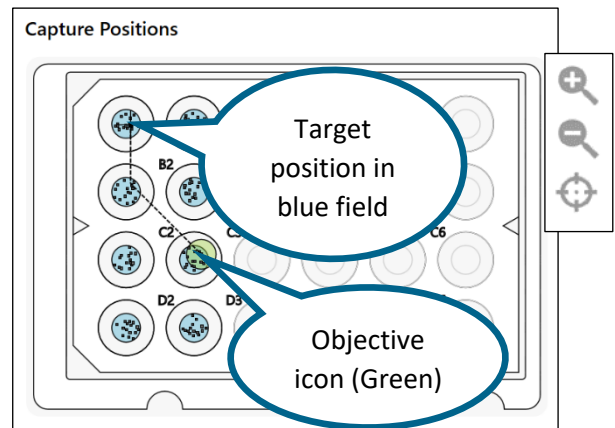


### Stage movement

The stage can be moved in three different ways:

- Click any desired position in a blue field in the Capture positions window.
- Enter the target coordinates in the Target coordinates text boxes.
- For smaller stage movements, use the XY-position arrows in the Control window for the XY position.

Adjust the step length for the stage movement by entering a suitable step length in the step length text box.



**Note that the step length applies to all directions of stage movement: X, Y and Z. Too big steps can crash the objective into the sample.**

Move the stage to one of the existing capture positions by clicking the position marker in the vessel map. To better see the Vessel map, the window can be moved by click-and-drag, and it can be zoomed in or out by click-and-scroll. The green marker indicates the current position of the objective,

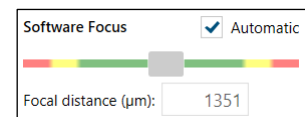
and the “+” symbolizes the new position. The window can also be zoomed in and out as well as reset to original size with the buttons found to the right of the Vessel map or double-clicking left mouse button.

## Focusing

### Focusing in theory

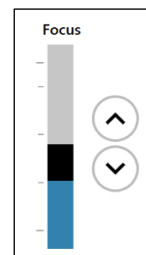
The software has pre-set focus settings for each vessel type. The settings are automatically selected when the vessel map is selected. The focus settings can also be activated with the Default Focus button.

The pre-set focus settings were calibrated when the HoloMonitor was installed, but sometimes focus needs to be adjusted by the user, e.g. if the cell culture vessel has a coating that makes autofocusing difficult. HoloMonitor M4 has both a coarse mechanical and a fine software focus. They need to cooperate for the image to be in focus. Use the mechanical focus, *i.e.* move the motorized stage, to move the cells to the approximately correct distance from the objective. The software focus then finds and calculates the cell images. The Software Focus indicator shows how well the software can focus an image at the current mechanical focus setting. The software can only find focus when the cell culture vessel is at the approximately correct distance from the objective.



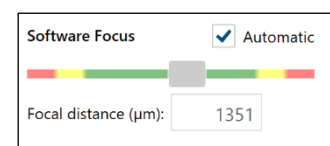
### Focusing in practice

The black box in the Focus bar represents the motorized stage. Move the black box up or down to move the motorized stage up or down. Click the two arrows for smaller stage movements up or down.



**Note that the step length applies to all directions of stage movement: X, Y and Z. Too big steps can crash the objective into the sample.**

By default, Automatic focus is activated. For good focus, the grey marker in the Software Focus indicator should be positioned within the green area, preferably in the middle of the green area. If the grey marker is too far to either the left or the right, move the motorized stage as described above in order to shift the marker to the middle.



Clicking the Default Focus button will replace the current focus with the pre-set focus settings.



Clicking the Stop button will immediately stop stage movement.

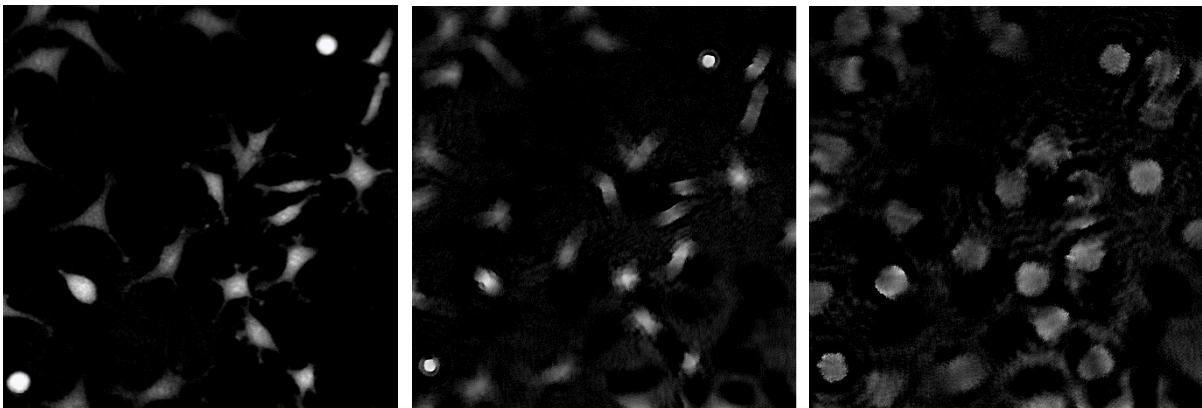
### Manual software focusing and when I went

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.

- Uncheck Automatic in the Software focus panel.
- First adjust the software focus settings by moving the grey marker in the Software Focus indicator until it is in the middle of the green area.
- Thereafter adjust the mechanical focus using the Focus bar. 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.

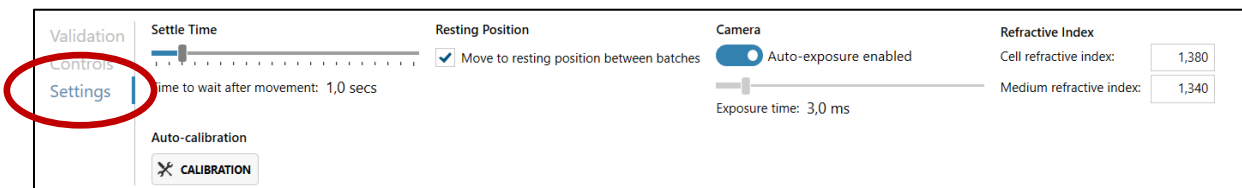
### Images in and out of focus

The images below show a holographic phase image that is in focus (left), slightly out of focus (center) and totally out of focus (right).



### Settings

In the settings tab, the stage movement settling time and resting position can be handled, as well as the exposure time of the camera. The Calibration function can be accessed here. Note that Calibration must be performed before the start of each experiment.



*Settle Time* is the pause between the last movement of the stage and the next capture. It allows cell culture medium and particle movement caused by the moving stage to slow down before the image is captured. If the Settle Time is too short, there may be image disturbances.

The *Resting Position* is a neutral position with no cells, where the laser light will be positioned during capture pauses. This decreases unnecessary light exposure. This function is activated by default.

*Camera exposure time* is set to auto exposure by default. It is possible to set the exposure time manually, as may be needed with extremely difficult samples.



*Refractive Index* is used by App Suite to calculate cell thickness. App Suite uses the difference in refractive index between the cells and the medium. The default refractive index values can be adjusted if the cells or medium have a different refractive index. There is a more detailed description of the calculations in the Analysis Features section in the back of the manual.

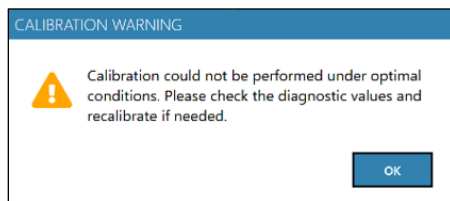
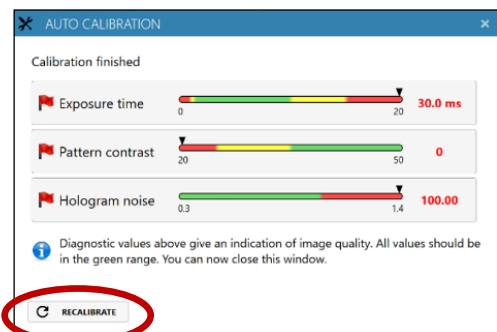
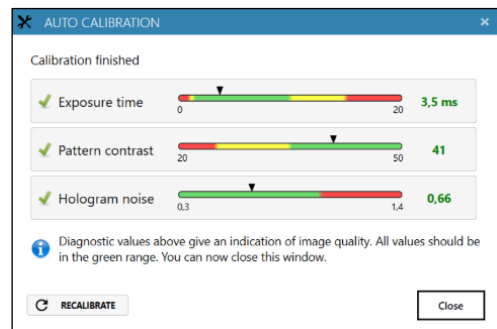
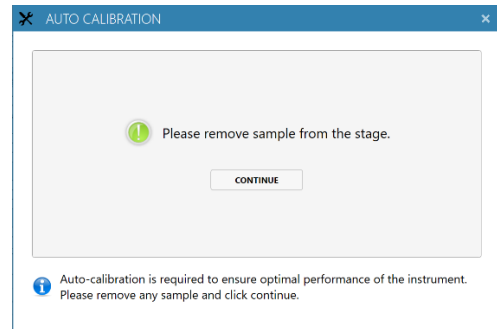
### Image calibration

HoloMonitor M4 must be re-calibrated before each use, especially when setting up an experiment. Auto-calibration gives a background image that is used by App Suite to lower background noise and increase image quality. The calibration procedure takes approximately one minute.

Start the calibration procedure by clicking Calibration and follow the instructions. The first instruction is to remove any sample from stage.

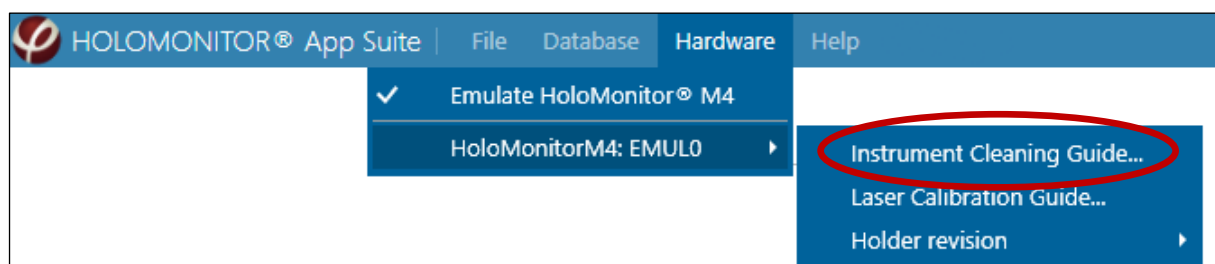
When the calibration is finished, the calibration wizard will display three diagnostic values measuring the image quality. All values (indicated by black arrow heads) should be in the green range.

If App Suite fails to perform an approved calibration, a warning will appear. In that case, please verify that HoloMonitor M4 is properly installed and then clean M4 thoroughly by following the Cleaning instructions (see below). After cleaning, click Recalibrate to refresh the Diagnostics values.



### Cleaning instructions

Go to the top menu, select Hardware and then the current HoloMonitor M4. Activate the Instrument Cleaning Guide and follow the instructions. You will probably have to repeat the cleaning 2-4 times





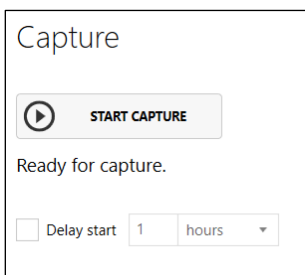
depending on how dirty the optics are. Afterwards, re-run the Calibration wizard. After successful auto-calibration, all values (black arrow heads) should be in the green range.



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

### Capture

In this page, image capture is started and monitored.



#### Start capture

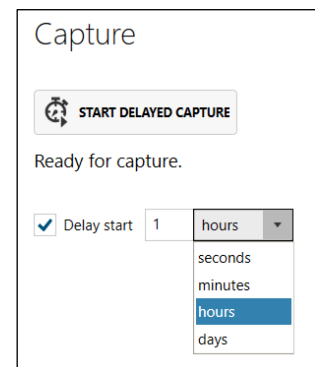
To start the experiment capture, click the Start Capture button (see image to the left).

#### Delaying Start Capture

The start of the experiment can be delayed by a timer (see image to the right).

During capture, progress information is

displayed (see image below).



### During capture

The Live View window will show the last captured image.

The progress information includes

- Number of timepoints completed out of total
- Number of images captured out of total
- Elapsed time
- Estimated time left
- Time to next capture

Results are calculated during the experiment and can be viewed while the experiment is running (See View Results, below).

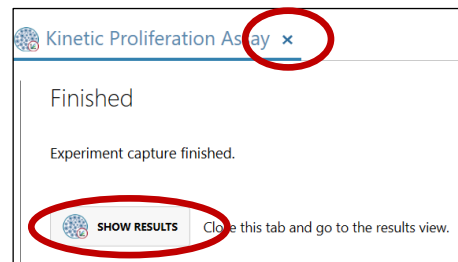


### Stop capture

The capture can be interrupted by clicking the Stop Capture button in the upper left corner. The experiment will then be interrupted and closed. A message appears stating that the capture is finished.

The results from guided assays can be accessed immediately with the Show Results button, or the experiment can be closed using the x-button beside the experiment name.

In-Depth assay results can only be accessed through the Experiments tab.



## Results for Guided Assays

The analysis of a guided experiment is automatic and starts when image capture begins. Hence, the results are ready for viewing even during capture. Experiment results are mainly presented as graphs and/or tables, the images can also be accessed. For further analysis, all results can be exported to Excel.

Note that even though the results are auto-generated, they need to be verified by the user.

### Results list

Click the Experiments tab to open a list of all accessible experiments with their results. A spinning red cog indicates that the experiment is ongoing and only preliminary results can be viewed.

A summary of the experiment is shown once an experiment is selected. The Experiment Summary shows data such as vessel type, type of experiment, data, duration, intervals, cell types, etc. The buttons below the Experiment Summary can be used to open, copy, export or delete the experiments.

The screenshot shows the HOLOMONITOR interface. On the left, the 'Experiments' tab is selected. The main area displays a table of experiments with columns for 'experiment title', 'Database', and 'Time started'. One experiment, 'KA1 Motility L929 etoposide', is highlighted. A callout bubble points to a spinning red cog icon next to the 'New Experiment' row, indicating it is in progress. Another callout bubble points to the 'Experiments' tab in the left sidebar. A third callout bubble points to a menu of actions (OPEN, COPY, EXPORT, DELETE) located below the experiment summary on the right. The experiment summary for 'KA1 Motility L929 etoposide' shows it is 'Fully completed' with a duration of 48 hours and 7 minutes.

experiment title	Database	Time started
Kinetic Proliferation Assay	Test db	2019-11-04 15:23
test	Test db	2019-10-15 18:58
New Experiment	Test db	2019-10-15 16:35
Motility L929 etoposide	KA1 L929 Motility AppSuite etopo	2019-03-29 14:46
arrow differentiation	Motility demo 4h bone marrow macrophages	2019-03-05 14:27
QC example 2	DB for AppSuite demo	2019-01-18 11:22
QC example 3	DB for AppSuite demo	2019-01-18 11:22
Proliferation L929	DB for AppSuite demo	2019-01-18 11:07
Dose response	DB for AppSuite demo	2019-01-18 11:07

**EXPERIMENT SUMMARY**  
KA1 Motility L929 etoposide  
Kinetic Motility Assay

Status: **Fully completed**  
Time started: **2019-03-29 14:46**  
Duration: **48 hrs**  
Capture interval: **7 mins**  
No. of timepoints: **405**  
No. of images: **7290**

Description: L929 cells treated with 0, 50 or 100 mikromolar etoposide  
Cell type: L929  
Vessel map: Sarstedt TC 96

**OPEN** Open the experiment along with its results in the same database  
**COPY** Copy the experiment along with its results so that it can be analyzed on another computer  
**EXPORT...** Export the experiment along with its results so that it can be analyzed on another computer  
**DELETE** Delete the experiment including all images and results

### View Experiment results

To view Experiment results, click the Open button. Several Experiments can be open in parallel.

### Copy an experiment

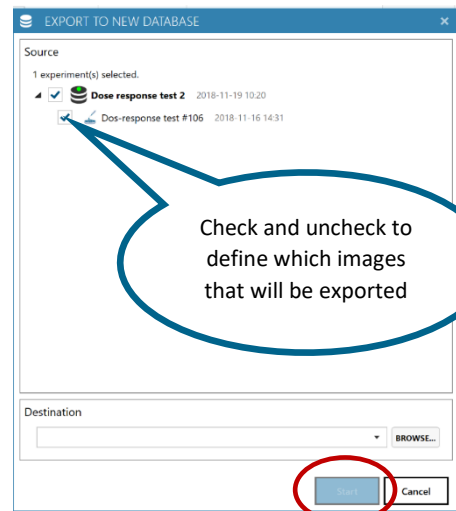
Click Copy (see image above) to make a copy of an experiment, including all settings, definitions and analyses. This is useful e.g., if the experiments need to be analyzed for two different treatment definitions.

### Export an experiment

Click Export (see image above) in order to make a backup or to create a copy of the experiment that can be analyzed on a different computer.

When the Export to New Database window opens, browse to the destination. Then create and thereafter select a new folder that will contain the exported experiment.

Click Start to export all database data connected with the experiment, including all image frames, settings, definitions and analyses.

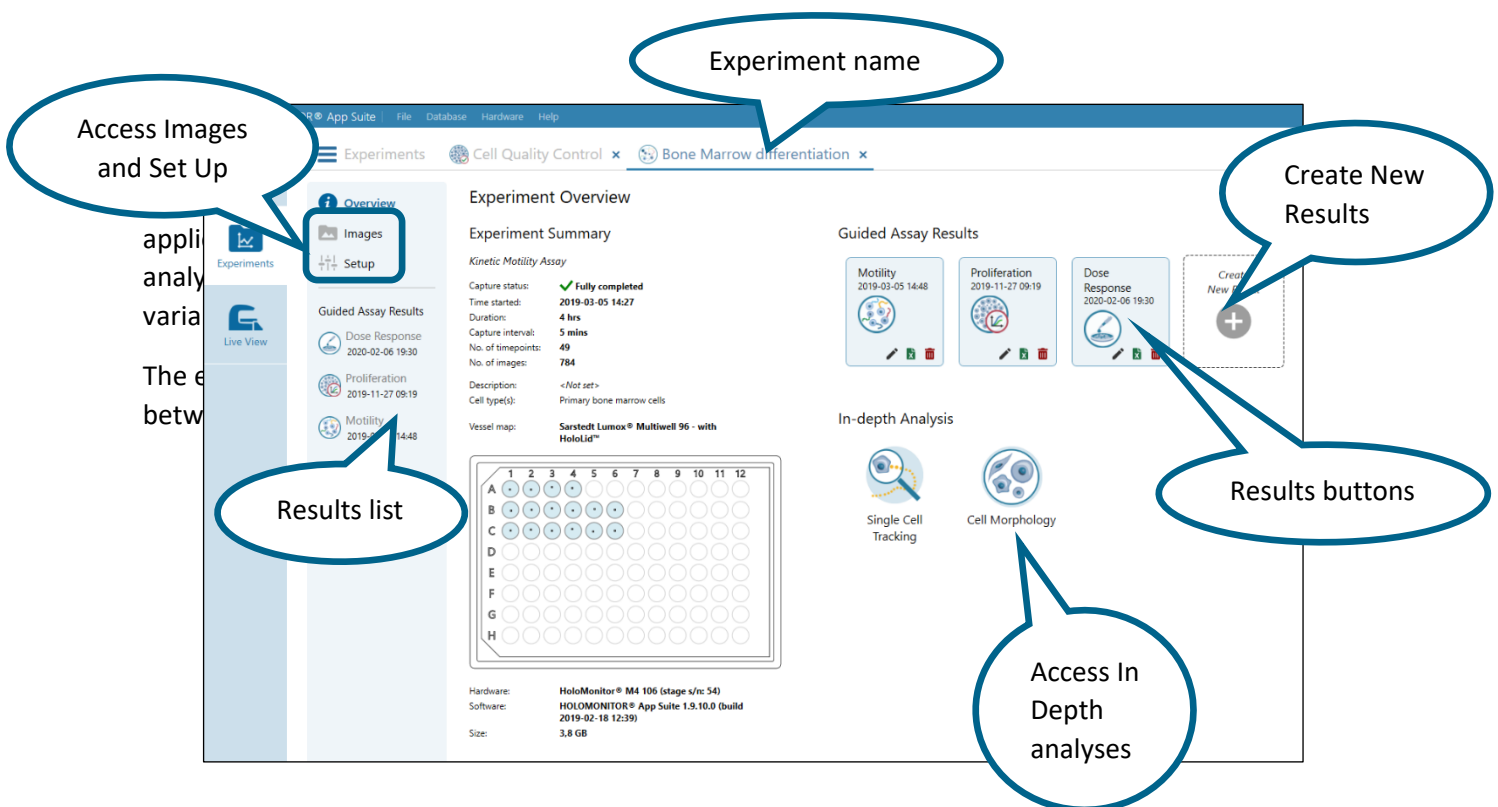


### Delete an Experiment

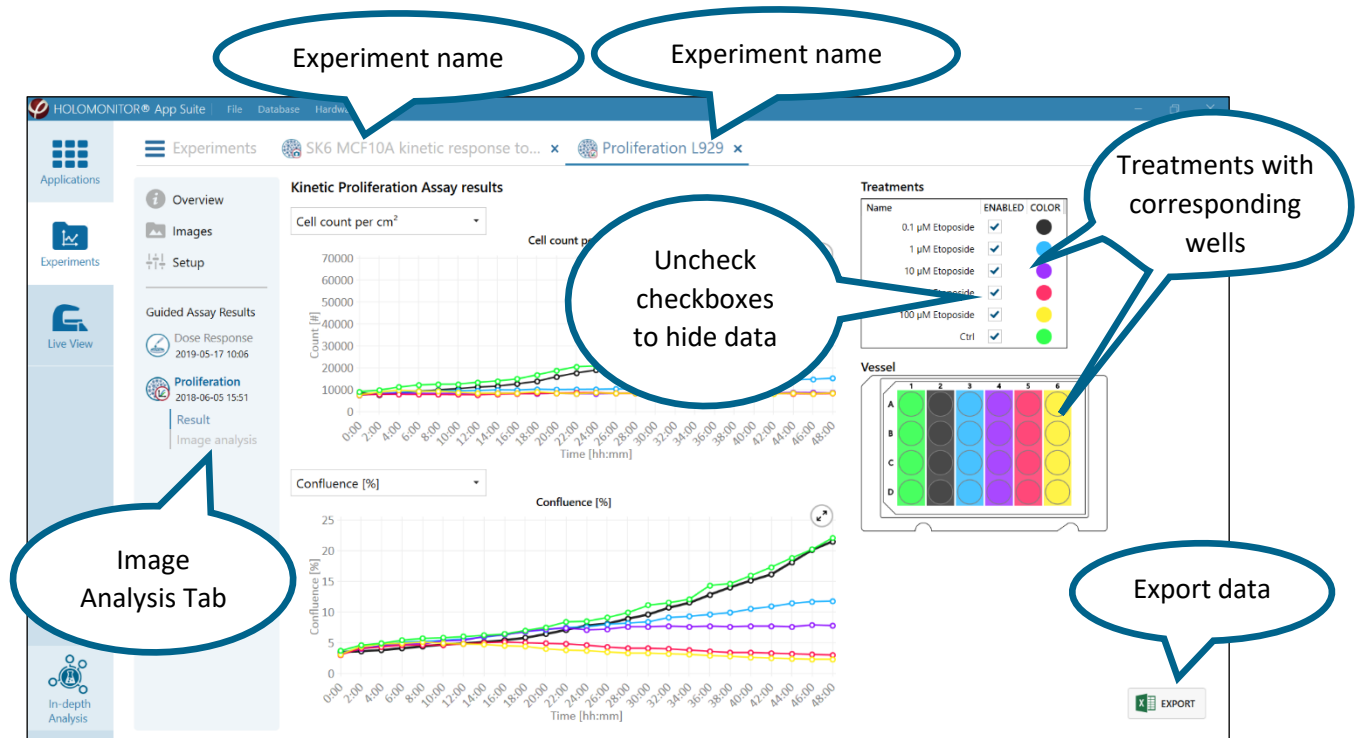
Click Delete (see image above) to delete the entire experiment with results, images and analyses.

### Experiment Overview

When an Experiment is opened, the Experiment Overview page is displayed. There, images can be accessed, and the Setup can be viewed and edited. Results can be accessed either in the Results list or by clicking the Results buttons. In the centre, experiment information is displayed. To the right, new results can be created.



When the results are displayed, the analyses of the image frames included in that experiment can also be accessed.



## Results display

### Treatments and Vessel

The experimental setup, with treatment(s) and cell culture vessel definitions are presented in Treatments and Vessel, respectively, to the right of the Results.

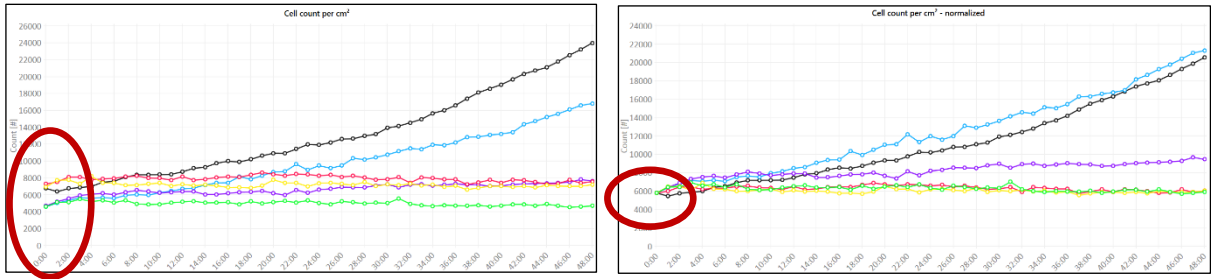
For the different treatments, the graph data are presented with the color they have in the diagram. Uncheck checkboxes to hide data in the diagram.

### Export results to Excel files

For further analysis, results can be exported to excel files by using the Export button.

### Normalized graphs

Graphs for Kinetic Cell Proliferation results can be presented as either standard (left graph below) or normalized (right graph below). The starting value for all normalized graphs equals the mean value for all treatments at time 0. This way the results can be directly compared to each other, even if there are small deviations in seeding caused, e.g., by pipetting errors,



### Image analysis

The basis for all results is the automatic image analysis, where cells are identified and counted. Also, the image quality is automatically evaluated by the software and low quality images are removed from the results.

In the Image Analysis tab, the settings for the image analysis underlying the results can be reviewed and adjusted. Bad quality images can be removed from the experiment. The images are ordered in a hierarchical way that matches the specific experiment setup.

If the graphs look irregular or spiky it is necessary to compare the analysed images with the results to see if e.g. anomalous image captures or faulty error analysis of the images cause the problem. In that case, it is possible to exclude images, capture positions or whole wells.

The screenshot shows the HOLOMONITOR App Suite interface. Key components are highlighted with callouts:

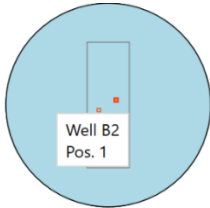
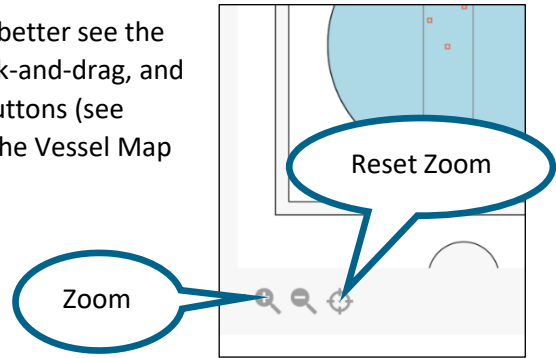
- Vessel Map:** A 4x6 grid of colored circles representing wells in a microplate.
- Capture Positions list:** A table listing image captures with columns for #, TIME, TREATMENT, CELL, POS., and SENS.
- Selected Image window:** A window showing a single image with detected cells overlaid in red and blue.
- Hierarchical image tree view:** A tree structure on the left side of the interface showing the organization of images by well and position.
- Image analysis:** A red circle highlights the 'Image analysis' option in the left sidebar.

#	TIME	TREATMENT	CELL	POS.	SENS.
1	0:02		C4	1	0
2	0:09		C4	1	0
3	0:16		C4	1	0
4	0:23	10	C4	1	0
5	0:30	10	C4	1	0
6	0:37	10	C4	1	0
7	0:44	10	C4	1	0
8	0:51	10	C4	1	0
9	0:58	10	C4	1	0
10	1:05	10	C4	1	0
11	1:12	10	C4	1	0
12	1:19	10	C4	1	0
13	1:26	10	C4	1	0
14	1:33	10	C4	1	0
15	1:40	10	C4	1	0
16	1:47	10	C4	1	0
17	1:54	10	C4	1	0
18	2:01	10	C4	1	0
19	2:08	10	C4	1	0
20	2:15	10	C4	1	0

### Image positions in the Vessel Map

All capture positions are displayed in the Vessel Map. To better see the positions in the Vessel Map, move the Vessel Map by click-and-drag, and zoom in or out by click-and-scroll or by using the zoom buttons (see image to the right). Use the Reset zoom button to show the Vessel Map in original size.

When a position is selected, the corresponding images are displayed in the Selected Image window.



To see the designation of a position, hover the cursor above a position marker, in this case Well B2, Position 1 (see image to the left).

### The Capture Positions list

Selections in the Capture Positions list makes it possible to look at all, or only part of the images included in the experiment. The list is organized as a tree view showing the experimental hierarchy. Unfilled arrows show that there is an underlying list that is hidden and can be expanded by clicking on it.

Selecting an item in the Capture Positions tree view shows all corresponding images in the Position Images list. If e.g., the experiment name is selected, all images included in the experiment will be displayed in the Position Images list. If a well is selected, all images captured in that well are shown.

It is possible to include or exclude images from a position or a well in the results by checking or unchecking the check boxes. Note that the corresponding changes to the graphs will only be created after clicking the Apply changes button.

A screenshot of the 'Capture Positions' list. The list is organized as a tree view. The root is 'Proliferation L929', which is expanded. Under it are 'Treatment' (expanded), 'Well A2', 'Well B2', 'Well C2', 'Well D2', 'Treatment 1 µM Etoposide', 'Treatment 10 µM Etoposide', and 'Treatment 50 µM Etoposide' (expanded). Under 'Treatment 50 µM Etoposide' are 'Well A5' (selected and expanded), 'Well B5', and 'Well C5'. Under 'Well C5' are 'Pos 1', 'Pos 2', and 'Pos 3'. Each item has a checkbox to its right. A callout bubble labeled 'Click white arrow to expand list' points to the arrow next to 'Well A2'. Another callout bubble labeled 'Use checkboxes to Include or exclude frames, wells, or treatments' points to the checkboxes in the list.

Click white arrow to expand list

Use checkboxes to Include or exclude frames, wells, or treatments



**Position images**  
(check/uncheck to include/exclude from analysis)

	#	TIME	TREATMENT	WELL	POS.	SENS.
<input checked="" type="checkbox"/>	1	0:00	Control	A1	1	0
<input type="checkbox"/>	2	0:07	Control	A1	1	0
<input type="checkbox"/>	3	0:14	Control	A1	1	0
<input checked="" type="checkbox"/>	4	0:21	Control	A1	1	0
<input checked="" type="checkbox"/>	5	0:28	Control	A1	1	0
<input checked="" type="checkbox"/>	6	0:35	Control	A1	1	0
<input checked="" type="checkbox"/>	7	0:42	Control	A1	1	0
<input checked="" type="checkbox"/>	8	0:49	Control	A1	1	0
<input checked="" type="checkbox"/>	9	0:56	Control	A1	1	0
<input checked="" type="checkbox"/>	10	1:03	Control	A1	1	0
<input checked="" type="checkbox"/>	11	1:10	Control	A1	1	0
<input checked="" type="checkbox"/>	12	1:17	Control	A1	1	0
<input checked="" type="checkbox"/>	13	1:24	Control	A1	1	0
<input checked="" type="checkbox"/>	14	1:31	Control	A1	1	0
<input checked="" type="checkbox"/>	15	1:38	Control	A1	1	0
<input checked="" type="checkbox"/>	16	1:45	Control	A1	1	0
<input checked="" type="checkbox"/>	17	1:52	Control	A1	1	0
<input checked="" type="checkbox"/>	18	1:59	Control	A1	1	0
<input checked="" type="checkbox"/>	19	2:06	Control	A1	1	0
<input checked="" type="checkbox"/>	20	2:13	Control	A1	1	0

Include selected images above  
 Show all images  
 Show images without warnings only  
 Show images with warnings only

405 images

### The Position Images list

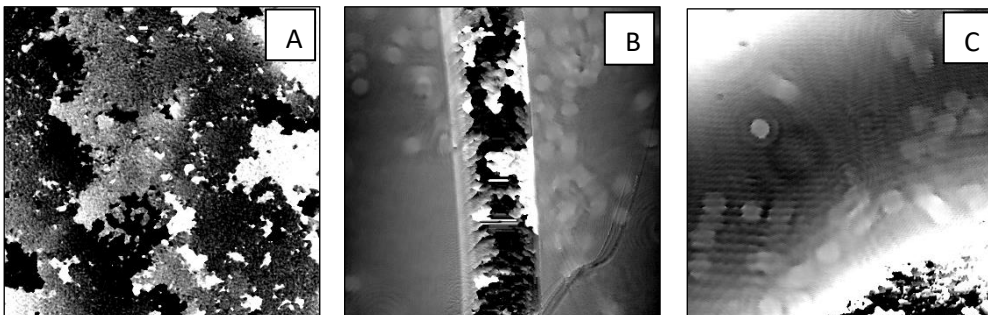
Images selected in the Capture positions list are displayed in the Position Images list. There, information relating to each frame is given and it is possible to include or exclude individual frames in the results by checking or unchecking the check boxes. The Image number and time of capture are given, as well as image treatment, well number and position number. The image of the currently selected position will be shown in the Selected Image Window. Image display functions are found below the Image List.

- Check Include selected images above to include data from all selected images in the Image list in the results.
- Activate Show all images to display all images from all positions in the experiment in the Image List.
- Activate Show Images without Warnings Only to display all images without a warning from all positions in the experiment.
- Activate Show Images with Warnings Only to displays only the images with a warning from all positions in the experiment

### Images with warnings

Sometimes the image quality is insufficient, or the image analysis is inaccurate. The software automatically tries to detect these frames and exclude those images from the results. There are two types of errors: quality and analysis. Images with low quality, due to, e.g., debris or high level of noise, are indicated with a red exclamation icon **!** in the Position Images list. Images with a faulty analysis, e.g. cell detection errors, are indicated with an orange exclamation icon **!**.

Below are examples of bad images which are typically excluded from an analysis. More examples are presented in the Troubleshooting section of this manual.

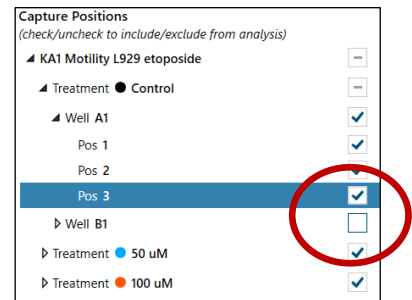


Sample examples with A. condensation B. a scratch and C. a corner of an air bubble, all of which cause insufficient image quality.



By activating Show Images With Warnings Only, it is possible to look at only the discarded images and determine whether it was a correct filtration. If the image quality or the analysis is indeed bad, no action is required. If the image is good enough to contribute to the data, even though it has been discarded, check the image box in the Position Images list, and the results will be added to the rest of the experiment.

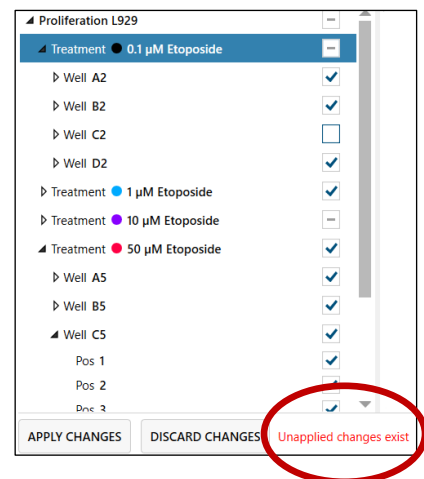
If the software has missed removing a bad quality image, it can be removed manually from the data. By activating Show Images Without Warnings Only, images included in the results are displayed. By unchecking the boxes in the Position Images list, data is removed from the results graph when the change is applied.



By unchecking the checkboxes beside the names of wells and positions in the Capture Position list, it is possible to remove entire sets of images from the results (see image to the right).

#### Apply changes

Checking and unchecking images or positions affect the data displayed in graphs and tables as well as exported data. To apply the changes and display them in the results graphs, click Apply Changes. To revert the changes, click Discard Changes. If there are any unapplied changes, a red warning text saying **Unapplied changes Exist** will appear beside the Apply and Discard buttons.

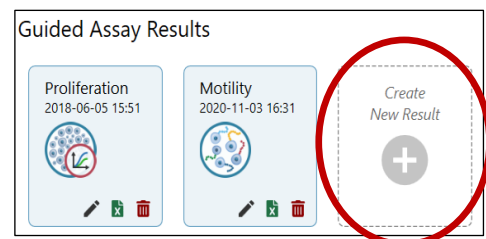


Note that changes made by checking and unchecking will not be saved and applied to the graphs and tables unless the Apply Changes button has been clicked.



Note that the changes will be applied to the current results, they will not be shown as a new result.

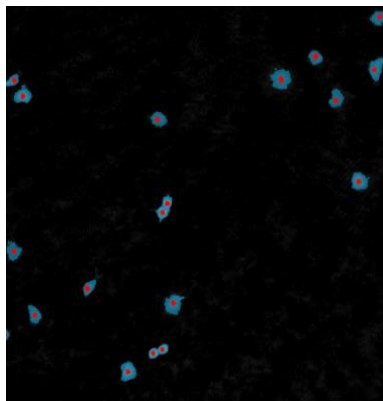
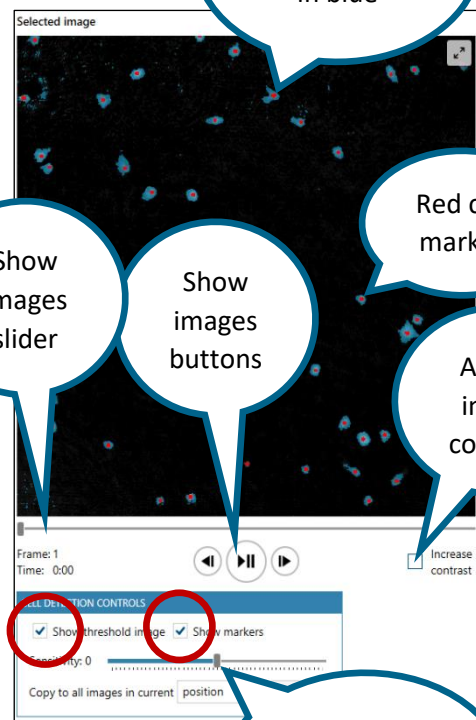
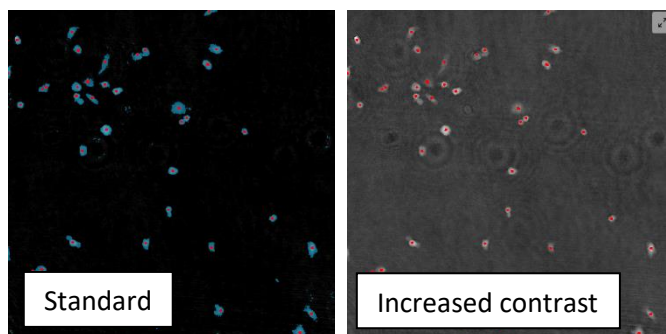
In order to keep the old results in parallel with new results, use the Create New Results button found in the Experiment Overview to create a parallel analysis (see Reanalyze results section for more information).



### Viewing the analyzed images

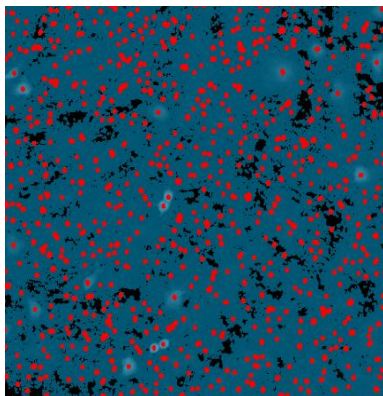
To view an image in the Selected Image window, first select the image in the Position Image list. Further images can be displayed by using the up and down arrow keys on the keyboard, by moving the slider below the Selected Image window or by using the start/stop button or the forwards or backwards buttons below the selected image (see image to the right).

Checking Increase Contrast results in an image with a brighter background which sometimes allows the user to better distinguish the cells.



### Automatic cell detection

The software automatically detects the cells in all images. The detection algorithm takes into account cell thickness, size and morphology to first separate the cells from the background, second split adjacent cells and third fine-tune the result. The algorithm identifies cells by setting a threshold that determines what is cell and what is background. Each identified cell is marked with a red dot. Unchecking Show Threshold Image and Show Markers in the Cell Detection Controls window will hide these parameters.



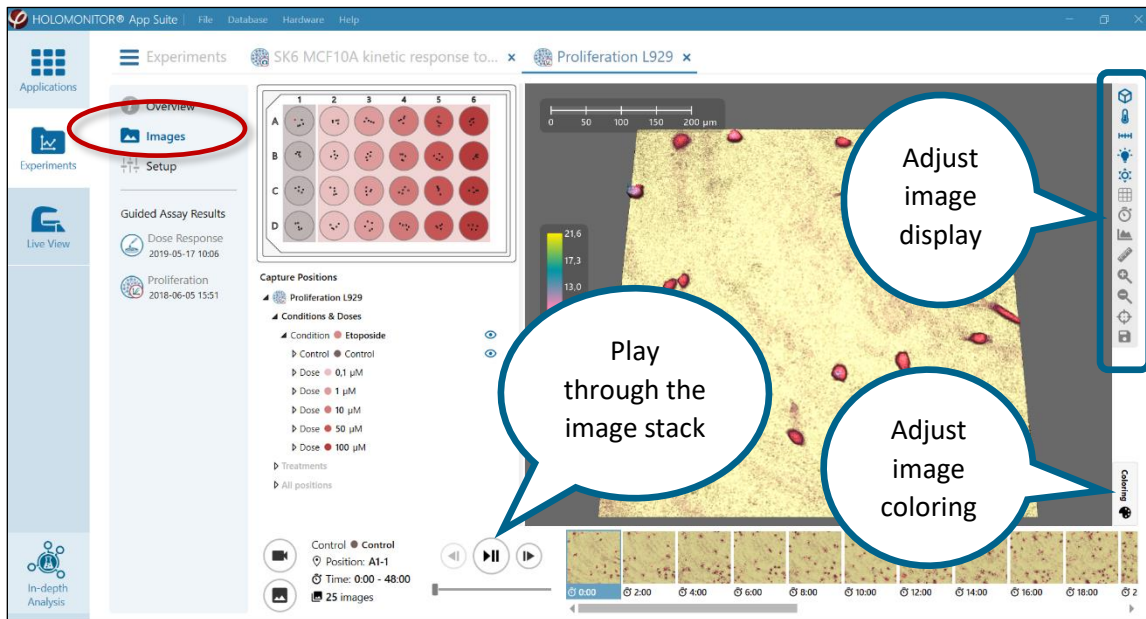
### Adjusting cell detection sensitivity

Usually, the cell detection algorithm identifies cells correctly, but if not, use the Sensitivity slider to perform a manual adjustment. To aid the adjustment, check Show Threshold Image and Show Markers in the Cell Detection Controls window. When a suitable sensitivity is found it is possible to copy the sensitivity value to all images captured at the current position, in the current well or in the current experiment. The examples show an image before (left) and after (right) threshold adjustment.

## Images

### View Images

The Images tab contains all images from the experiment, ordered in a hierarchical way that matches the specific experiment setup. Here you can, for example, look at your images and playback the timeseries for individual capture positions. The images can also be adjusted in numerous ways, including addition of artificial coloring. Colored still pictures and Videos can be created and exported in standard formats.



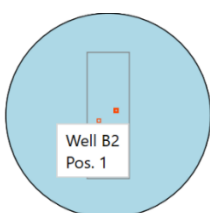
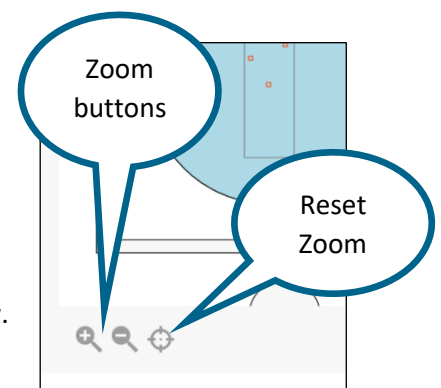
### Move or zoom the captured image

- To zoom the cell image, 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.

### Viewing Image positions


All capture positions are marked in the vessel map. To better see the positions in the Vessel map, move the window by click-and-drag, and zoom in or out by click-and-scroll or by using the zoom buttons (see image to the right). Use the Reset zoom button to show the Vessel map in original size and position or double-click left mouse button. Moving the vessel map will not affect the actual position of the motorized stage.

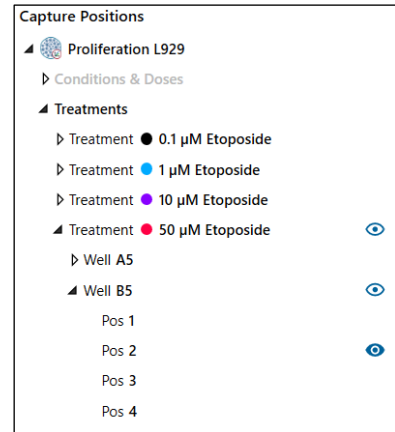
Select a position to view the corresponding images in the Image window.



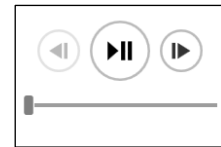
To see the designation of a position, hover the cursor above a position marker, in this case Well B2, Position 1 (see image to the left).

### The Capture Positions list

The Capture Positions list makes it possible to look at all, or only part of the images included in the experiment. The list is organized as a tree view showing the experimental hierarchy. Selecting an item in the Capture Positions tree view shows all corresponding images in the Position Images list, e.g. if a well is selected, all images captured in that well are shown. If the experiment name is selected, all images included in the experiment will be displayed in the Position Images list, if a treatment is selected all images included in that treatment will be displayed etc. The filled eye icon  indicates which images are currently displayed. To see a time lapse showing one position, ensure that only one position is selected.



Images can be displayed by moving the slider below the selected image or by using the start/stop button or the forwards or backwards buttons below the selected image (see image to the right).




### Export images or a movie

Select the position from which a time lapse movie will be created from either the Capture Positions list or by clicking the desired position in the vessel pattern.

Adjust the images by zooming, moving or flipping them and add colors, scale bar, clock etc. The adjustments will automatically be applied to all images.

### Export a movie

Click the Create a Movie button  to open the Movie Export window (see image to the right). Select image presentation format and frame rate in the finished movie, and then click Export.

Selecting 2D Images uses the current image and color but shown in 2D with no overlays or effects. Selecting 2D Plain Images uses a 2D image with greyscale coloring adapted to each image.


Click Advanced functions to adjust movie file type, encoding method and Quality level.



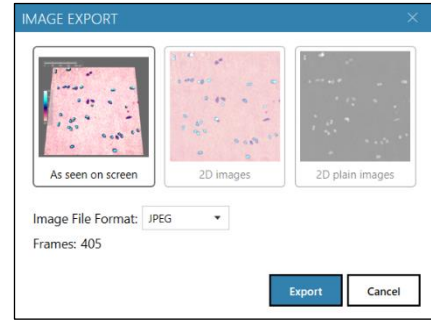
**Quality based VBR:** The quality and size of the exported movie file depends on a user-set target quality level for the movie,

**Unconstrained VBR:** The quality and size of the exported movie file depends on a target bitrate (amount of data per second) set by the user.

## Export images

Click the Export Images button  to open the Image Export window (see image to the right). Select image presentation format and image file format for the exported images, and then click Export.

Selecting 2D Images uses the current image and color but shown in 2D with no overlays or effects. Selecting 2D Plain Images uses a 2D image with greyscale coloring adapted to each image. Jpg, png, bmp and gif files are available.

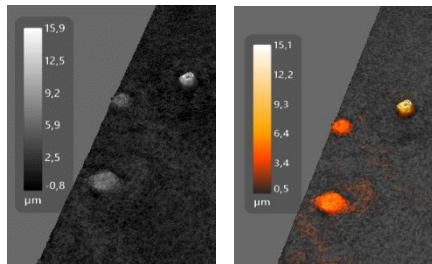


## Adjusting image presentation

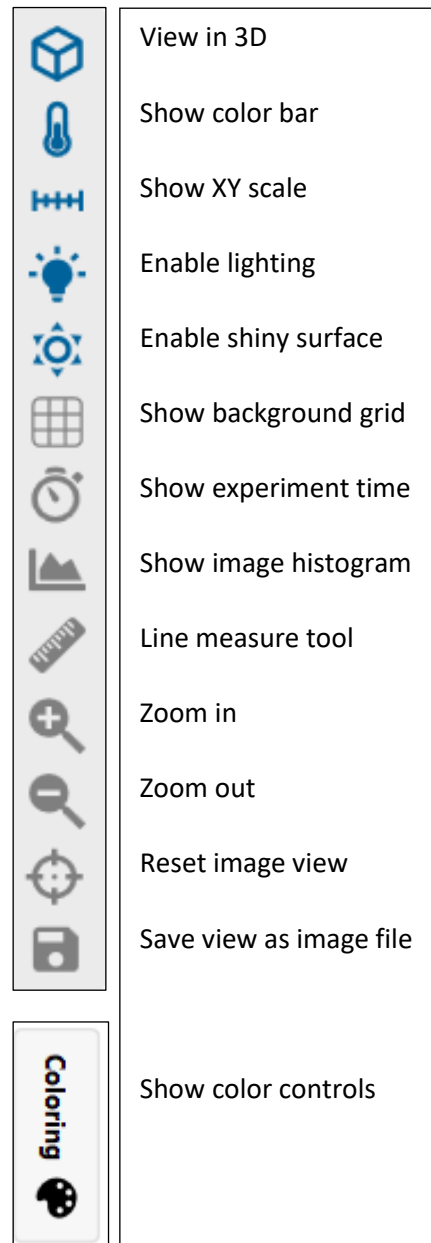
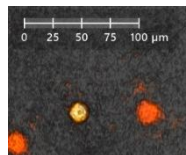
To the right of the 3D image view window there are several functions that adjust the presentation of the captured image (see image to the right). Blue functions are active while gray functions are inactive. All adjustments will be present in exported movies and images, when “As seen on screen” option is selected.

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

*Show color bar* adds a scale that correlates the image coloring with cell thickness (see images below).



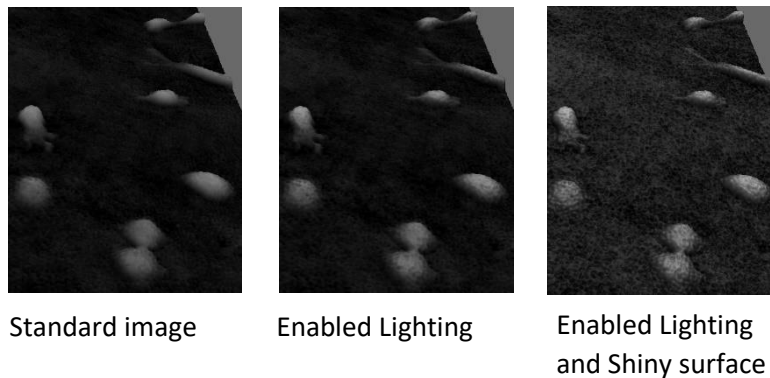
*Show XY scale* displays a horizontal scale bar representative of the distance in X and Y (See image to the right).



*Enable Lighting* applies an artificial light source to the image which may sometimes render an improved image (See image below).

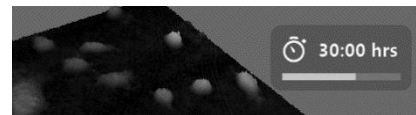


*Enable Shiny surface* shows the rendered surface with a reflective visual effect. Shiny surface is only active when Lighting is active.

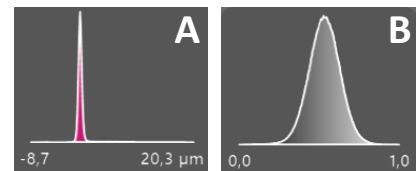


*Show background grid* adds a grid in the background.

*Show experiment time* adds a clock to the image, showing the experiment duration at image capture. (See image to the right).




*Show image histogram* shows a histogram overlay of the pixel values in the image. For a phase image this equals a histogram of the optical thickness values (A) and for other image channels such as camera it shows a histogram of the pixel intensity values (B).

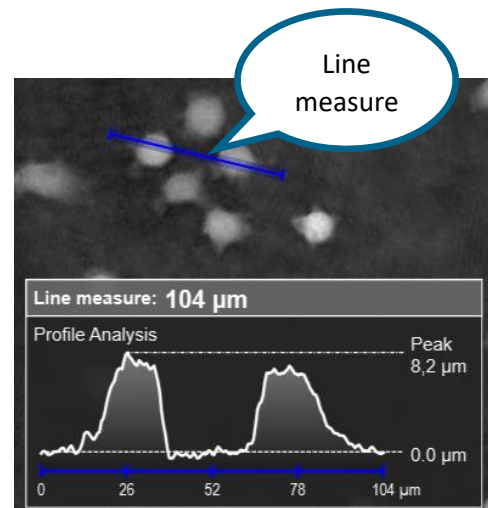


*Line measure tool* is a simple tool for interactively measuring distances and cells. Once the tool is activated, the measurement is started by left clicking and releasing anywhere in the image. The measurement is finished by left clicking and releasing at a new point in the image.

It is possible to move and flip the image while the measuring function is activated.

The data can be saved as an image with the Save View button  .

When the line measure tool is clicked again, the measuring function is deactivated.



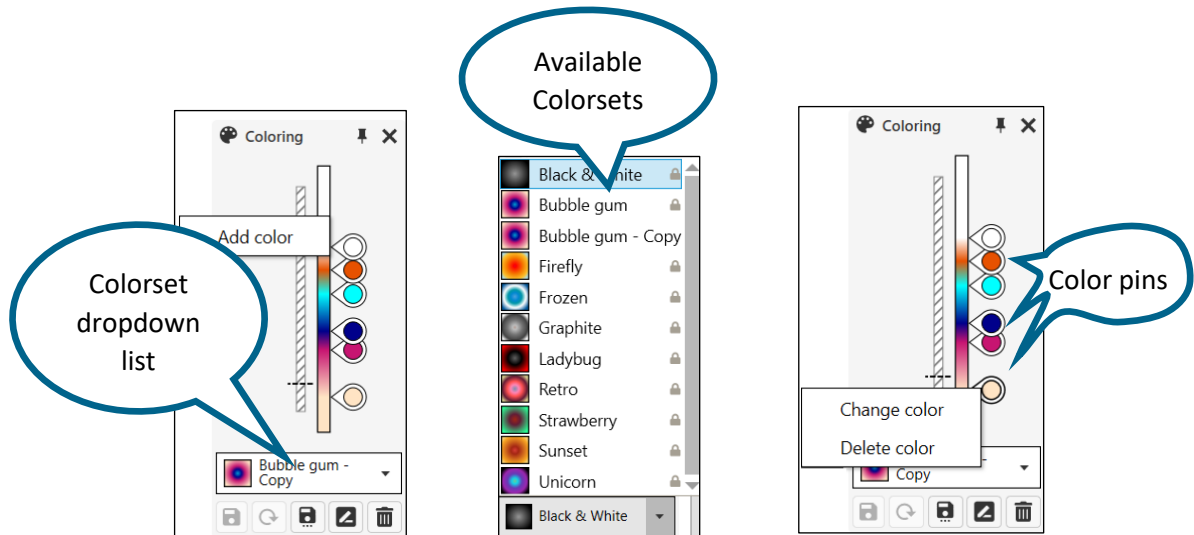
*Zoom in and Zoom out* will increase or decrease image view.

*Reset image view* will return the image view to default view and center position.

*Save view as image file* saves the image in the 3D window exactly as it is shown as bmp, gif, jpg or png files.






*Show color controls* opens the Coloring window, which is used to adjust the color balance of the window and to color the image relatively to the thickness of the cells.

A set of colors that are saved together is called a Colorset. The default colorset for any image is Black & White. There are a number of pre-set colorsets to choose from and it is easy to create new, personal colorsets. Once a colorset has been selected, it will automatically be applied to all captured images in that experiment.



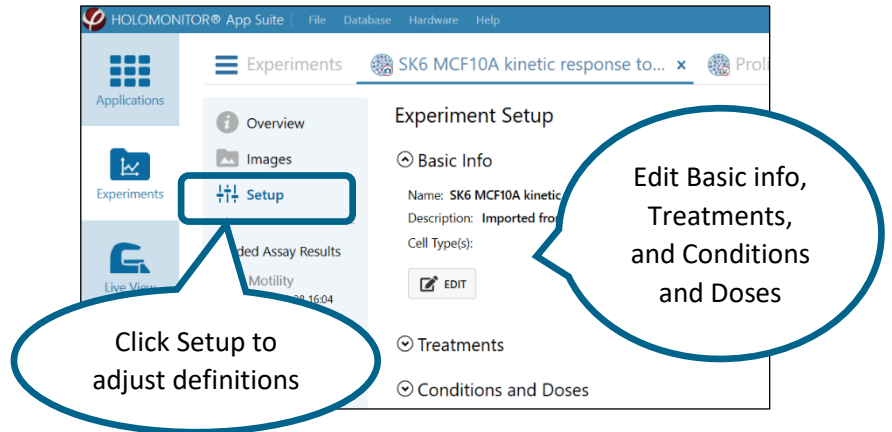
- Switch to a pre-existing colorset by using the Colorsets dropdown list (see left image above).
- Click-and-drag to move the color pins to a suitable position in the color set.
- Create new color sets by right clicking in the Coloring window and then click Add Color (See left image above).
- Change or delete colors by right clicking the color pin and select Change or Delete (see right image above).

Use the buttons below the Coloring window to

-  Save a new Colorset
-  Go back to the last saved settings
-  Save the coloring preset to a new name
-  Rename the coloring preset
-  Delete the coloring preset

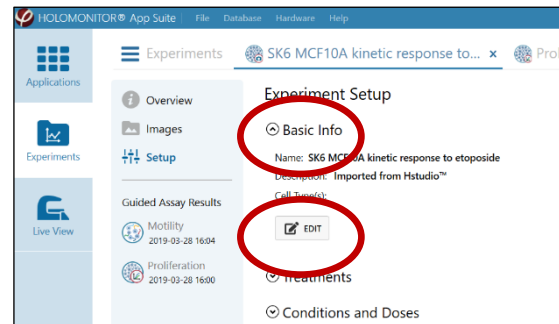
## Setup

Editing Experiment Setup, Treatments, Conditions, and doses  
All results are grouped and presented according to the treatments or conditions and doses that were defined when the experiment was set up. However, if needed, the definitions can be altered afterwards by clicking Setup.



## Basic Info

To add or edit experiment details, click Basic Info and then Edit to open the Edit window. Enter the changes and then Save.

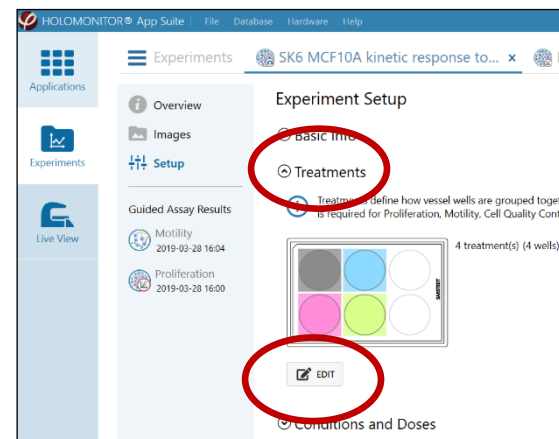


## Treatments

To add or edit Treatments, click Treatments and then Edit to open the Edit window.

The different treatments assigned to each well in e.g., motility and proliferation experiments can be modified. Select the treatment that needs to be changed from the list and use the buttons below the Treatments list to Rename or Delete the treatments.

If e.g., a dose-response experiment is converted to a motility experiment, treatments can be added to the setup.

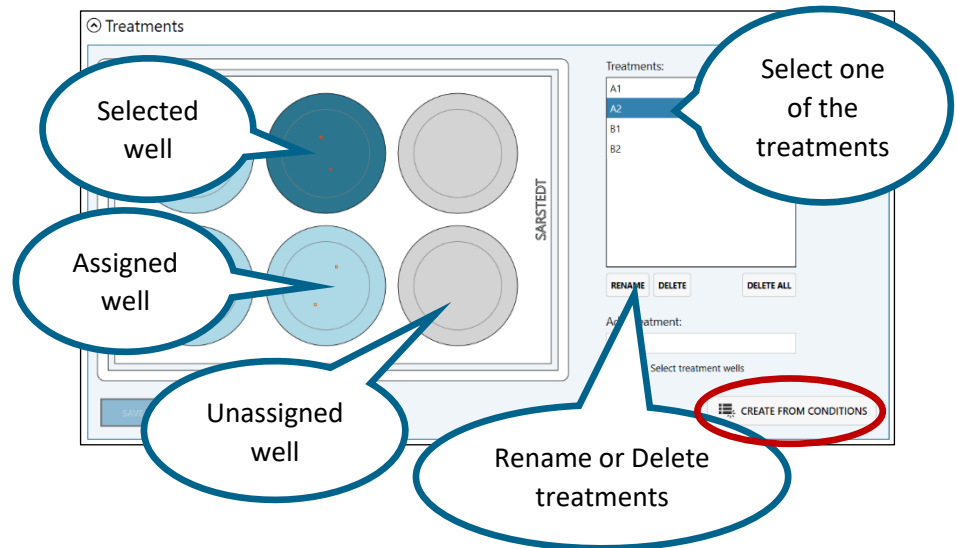




In order to Add a new Treatment, there must be unassigned wells (grey). Select the unassigned well which will then turn dark blue, enter a new name in the Add Treatment textbox and click Add.

If the experiment was originally set up for the dose-response application, convert the setup to treatment by clicking the Create from conditions button.

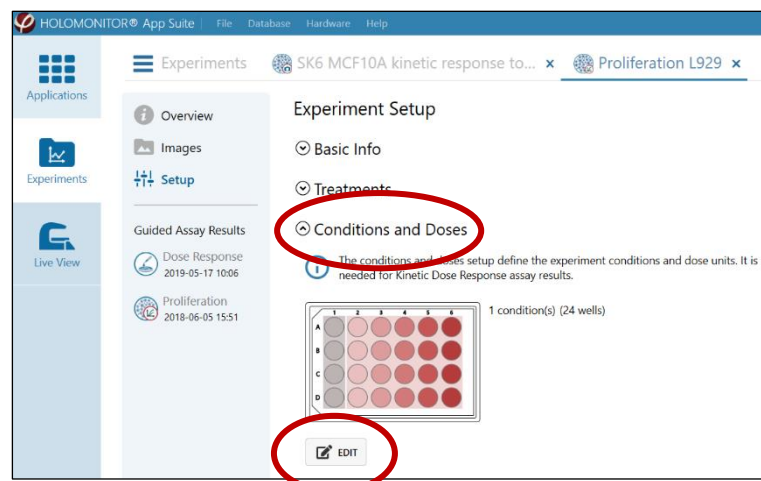
Click Save to add the changes and close the Edit function.



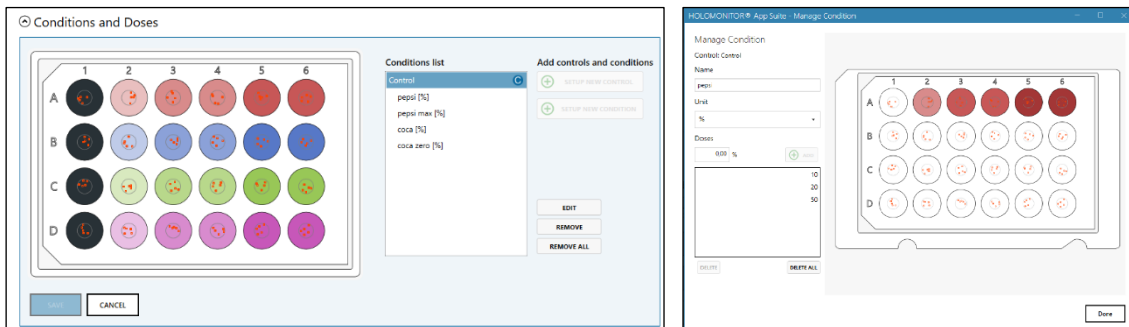
Note that editing treatments will cause previously saved settings and results to be deleted. In order to create parallel treatment set ups, first create a copy of the experiment.

### Conditions and Doses

In Conditions and Doses, the conditions and doses for dose-response curves can be edited. Select the item in the Conditions list that needs to be changed, either a control or a treatment, and use the buttons beside the list to Edit or Remove. Note that a Control must be set before any conditions can be added.



Clicking Edit will open Conditions and Doses (below left) where the conditions can be selected from a list. Highlighting an item and clicking Edit will open Manage Control or Manage Conditions (see image below right). In the Manager, the control or the selected condition can be edited, e.g., the name can be corrected, or the Unit and the Doses can be adjusted or added.



Note that one experiment can have more than one control, e.g., if there are two different cell lines. Use Setup New Control to add more controls. To setup new controls there must be unassigned wells. Note that the controls must have unique names, otherwise it will not be possible to save them.

Select one of the controls and then click Setup New Conditions in order to add the associated conditions. Note that the controls must have unique names, otherwise it will not be possible to save them.

Click Done to add the changes and close the Edit function.

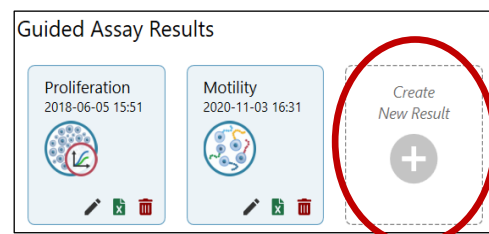


Note that editing conditions will cause previously saved settings and results to be deleted. In order to create parallel treatment set ups, first create a copy of the experiment.

*Re-analyze data using the same or a different type of analysis*

#### Create New Result

Experiments can be utilized for more than one analysis of the same type of results, or for more than one type of results. E.g. a proliferation assay can be reanalyzed for proliferation with results from certain wells excluded, or the proliferation data can be used to create a dose response curve if there are enough different treatments. Also, dose response experiments can be used for e.g. proliferation assays.



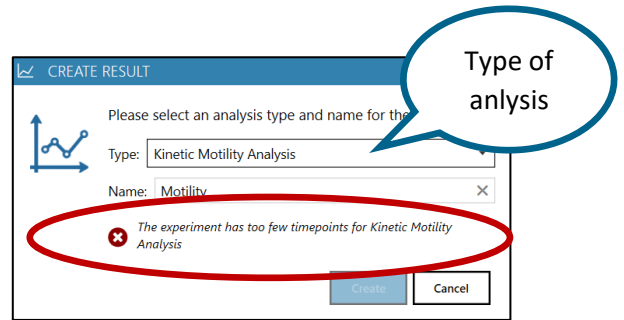
Click the Create New Result button to open the Create Result window.

### Select Assay Type

If the experiment data setup does not support the analysis type requested, regarding the treatment or conditions setup definitions, a warning will appear. E.g., as a QC experiment has only one time point it cannot be converted to a kinetic experiment.

Note! Before using a proliferation experiment to extract motility data, ensure that the timepoints are frequent enough to provide accurate data.

An experiment set up for dose response can be reanalyzed as e.g. a proliferation assay, but then conditions must be converted to treatments and vice versa. In that case, go to Setup and edit treatment or conditions.

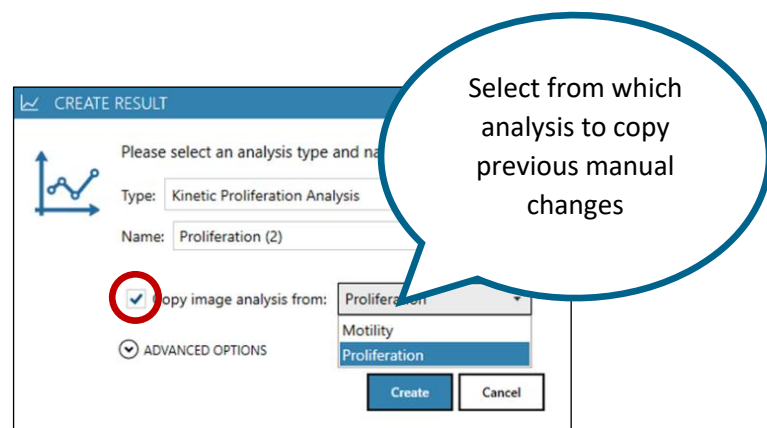


### Copy image analysis settings from a previous result

Usually, manual changes will have been performed on the experimental results, such as including/excluding images, or change Cell Detection Control settings.



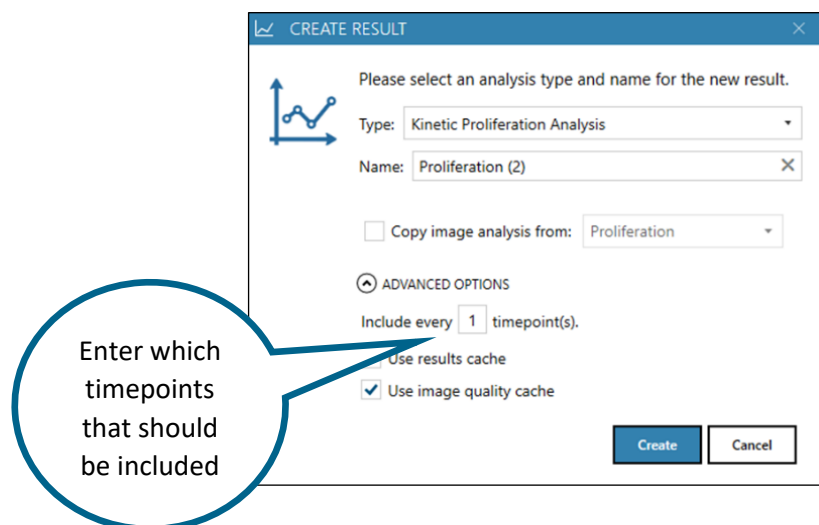
Note that when creating a new result, the image analysis settings are set to the default values of the experiment. No changes that have previously been applied will accompany the new results.



To re-use manual changes made for a previous analysis, check “Copy image analysis from” and select which analysis result to copy the settings from.

### Use fewer timepoints in the analysis

Click Advanced options to present these alternatives. The number of timepoints included in the analysis can be adjusted, allowing the results to comprise fewer time points. E.g. every third or every tenth timepoint can be included.



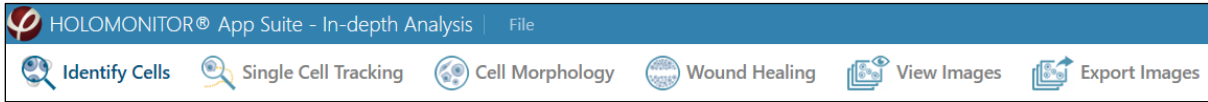
### Use stored calculations to display results and images

Click Advanced options to present these alternatives. When Results cache and Image Quality cache are checked, the software will use stored calculations when displaying the results and the images. This is not normally something a user needs to uncheck, but for some special cases and troubleshooting it might be necessary to force re-calculation of results or image quality. Note that not using the Results Cache and previous Image Quality calculations requires a lot of computing time.

## In-Depth Analysis

### Software layout

This part of the software comprises six tools. They can be used with both App Suite and Hstudio data.



*Identify cells* identifies and out-lines cells in images and time-lapses.

*Single Cell Tracking* tracks individual cells in time-lapses. Results for both movement and kinetic morphology changes will be displayed. Cell divisions are tracked and displayed in cell family trees

*Cell Morphology* analyses cell morphology in images and time-lapses, as well as displays and exports the results.

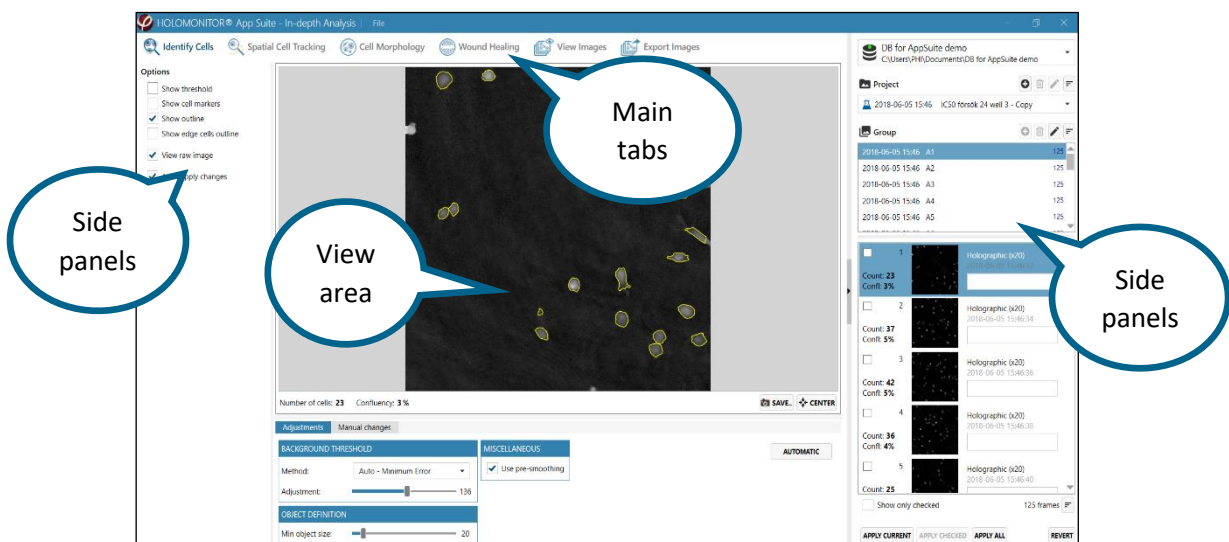
*Wound Healing* analyses gap closure in time lapses for wound healing.

*View images* allows the user to view and color captured images and time-lapses and to adjust autofocus settings.

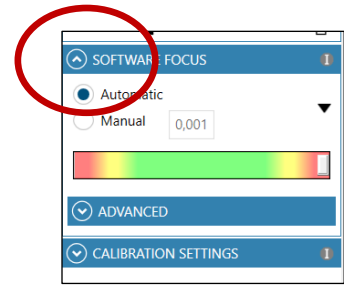
*Export images* visualizes and exports images and time-lapse movies.

### View area and side panels

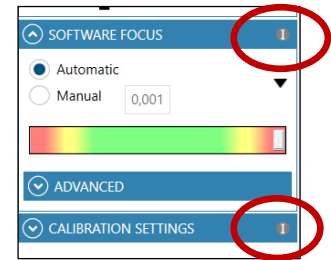
The View area is the main viewing area where cell images and cell data are shown. Basic functions controlling the View area are found in the Side panels to either side.



Some of the side panels can be collapsed, and they can be expanded by clicking the arrow tip found in every collapsible side panel header.



Information concerning the different side panels can be found by clicking the Information buttons which are placed in the side panel headers.

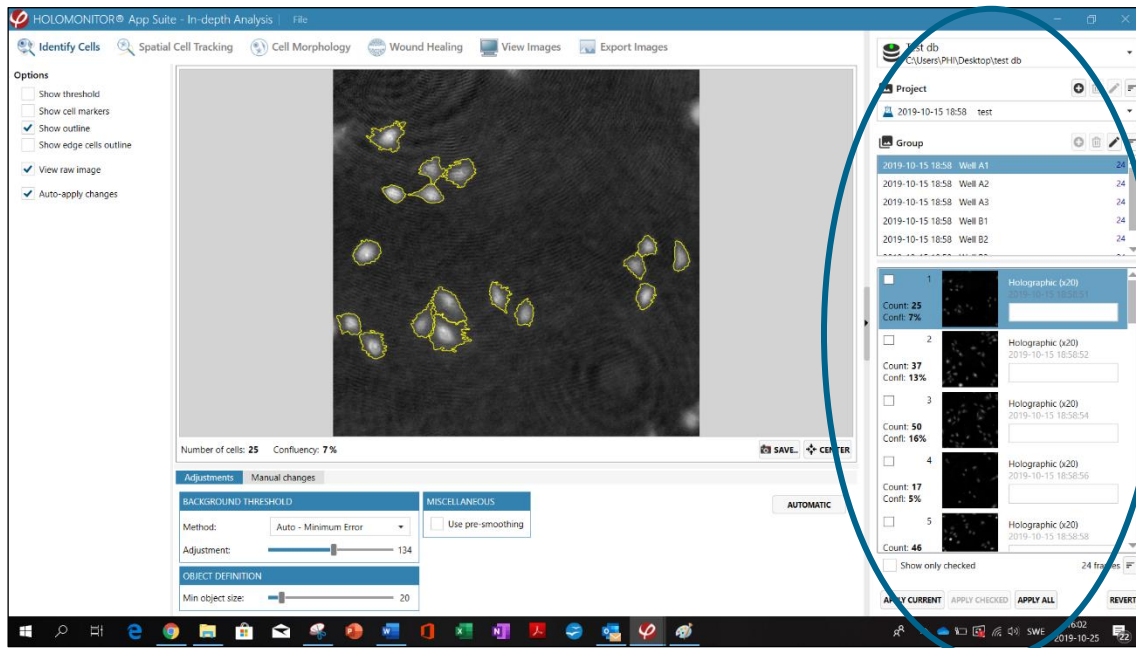


### The Image Frame list

Images are grouped in Image Groups, which are organized into Projects, which in turn are stored in a Database.

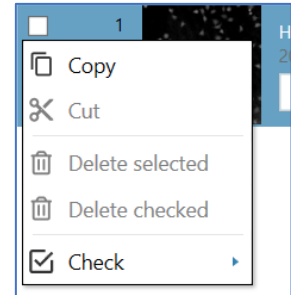
In all the Main tabs, the right-side panel shows the Image Frame list as well as the corresponding Database. The list controls which Group of images that is currently viewed or analyzed.

For data captured with App Suite, a Group comprises images from one position in a time-lapse sequence. For imported Hstudio data, a Group may also comprise images captured at various cell culture positions.



### Copy, Cut or Delete frames

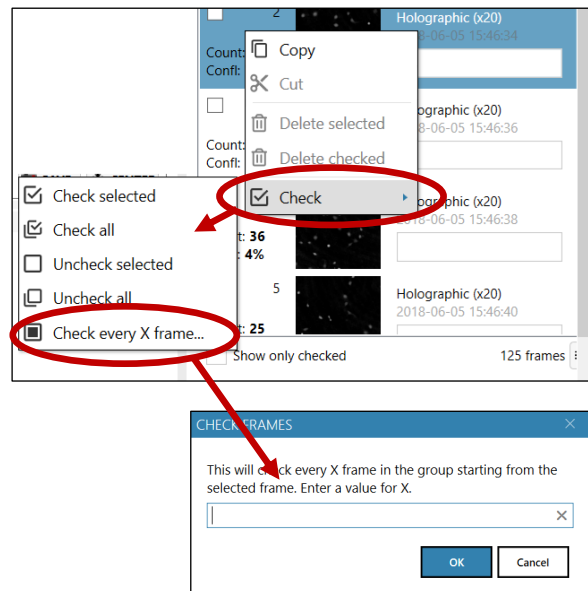
Right click in the Image Frame list to copy App Suite or Hstudio image frames.



The other functions, i.e., Cut or Delete Image Frames, work only with data of Hstudio origin, which has not been imported into App Suite and thereby been converted to App Suite data. As App Suite organizes image frames differently than Hstudio, an App Suite-generated Image Frame list must be edited in the App Suite Results tool. This also applies to data of Hstudio origin which has been imported into App Suite and thereby been converted to App Suite data organization.

### Use Check to Sort the Image Frame list

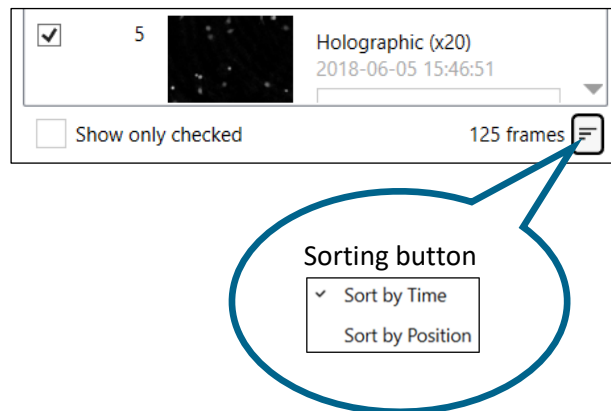
The Check options can be used to analyze subgroups of images. Right click in the Image Frame list to access the Check options. Especially the Check every X frames is a very useful tool in combination with the Show only Checked function which is found below the Image Frame list.



### The sorting button

In addition, the Image Frame list can be sorted either based on image frame number or on time of capture.

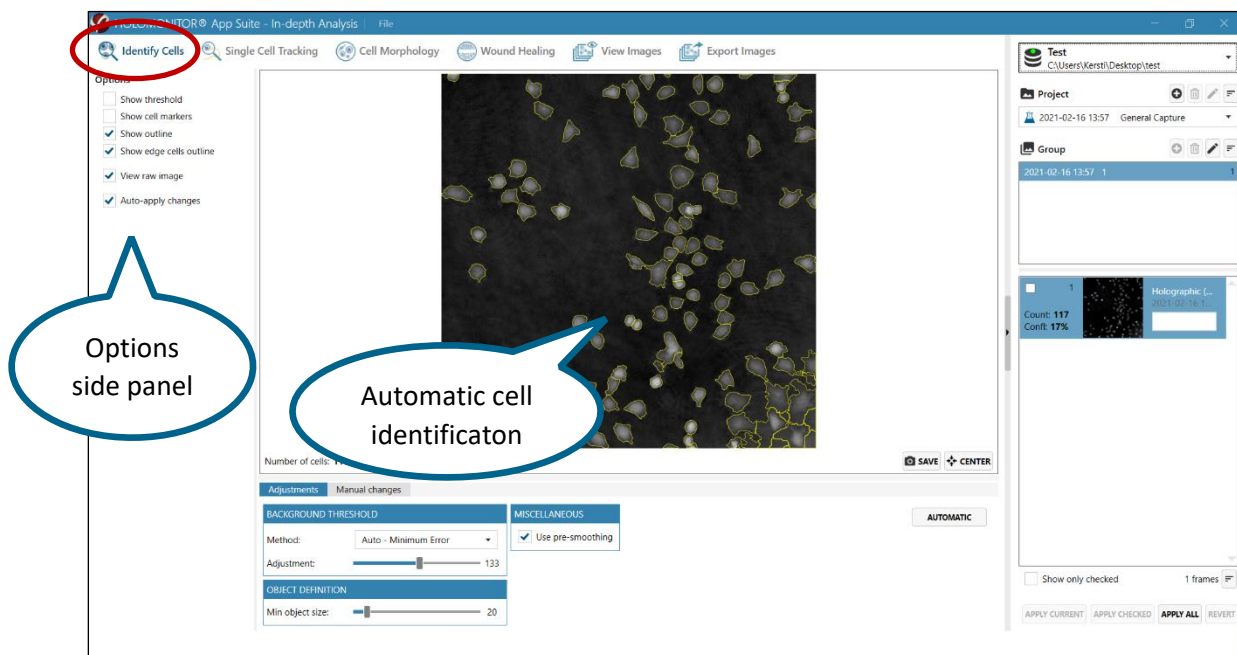
This function is mainly useful for Hstudio data, as App Suite data is already sorted by time and only shows one position.



## Identify Cells

Identify Cells is the first step before the Single Cell Tracking or Cell Morphology analyses. The more careful and precise the identification, the better-quality data can be extracted, and the smoother and faster the analysis will go.

Choose the Identify Cells tab and then select an image in the Image Frame list. The software will automatically suggest a cell identification.



## Change how identified cells are displayed

Adjust cell and image display in the Options side panel by unchecking and checking.

*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* outlines cells touching the image edges.

*View raw image* displays the image without using pseudo coloring.

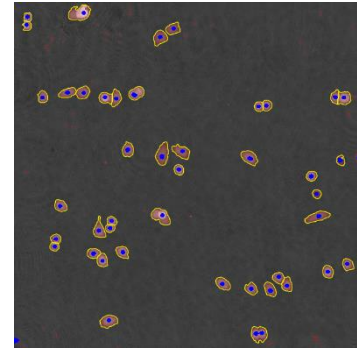
*Auto-apply changes* automatically applies adjustments when switching to another image in the Image Frame list.



## Cell Identification Adjustments

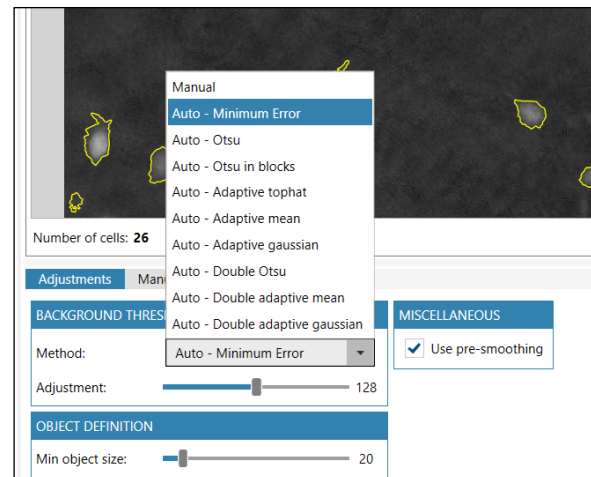
These adjustments encompass the entire frame and can be applied to other frames. The cell identification adjustments are performed in three steps:

1. Determine which threshold calculation method that is optimal to determine which objects that will be identified as cells for the current cell culture.
2. If needed, use the adjustment slider to adjust the threshold setting.
3. If needed, use the Min object size slider to ensure that every cell has a cell marker.



### 1. Different threshold calculation methods

The background threshold determines which objects that will be identified as cells in the image. There are several methods to automatically calculate the Background threshold in the Adjustments tab. 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. The software uses the Minimum error method by default.



*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 the Otsu method. This is a form of adaptive threshold.

*Adaptive tophat* uses morphological filtering prior to thresholding to amplify cell-alike objects.

*Adaptive mean* sets an adaptive threshold using a mean filter.

*Adaptive gaussian* sets an adaptive threshold using a Gaussian filter.

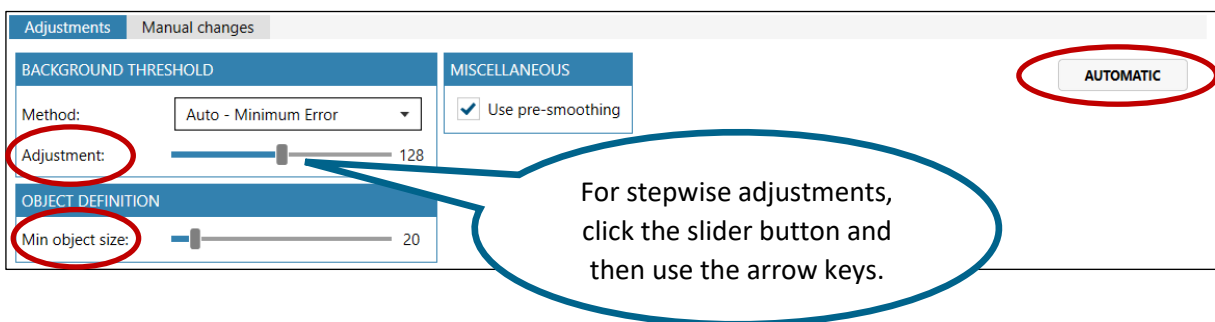
*Double Otsu* is a threshold method where both a wide and a narrow threshold mask are used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. Double Otsu uses double thresholding with Otsu global threshold as mid-level threshold.

*Double adaptive mean* is the same as Double Otsu, but with two adaptive mean threshold masks.

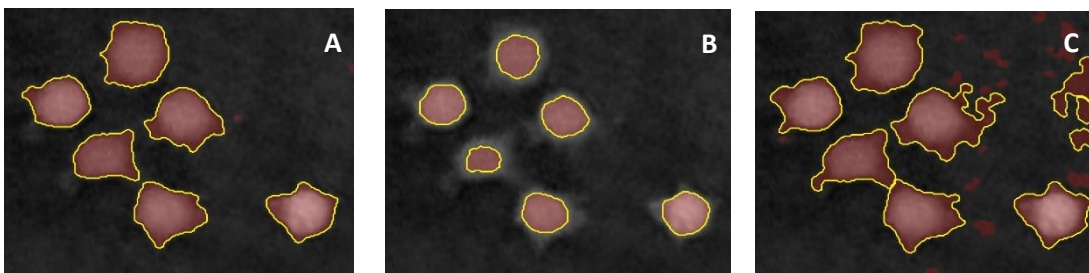
*Double adaptive gaussian* is the same as Double Otsu, but with two adaptive Gaussian threshold masks.

### 2. Adjusting background threshold

In addition to the different automatic calculation methods for cell identification, the user can adjust the settings using the Background Threshold Adjustment slider in the Adjustments tab, which is found below the View area.



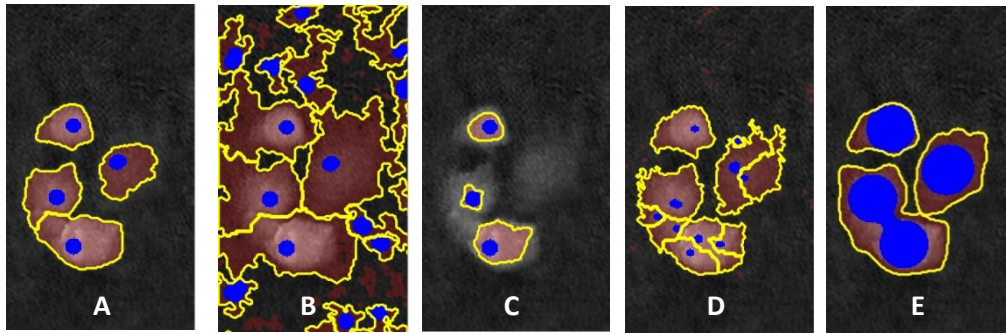
Adjusting the threshold between cell and background with the slider will adjust the minimum thickness of objects considered to be cells. Image A shows correctly thresholded cells, image B cells with a too high threshold, and Image C with a too low threshold.



### 3. Adjusting object definition, i.e. cell size

The minimum cell size is adjusted by using the Min object size slider (see image above). The goal is to have one blue marker in every object considered to be a cell (see image A below). The slider values do not have a unit, but they are related to the diameter of the largest circle that can fit into the cell.

## Common cell identification problems



- A. Threshold is OK, Object Definition is OK
- B. Threshold is set too low, too much of the background is counted as cell area
- C. Threshold is set too high, too little of the cell is included in the cell area
- D. Threshold is OK, Object Definition set too low, the cells are segmented into smaller units
- E. Threshold is OK, Object Definition set too high, the cells are merged into larger units

### Pre-smoothing

When Use pre-smoothing is activated, the cell edges will be smoothed.

### Default values

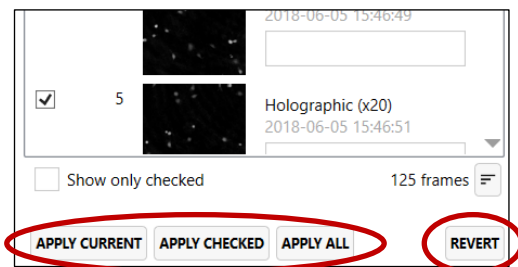
Clicking Automatic will restore the default values for automatic cell identification.

### Apply cell identification adjustments

Threshold and object size adjustments will be saved automatically when Auto-apply changes is activated. To apply the same adjustments to other image frames, use the Apply buttons below the Image Frame list

### Revert Cell identification adjustments

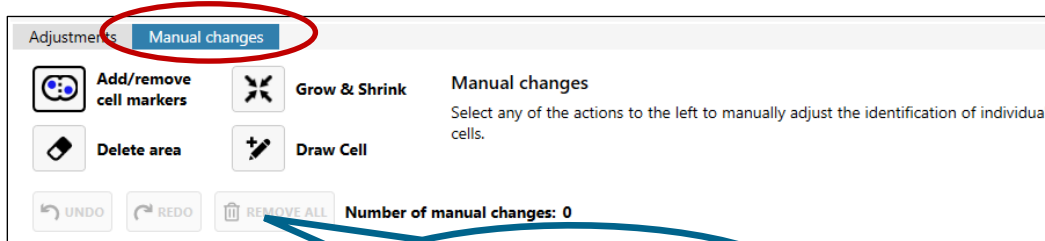
Use the Revert button to revert any changes back to saved settings for the current frame.



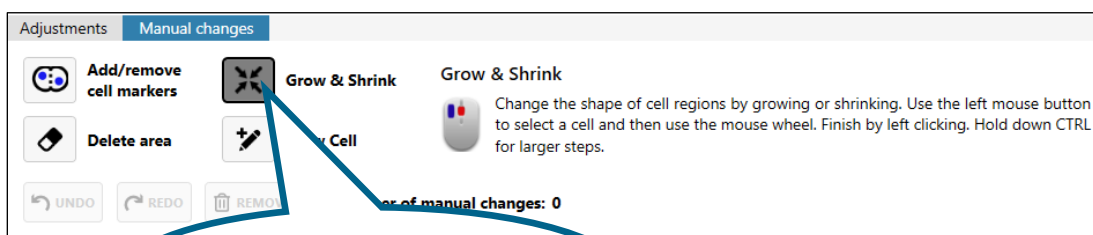
### Manual identification adjustments for individual cells

The identification of individual cells may be manually adjusted. It is possible to add, remove and delete as well as enlarge or shrink individual identified cells. These changes are not possible to apply to other frames, as they are only valid for that specific cell in that specific frame. These functions are found in the Manual changes tab below the View area. Each function is carefully described when the function is activated.

There are buttons that enable the user to edit the changes. It is possible to undo the changes step by step, to redo an undone step and to remove all adjustments.



Use these buttons to edit the manual changes



Activated function with corresponding description

*Add/remove cell markers* adds or removes cell markers within the current threshold regions to split cells or merge existing ones. Use the left mouse button to add cell markers and the right to remove.

*Grow & Shrink* changes the shape of cell regions by growing or shrinking them. Select a cell marker using the left mouse button and then use the mouse wheel to grow or shrink the cell marker. Finish by left clicking. Hold down CTRL for larger steps.

*Delete Area* removes threshold mask and marker from cell area. It can be used to remove small objects or disturbances created by floating cells that cannot be removed by other adjustments. Left click to select.

*Draw Cell* outlines any cell manually. First right-click to clear area and then left-click to start drawing. Continue setting points by left clicking, and finish by clicking the starting point. Clicking right cancels.



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

## Single Cell Tracking

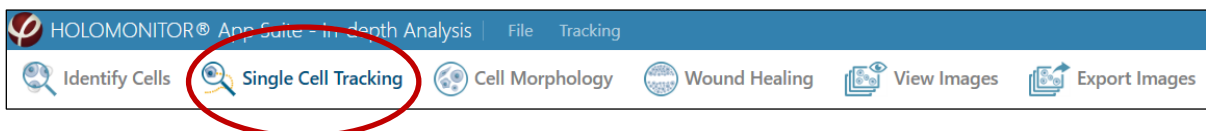
Cells can be tracked through a time-lapse sequence and analyzed for both movement and morphological changes over time. The software also tracks cell divisions and supplies cell family trees.



Note that the cells must be identified in the Identify cells tab before using the tracking functions.

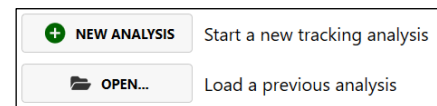


Note that images must be captured with sufficient frequency for the software to be able to perform a correct cell tracking. Use the App Suite protocol Pre-study for Kinetic Motility to determine the capture frequency for each cell line.



### Start a new tracking

Choose the Single Cell Tracking tab. If tracking has not been performed previously, a tab will appear with the choices of starting a new analysis or opening a previous. When selecting New analysis, a request to add image frames to the analysis appears. When images have been added, all the tracking functions can be accessed.



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

A short summary of how many cells that have been tracked, and how many cell families that have been detected is given immediately.

The screenshot shows the 'Single Cell Tracking' interface with several callouts:

- List of added frames:** A list of 20 cells (Cell 1 to Cell 20) with checkboxes and colored dots.
- Cell tracking summary:** A central panel showing a dark image with yellow and green spots representing tracked cells.
- Time-line slider:** A horizontal slider at the bottom of the image.
- Sorting button:** A dropdown menu with options 'Sort by Time' and 'Sort by Position'.
- ADD ALL:** A button at the bottom right of the interface, circled in red.

### Add image frames to the analysis

Add image frames to the analysis by using the Add buttons, found below the Image Frame list. Sometimes not all images are needed to get good results, and then it is timesaving to analyze only the frames that are needed. The frames can be chosen by selection, sorting or Check options. Added image frames are listed in the Frame list.

### Image selection

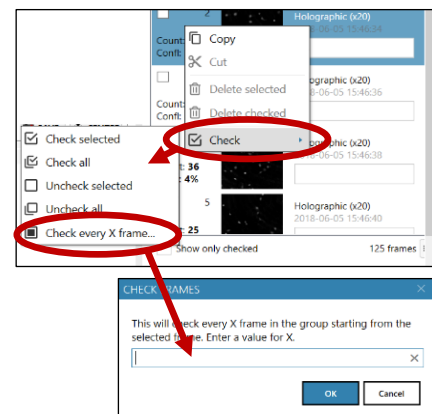
Several images can be added simultaneously if they are all selected. The shift key can be used to select several consecutive images and the Ctrl-key to select non-consecutive image frames.

### Image sorting

The Image Frame list can be sorted by time or position using the sorting button (see image above). This function is mainly useful for Hstudio data.

### Check options

The Check options can be used to analyze subgroups of images. Especially the Check every X frames is a very useful tool in combination with the Show Only Checked function which is found below the Image Frame list. Access the Check options by right clicking in the Image Frame list (see image to the right).



### Tracking

All cells that have previously been identified as cells are automatically tracked by the software. Each identified cell is marked with a small orange +. When cells leave the field of view, the tracking is automatically discontinued. When cells enter the field of view, they are automatically tracked.



### Cell tracks

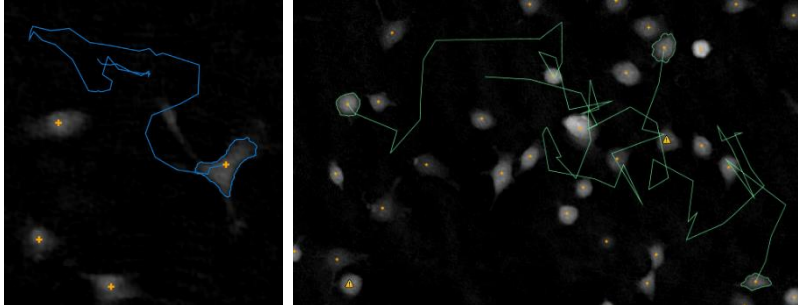
When the Timeline slider below the View area is moved by clicking arrows or dragging the black box, trails showing the cell movements will be displayed. Use the checkboxes to hide or shorten the cell trails and to hide the cell outlines.





### List of tracked cells

All tracked cells are shown in a list. The cells are numbered and color-coded. To show only specific cell trails, use the checkboxes, or the Include-Exclude buttons. That way the trails of, e.g., an individual cell or cell family can be shown.



Tracked cells Warnings (196) Frames

Include/exclude tracked cells

- Cell 1
- Cell 2
- Cell 3
- ▶  Cell 4
- Cell 5
- Cell 6
- Cell 7
- Cell 8
- ▶  Cell 9
- Cell 10
- Cell 11
- Cell 12
- Cell 13
- ▶  Cell 14
- Cell 15
- Cell 16
- Cell 17
- Cell 18
- Cell 19
- Cell 20

INCLUDE ALL     EXCLUDE ALL

- Cell 1
- Cell 2
- Cell 3
- ▶  Cell 4
  - Cell 4-1
  - ▶  Cell 4-2
    - Cell 4-2-1
      - Cell 4-2-1-1
      - Cell 4-2-1-2
      - Cell 4-2-2
- Cell 5
- Cell 6

### Cell families

In the tracked cell list, cell families are numbered and color-coded for easy identification. For example, when Cell 4 divided, the daughter cells were named Cell 4-1 and Cell 4-2, then when Cell 4-1 divided, the daughter cells were named Cell 4-1-1 and Cell 4-1-2. All the cells in a family have the same color.

### Cell tracking warnings

When the cell volume deviates from the previous image, the software will give a warning. The warnings are listed in the Warnings tab, found beside the image list.

For each warning, time and cell identity is given. Clicking the warning will display the corresponding image frame. The corresponding cell will be filled with color and have a warning triangle.

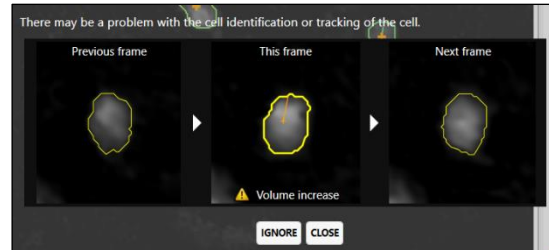
Tracked cells
Warnings (472)
Frames

Ignore	Volume increase	0:05	Cell 23
<input type="checkbox"/>	Volume drop	0:05	Cell 2
<input type="checkbox"/>	Volume increase	0:05	Cell 10
<input type="checkbox"/>	Volume drop	0:05	Cell 12
<input type="checkbox"/>	Volume increase	0:05	Cell 14
<input type="checkbox"/>	Volume increase	0:10	Cell 2
<input type="checkbox"/>	Volume increase	0:10	Cell 12
<input type="checkbox"/>	Volume increase	0:10	Cell 14
<input type="checkbox"/>	Abnormal end	0:10	Cell 24
<input type="checkbox"/>	Volume drop	0:10	Cell 34
<input type="checkbox"/>	Volume increase	0:15	Cell 12
<input type="checkbox"/>	Volume increase	0:15	Cell 25
<input type="checkbox"/>	Volume increase	0:15	Cell 28
<input type="checkbox"/>	No vol. drop division	0:15	Cell 30-1
<input type="checkbox"/>	Volume drop	0:15	Cell 34
<input type="checkbox"/>	Volume drop	0:15	Cell 38

Sensitivity:  Medium

Show icons in image  
 List ignored warnings  
 Show warnings for included cells only

When the warning triangle is double-clicked, 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 apoptosis or cell division, click Ignore to ignore the warning.
- The warning can be caused by cell misidentification and can be corrected using Adjust Cell Identification.
- The warnings can also be caused by cell tracking errors which are corrected using Adjust Tracks.
- In case the warning concerns a cell division, the daughter cell tracking can be adjusted as described below using Adjust Tracks.

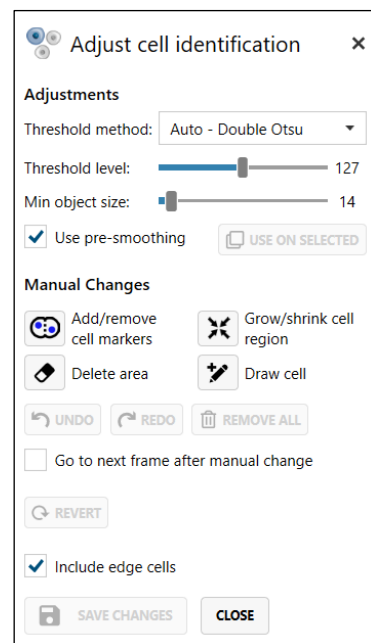
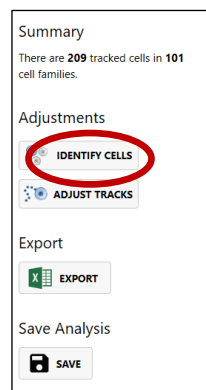
### Adjust cell identification

If the warning is caused by a cell identification error, click Identify Cells to open the Adjust Cell Identification tab. After adjusting the identification, save changes and close the tab.

*Use On Selected* will apply Adjustments to all selected frames in the Frames list.

Checking *Go to the next frame after manual change* will automatically skip to the next frame after a manual change to a cell has been done, allowing for smoother application of manual changes that have to be performed on consecutive frames on a single cell.

*Include edge cells* will include cells touching the edge of the image frame in the analysis for all frames.



All other functions in this tab are equal to the functions in the Identify cells tab. The functions are described in detail in the Identify cells section of the manual.



Note that all changes made in the Adjust Cell Identification tab will be applied globally to all results, thus changing other analyses that may have been done with the same data.



## Adjusting tracks

If the warning is caused by a cell tracking error, click Adjust Tracks to open the Adjust Tracking tab. After adjusting the tracking, save changes and close the tab.

*Move track* is used to transfer the tracking from one cell to another. Click in the image on the tracked cell that needs to be adjusted. Then click on Move Track, and thereafter click the cell to which tracking should be transferred.

*Flip tracks* is used to flip tracks of two cells. Click in the image on the cell that needs to be adjusted. Then click on Flip Tracks, and thereafter click the other cell in the switch.

*Join tracks* is used to connect two track segments. Click on the tracked cell in the last image containing the first tracking segment, then click on the cell to join with in the next image.

*Discontinue track* is used when a cell track needs to be terminated. After selecting the cell to be changed, click Discontinue Track. The tracking will be discontinued from the present frame and onward.

*Add to tracks* is used to add cells to an existing track. The cells are added frame by frame. Start by selecting a cell and click the button, then follow the instructions.

*Add track* is used to create a new track by stepwise adding cell by cell. Start by selecting a cell and click the button, then follow the instructions.

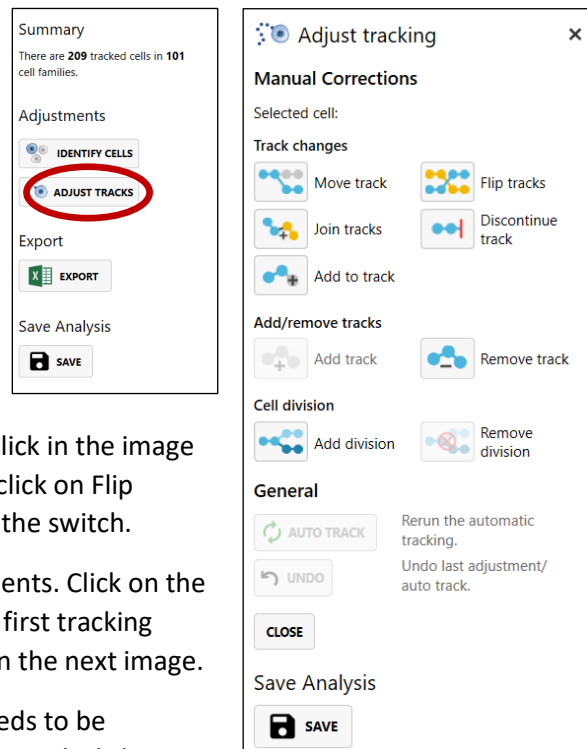
*Remove track* is used to remove an entire track. Select a cell and click the button.

*Add division* is used to add a division that was not recognized by the software. Start by selecting a cell and click the button, then follow the instructions. In some cases, there will be more than two daughter cells. Check Allow more than two children to connect more daughter cells to the mother cell.

*Remove division* is used to remove an erroneously identified division. The cells will be tracked as unrelated individuals from the present frame and onward.

*Auto track* is used to re-run the automatic tracking while incorporating all manual changes to, e.g., divisions and removals.

*Undo* is used to undo up to 10 consecutive actions.

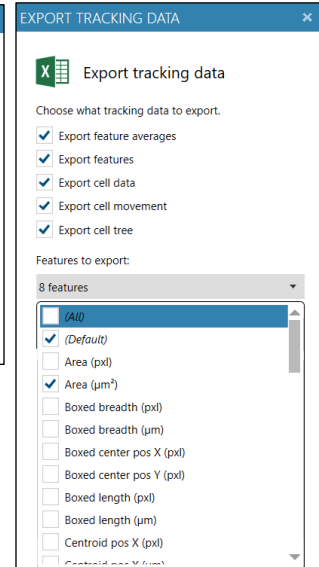
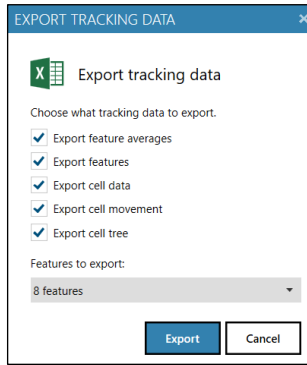


## Export

Click the Export button to export the tracking data.

Options for export include exporting only cell feature averages, all cell features, individual cell data, cell movement data and cell family trees.

There is an option to decide which features to export.



Note that only data from the cells and cell families that are checked in the Tracked Cells list will be exported.



## Save Analysis

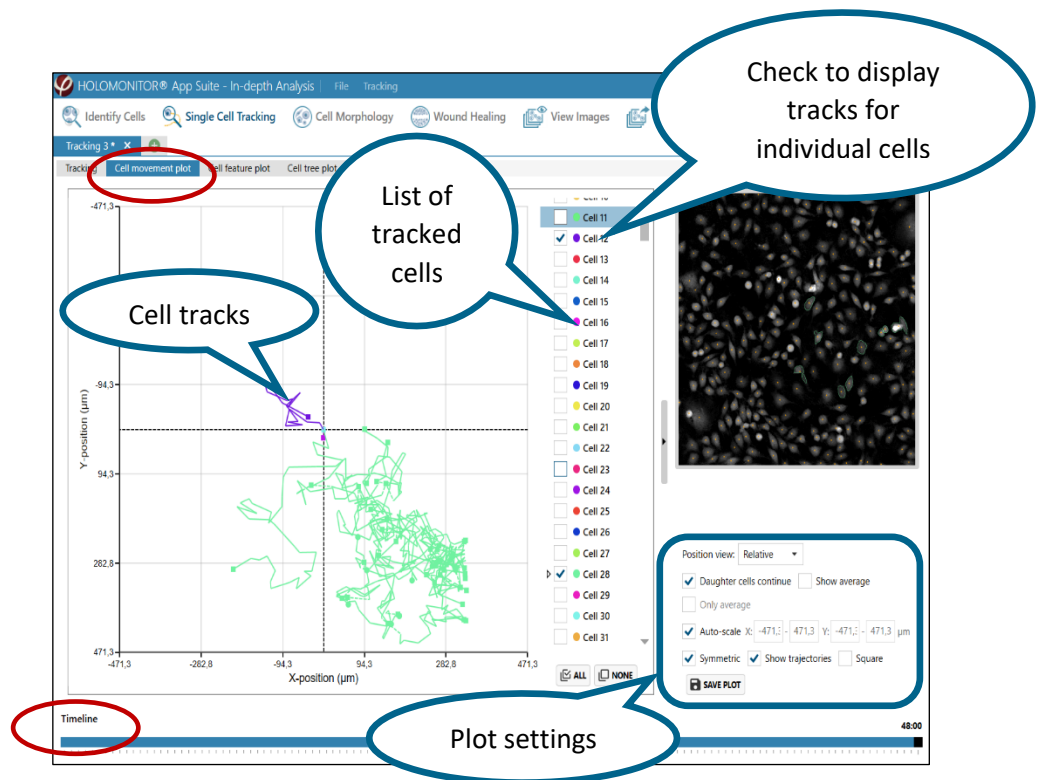
Click the Save button to save the analysis. The analysis can then be re-opened for continued use at a later timepoint.



Note that to open a saved analysis, the database data used for the analysis must be accessible to App Suite.

## Plotting cell movements

In the Cell Movement Plot tab, cell movements are given as x- and y-coordinates over time. Initially, no movement is displayed, but by checking the boxes next to the cell names, individual cell tracks or cell family tracks can be displayed.



## Plot settings

The plot settings are found below the Image view.

### Position view

The position view can be Relative or Absolute. With Relative view, the origin corresponds to the starting point (X=0, Y=0) for cell movement. With Absolute view, the cell movements are shown as in the image frame.


Position view: Relative ▾

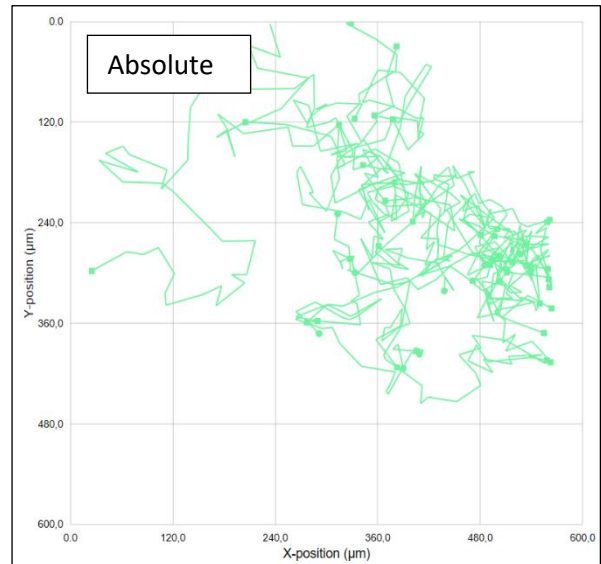
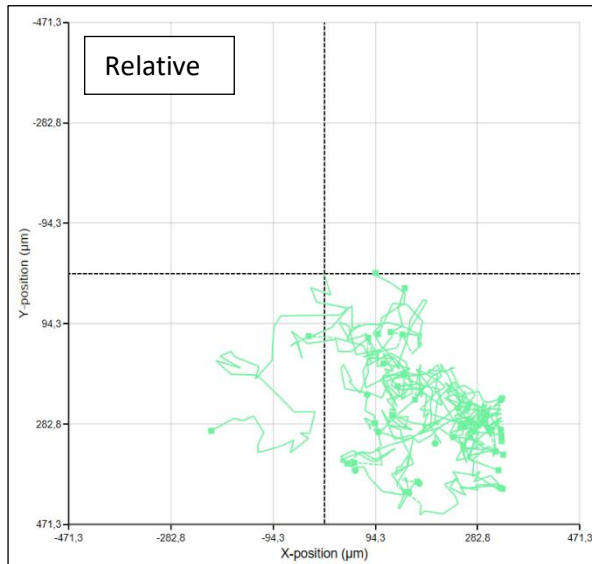
Daughter cells continue  Show average

Only average

Auto-scale X:  -  Y:  -   $\mu\text{m}$

Symmetric  Show trajectories  Square

 **SAVE PLOT**



### Daughter cells continue

With this function checked, only the tracks of the mother cell will begin at the origin. The tracks of daughter cells will be a continuation of the mother cell tracks.

### Show average

With this function checked, the average track of all selected cells will be displayed.

### Only average

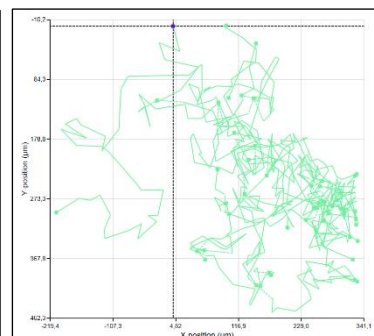
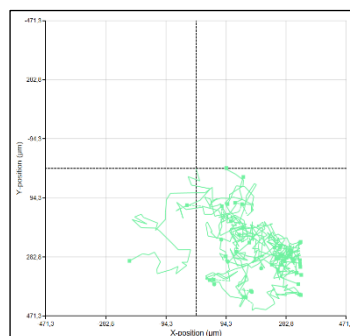
With only average checked, all other tracks will be hidden.

### Auto-scale

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.

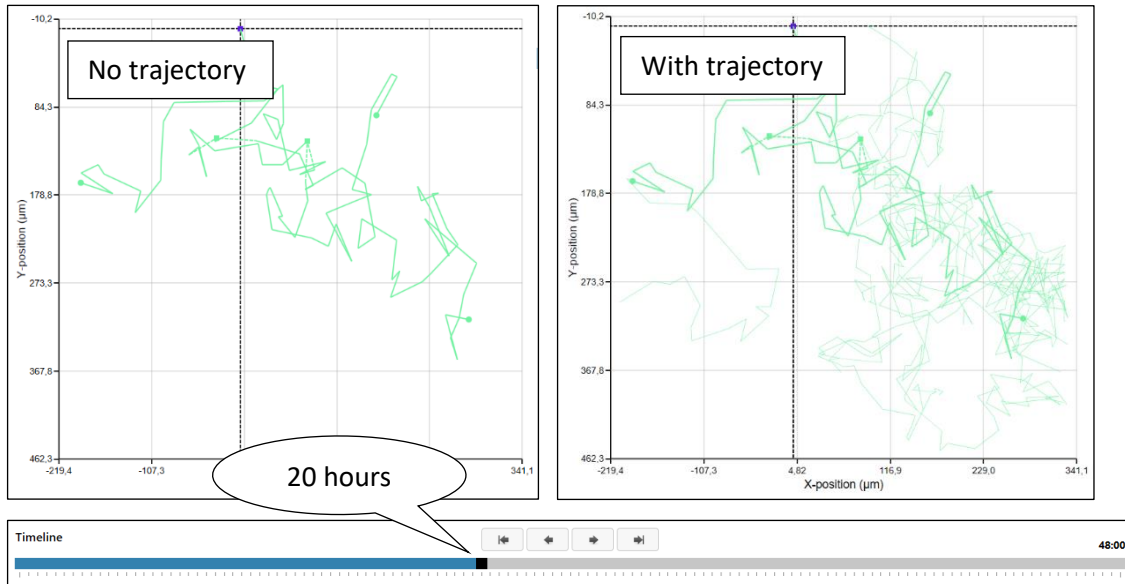
### Symmetric

With Symmetric checked, origin (X=0, Y=0) will be in the center of the plot. With Symmetric unchecked, the plot will autofit to the cell trajectory.



### Show trajectories

When sliding the time slider, the cell tracks for the time that has passed will show in a darker color than the future trajectory of the cells. In the example below, the tracks show in darker green up to 20 hours of the experiment, and thereafter the trajectories are shown in light green.



### Square

Checking Square shows a plot that is square, meaning X and Y axis min-max values are the same.

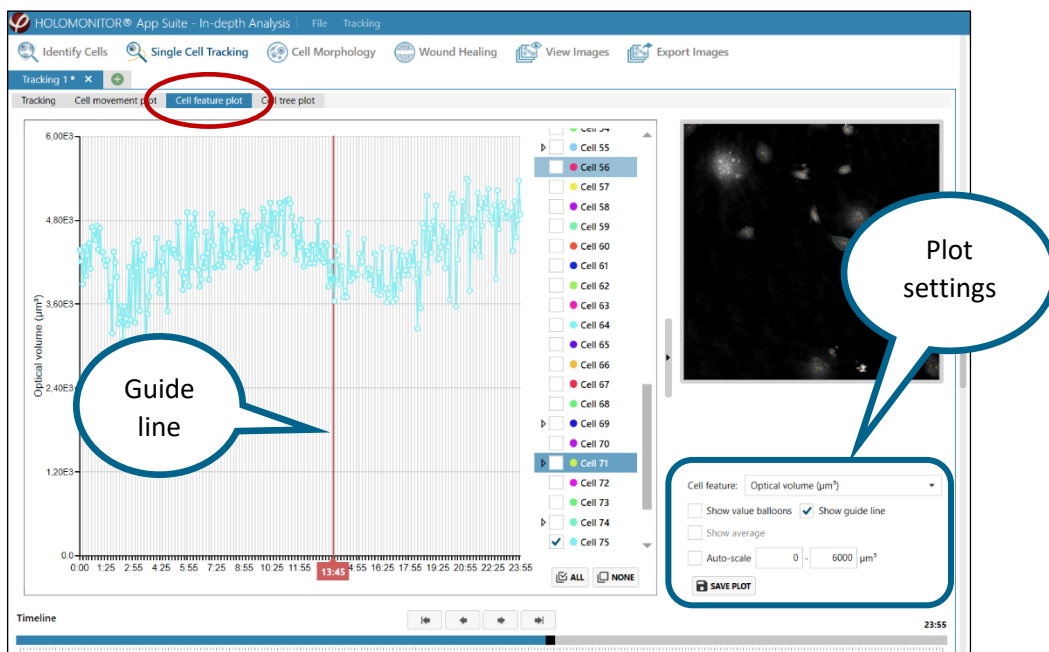
### Save plot

The plot can be saved in the .png, .jpg, bitmap, or .gif formats.

### Auto-calibration

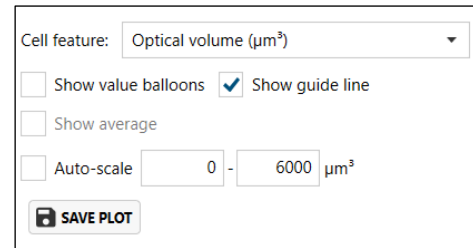
### Tracking cell morphology

Once the cell movement has been tracked, cell morphology can be followed over time by using the Cell Feature Plot tab.



## Plot settings

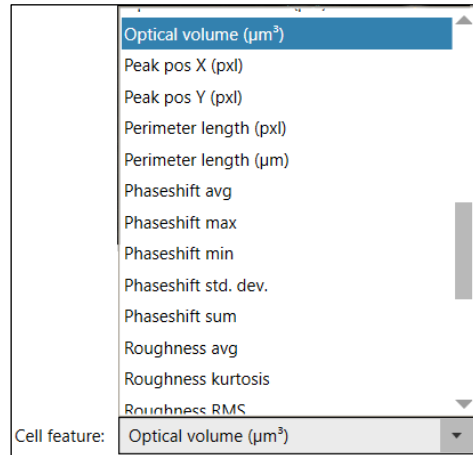
The plot settings are used to adjust the plot display.



## Cell feature

Use the Cell Feature drop down list to choose from a range of different morphological features.

For the full list of features and their descriptions, see [Fell Hittar inte referenskälla. Fell Hittar inte referenskälla.](#) in the back of the manual.



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

*Show guideline* is a vertical line that displays the current time-point.

*Show average* displays the average track of all selected cells.

*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 feature graph in the .png, .jpg, bitmap, or .gif format.

## Cell tree analysis

In the cell tree plot tab, cell trees are constructed based on the tracking of cell divisions. Other occasions are noted as well.

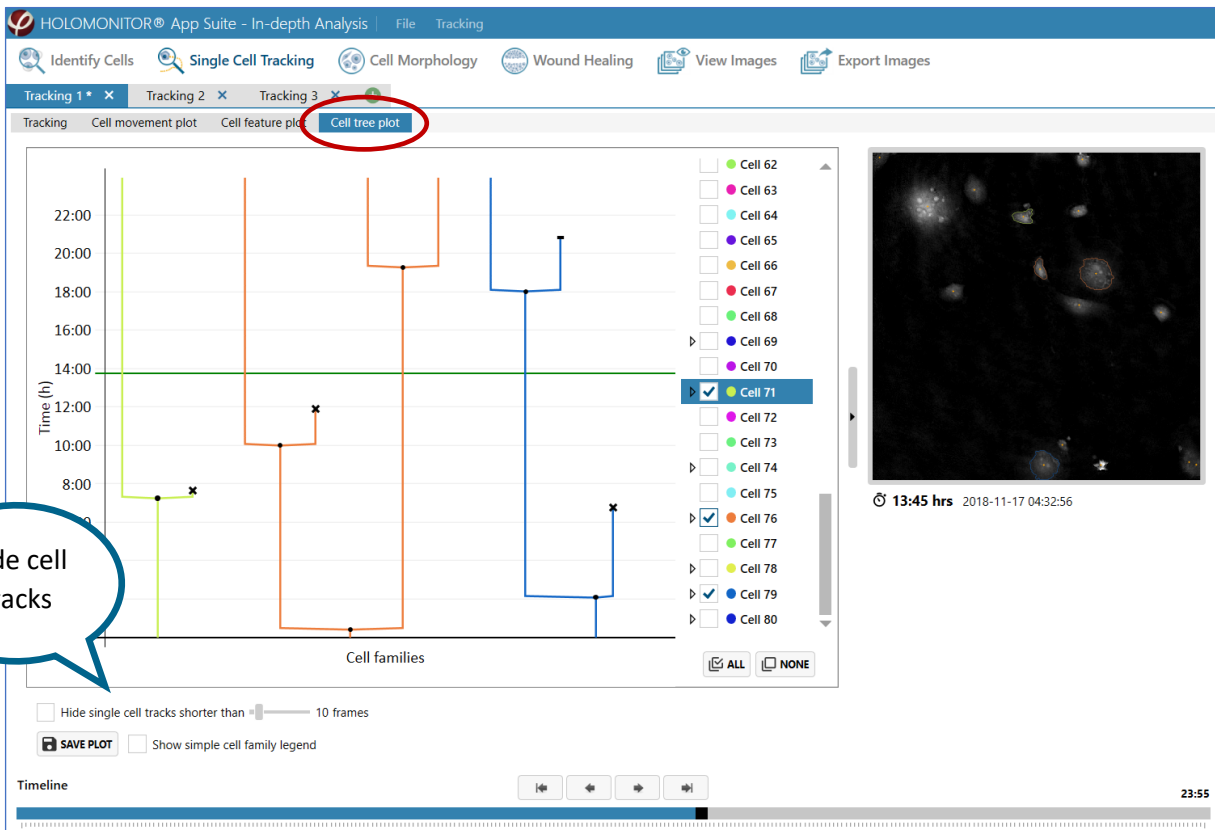
+ symbolizes that the tracking of the cell ends. May be caused by, e.g., cell death or cell engulfment.

- symbolizes that the cell is traveling out of the frame, and track is therefore discontinued


• symbolizes cell division

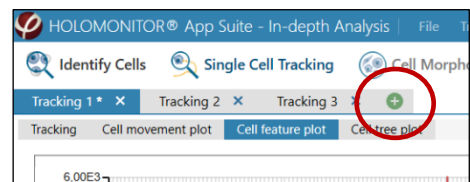
In the tracked cell list, cell families are numbered and color-coded for easy identification. When Cell-4 divided, the daughter cells were named Cell 4-1 and Cell 4-2. When Cell 4-1 divided, the daughter cells were named Cell 4-1-1 and Cell 4-1-2 and so on. All the cells in a family have the same color.

There is a possibility to hide cell tracks which are present during less than 2-100 frames. This is set by using a slider.



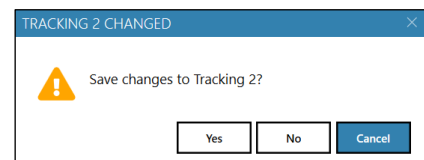
### Multiple analyses

Several time-lapses can be analyzed in parallel. Click the  button to open a tab for new analysis.

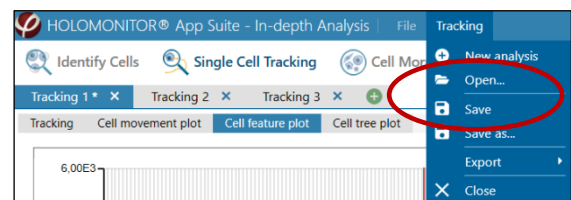


### Save or open tracking analyses

When the analysis is closed, a choice to save the changes to the tracking appears. The saved tracking can be opened as long as the database data are available.



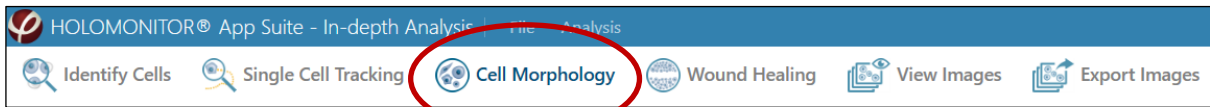
There is a Save option in the top menu as well, and here saved trackings can be opened.



Note that the analyses are saved as separate files to the computer. They are not integral parts of the software and need to be backed up separately.

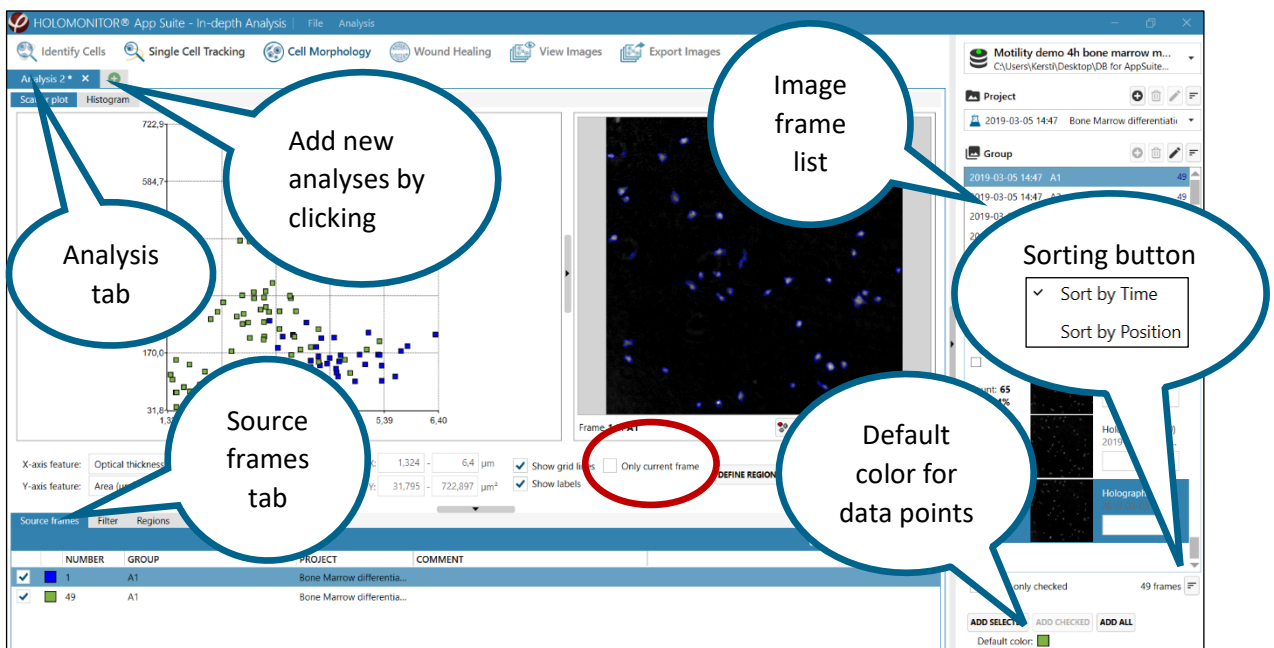
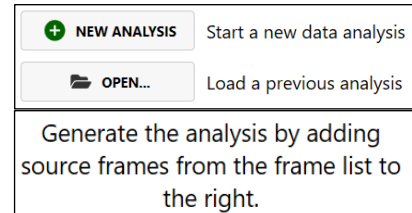
## Cell Morphology

In the Cell Morphology tab, data for both individual cells and cell populations are given as scatter plots and histograms.



### Start a new analysis

Choose the Cell Morphology tab. If an analysis has not been performed previously, a tab will appear with the choices of starting a new analysis or opening a previous. When New analysis is selected, a request to add image frames to the analysis appears.



### Add image frames to the analysis

Select a Project and a Group in the Image Frame list. Then add image frames by using one of the Add buttons, found below the Image Frame list. Added image frames are listed in the Source frames tab.

Sometimes not all images are needed to get good results, and then it is timesaving to analyze only the frames that are needed. The frames can be chosen by selecting, sorting or checking options.

#### Image selection

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.

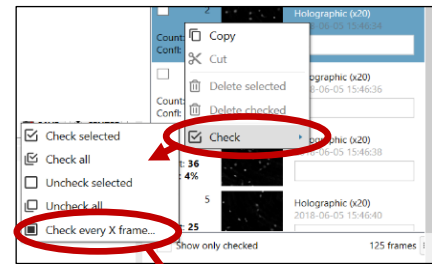
#### Image sorting

The Image Frame list can be sorted by time or position using the sorting button (see image above). This is useful for Hstudio data.



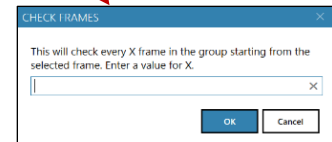
### Check options

The Check options can be used to analyze subgroups of images. Especially the Check every X frames is a very useful tool in combination with the Show Only Checked function which is found below the Image Frame list. Access the Check options by right clicking in the Image Frame list (see image to the right).



### Sorting data from the Hstudio software

An Image Frame list created in Hstudio can be sorted either based on image frame number, or using the sorting button, capture position or time of capture. The check options can also be used.




### Display data from only one frame

If several frames are added to a plot, the data from all frames will be displayed in the same plot. To display data from only one frame of many added frames, check Only Current Frame. In that case, data from the other frames is displayed when the frame is selected in the Source frame list.

### Add data from different experiments or treatments

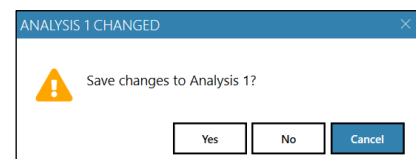
Data from different experiments and treatments can be added to the same analysis if they are stored in the same database.

### Multiple analyses in parallel

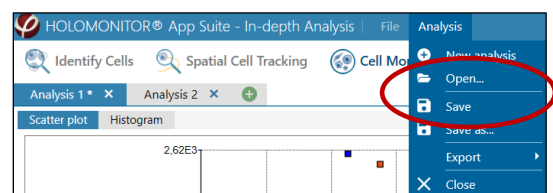
Several analyses can be performed in parallel. Click the  button to open a tab for an additional new analysis.

### Save or open morphology analyses

When the analysis tab is closed, a choice to save the changes to the analysis appears. The saved analysis can be opened as long as the database data is available.



There is a Save option in the top menu as well, and there it is possible to open saved analyses. Note that the analyses are saved as files to the computer, and are not integral parts of the software. They need to be backed up separately.

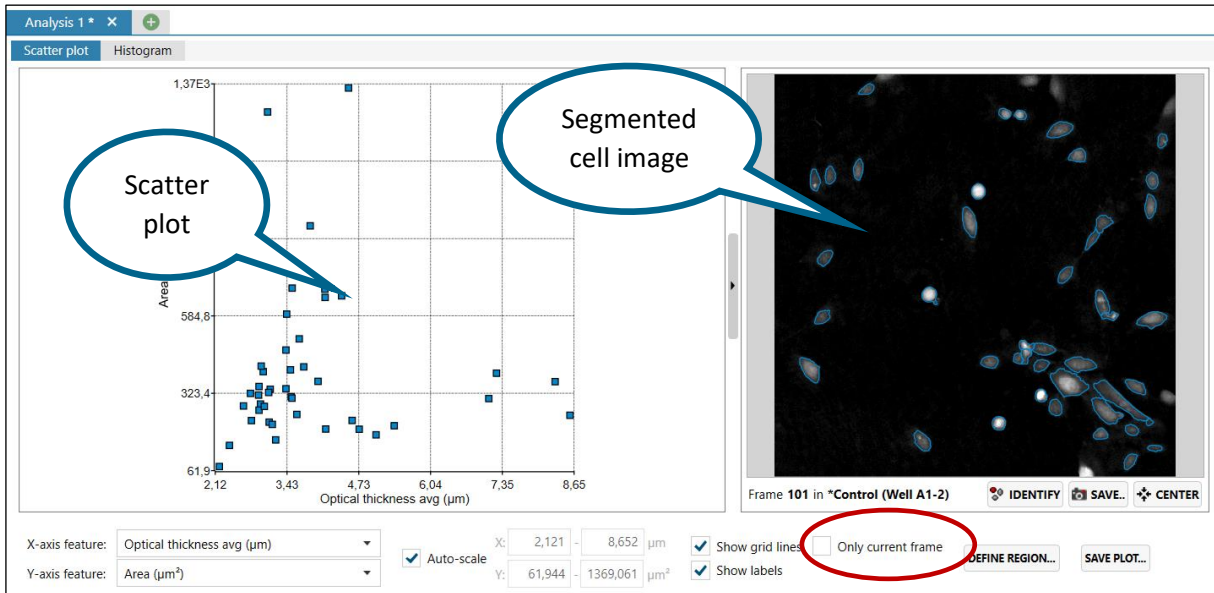




### Display results as scatter plot

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.

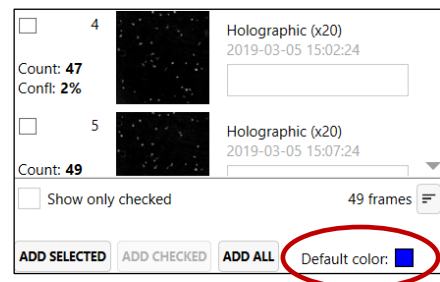
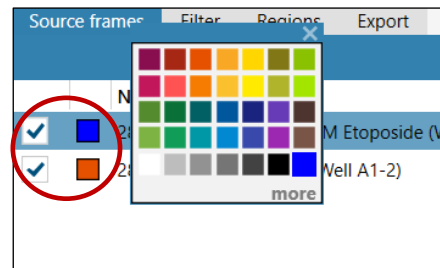
If several frames are represented in the plot, the data points from all images will be displayed in the plot simultaneously, while the frame that is selected in the Source frames tab is displayed in the viewer.



### Data point coloring

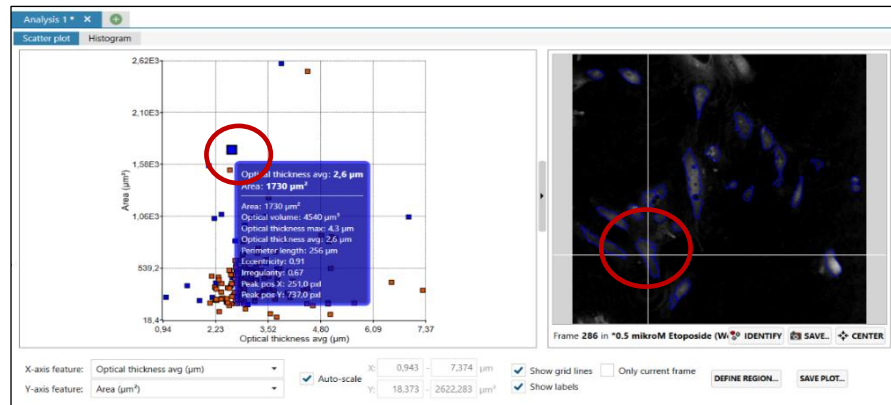
The data points will be displayed in the plot with the color shown in the color box in the Source frames tab, found below the Scatter plot tab. By default, all data points from all frames are set to be shown in blue. Thus, to separate between frames and/or time points, it is useful to change the frame color. To change the display color, left click the color box for the added frame and select a different color.

By changing the default color before adding the frames, they will have the designated color when added.

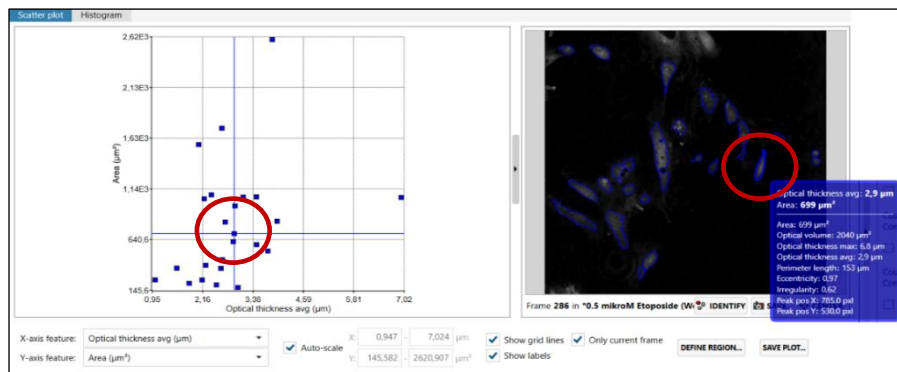


*Identify which data point corresponds to which cell*

To identify the cell that is connected to a certain data point in the scatter plot, hover with the mouse cursor over the data point. 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.

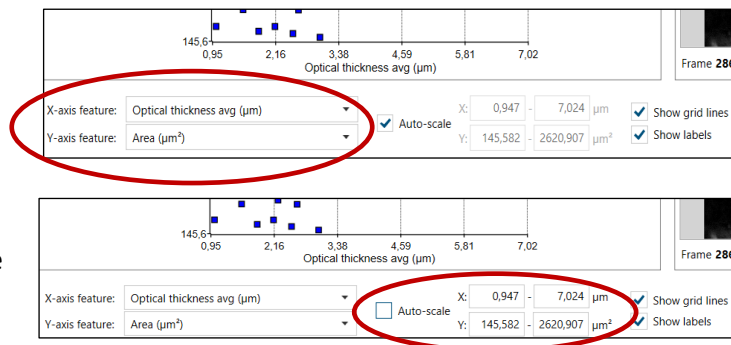


It works the other way around as well. When hovering over a certain cell in an added image frame, the data point representing that cell will be identified in the scatter plot, and data for that cell will be displayed.



*Change the scatter plot axis features*

The features shown for each axis 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 features, see the Cell Morphology Features at the back of the manual.



*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.

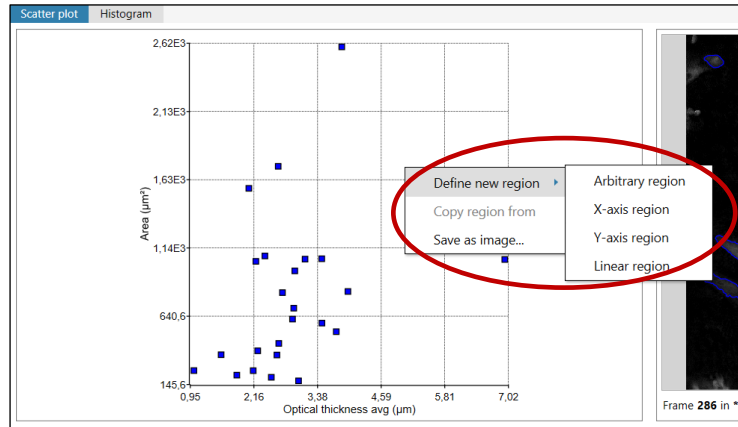
Alternatively, the scatter plot area can be changed with the mouse scroll button. Left click in the plot and scroll using the mouse scroll button to zoom in or out.

To move the plot area in the scatter plot, left click in the plot, hold and drag.

### Create plot regions

The scatter plot diagram can be divided into regions to separate out data from certain cells or cell populations, comparable to gating performed using flow cytometry. To open a region menu, right-click while hovering over the plot. 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.



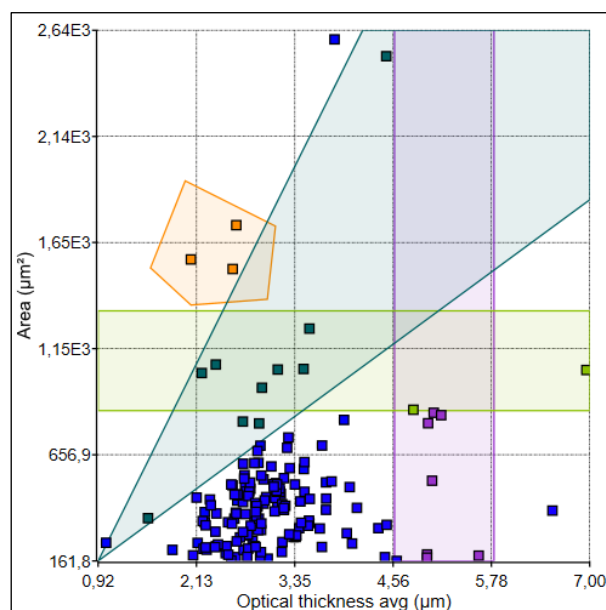
Select a region type by clicking it. There are four region types:

*X-axis region* (purple) where the x-axis values are the region cut off values. First mouse click in the plot determines the starting point and second click the end of the region.

*Y-axis region* (light green) where the y-axis values are the region cut off values. First mouse click in the plot determines the starting point and second click the end of the region.

*Linear region* (dark green) where clicking in the diagram results in straight lines that cut off the region from origin ( $X=0, Y=0$ ). First mouse click in the plot determines the starting point and second click the end of the region.

*Arbitrary region* (yellow) where repeated clicking in the diagram results in an irregular figure that outlines the region. First mouse click in the plot determines the starting point. Every further 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.



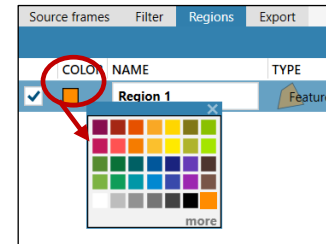
### The Regions tab

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

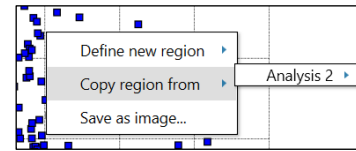
COLOR	NAME	TYPE	NO. OF CELLS COVERED	PART OF POPULATION (...)
<input checked="" type="checkbox"/>	Region 1	Feature-region	3	1,83
<input checked="" type="checkbox"/>	Region 2	Feature-region	8	4,88
<input checked="" type="checkbox"/>	Region 3	Feature-region	8	4,88
<input checked="" type="checkbox"/>	Region 4	Feature-region	10	6,10

The regions are given unique colors. Click the color box beside the region name to change the color.

Regions can be copied from one analysis to the next by using Display from other in the Regions tab or by right clicking the mouse on the scatter plot and selecting Copy Region From.



COLOR	NAME	TYPE	NO. OF CELLS COVERED	PART OF POPULATION (...)
<input type="checkbox"/>	Analysis 1 : Region 1	Feature-region	...	...
<input type="checkbox"/>	Analysis 1 : Region 2	Feature-region	...	...
<input type="checkbox"/>	Analysis 1 : Region 3	Feature-region	...	...

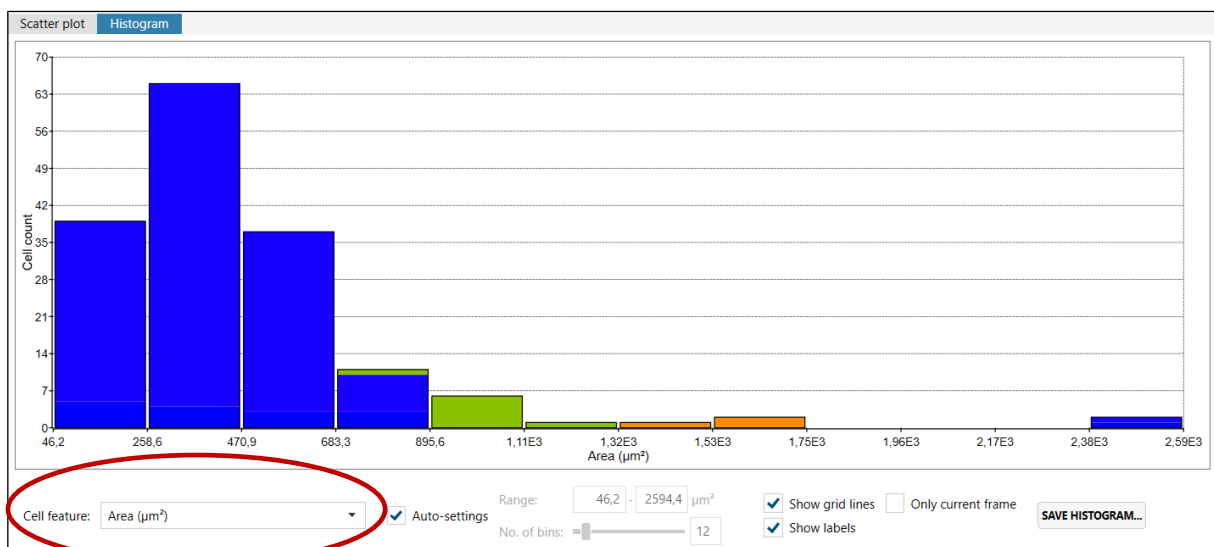


### Display results as histograms

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, or several frames with different colors, they will show up with the appropriate color in the histogram as well.

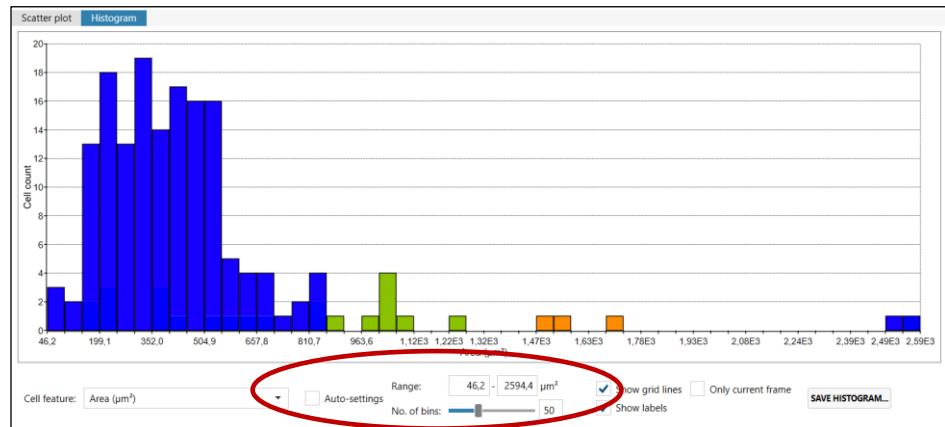
### Change the histogram axis units

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 features is found in the Cell Morphology Features at the back of the manual.



### Change the histogram x-axis intervals and bins manually

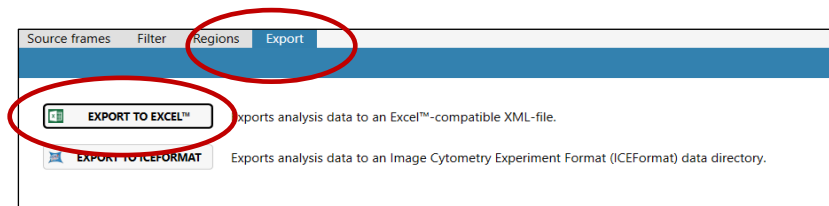
The histogram x-axis interval is set automatically to fit in all values from all added frames. To set the histogram x-axis interval manually, Auto-settings must be unchecked. Enter the minimum, maximum values and the number of bins.



### Export cell data

In the Export tab, cell data from the current plot can be exported to an Excel-file.

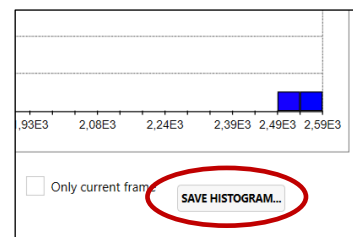
- Click one of the Export buttons to export all cell data. A file browser window will open. The file will automatically be named with date and a code, but it is possible to rename the file.
- Save the file to an appropriate place. All data relating to the image frames added to the current plot are exported.



### Save images, plots and histograms

Images of the current cell image, plot and histogram can be saved as bitmap, GIF, JPEG, PNG or TIFF by using the Save, Save Plot and Save Histogram buttons.

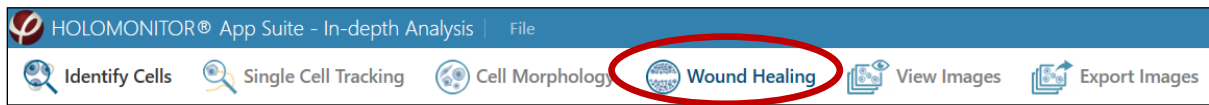
- Click the respective Save, Save plot or Save histogram button. A file browser window will then open.
- The file will automatically be named; however, it is possible to rename the file.
- Decide which image format you want (bitmap, gif, jpeg, png, tiff).
- Save the file to an appropriate place.



## Wound Healing

The Wound Healing, or scratch assay, measures gap closure in densely growing cell cultures.

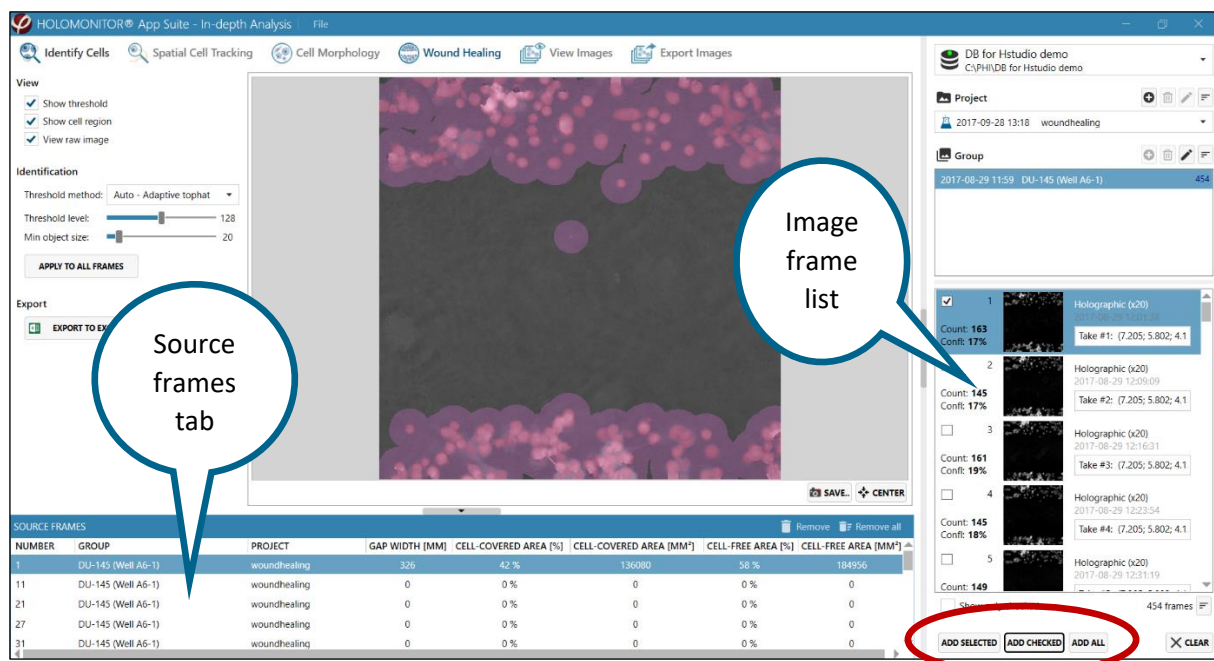
Note that there is no need to identify the cells in the frames before a Wound Healing analysis.



### Start a new analysis

Choose the Wound Healing tab. If an analysis has not been performed previously, a tab will appear with a request to add image frames to the analysis.

Begin by adding frames from the frame list to the right.



### Add images

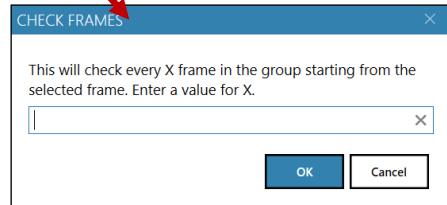
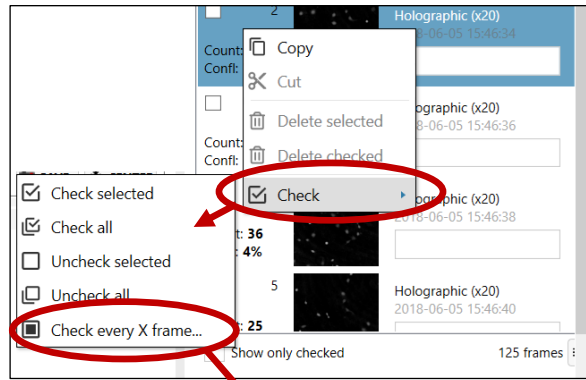
Select a Project and a Group in the Image Frame list, which is found to the right of the Viewing area. Then select image frames and transfer them to the analysis by using the Add buttons, found below the Image Frame list, or by drag and drop into the source frames list.

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.



Often, for wound healing analyses, only images captured every half hour, or every hour are needed. As an example, if images have been captured every five minutes, but only every 30 minutes (=every 6 images) are needed, use the check-functions to select every 6<sup>th</sup> image.

- Select the first of the images.
- Right click in the Image Frame list, select Check and thereafter Check every X frame. In the example above, 6 would then be entered in the Check frames window.
- Use the Add Checked function to add the checked images to the analysis.

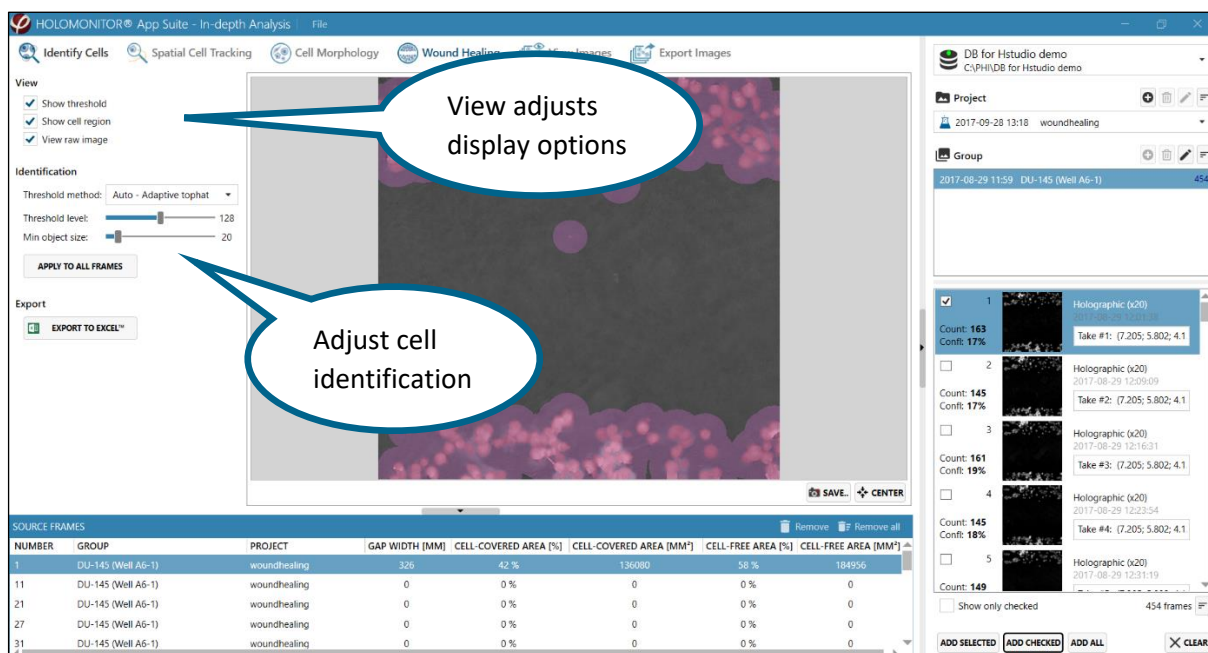


The images that are included in the analysis are listed in the Source frames tab, found below the Viewing window.

SOURCE FRAMES							
NUMBER	GROUP	PROJECT	GAP WIDTH [MM]	CELL-COVERED AREA [%]	CELL-COVERED AREA [MM <sup>2</sup> ]	CELL-FREE AREA [%]	CELL-FREE AREA [MM <sup>2</sup> ]
1	DU-145 (Well A6-1)	woundhealing	326	42 %	136080	58 %	184956
11	DU-145 (Well A6-1)	woundhealing	0	0 %	0	0 %	0
21	DU-145 (Well A6-1)	woundhealing	0	0 %	0	0 %	0
27	DU-145 (Well A6-1)	woundhealing	0	0 %	0	0 %	0
31	DU-145 (Well A6-1)	woundhealing	0	0 %	0	0 %	0

### Masking the gap

When the image frames have been added to the analysis, App Suite will suggest a mask that covers the cells but leaves the gap free. This mask is used to determine the cell free area. If the suggested mask is suitable, click Apply to All Frames to activate the identification.



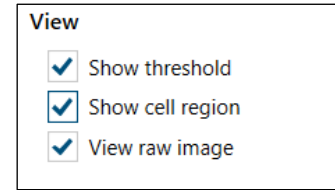
### Adjust the display options

Use the View panel to adjust the display options.

*Show threshold* displays the identification of cells.

*Show cell region* displays the mask that covers the cells

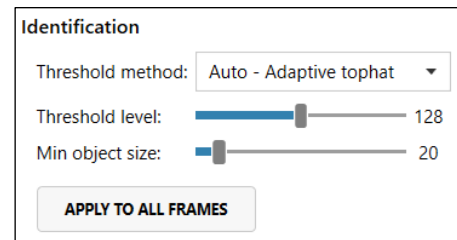
*View raw image* removes all colorsets and other image adjustments.



### Adjust the cell identification

App Suite automatically suggests a cell identification using the Adaptive Tophat threshold method. If the cell identification is not adequate, adjust the threshold method or level or minimum object size.

There are several threshold methods. The threshold setting determines which objects will be identified as cells in the image. The different threshold methods will result in slightly different thresholds. It is advisable to try out several methods to find the one that works best for every type of cell sample. The method that suits the sample best is the correct method to use.



*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 tophat* uses morphological filtering prior to thresholding to amplify cell-alike objects. It is the default method.

*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.

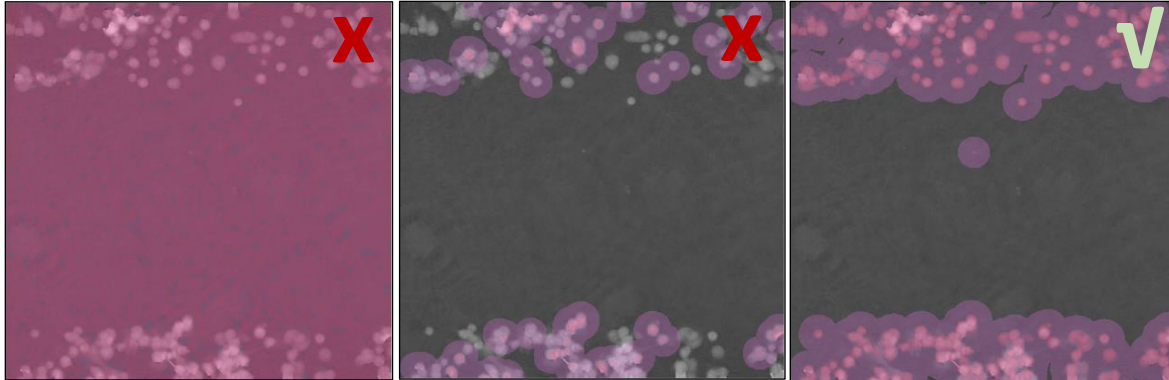
*Double adaptive mean* is the same as Double Otsu, but with two adaptive mean threshold masks.

*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 using the sliders in the Identification panel. The threshold 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. The slider values do not have a unit, but they are related to the diameter of the largest circle that can fit into the cell.

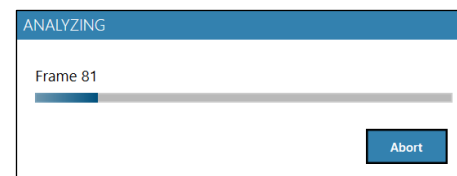
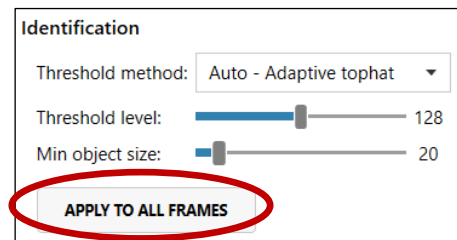


When the sliders are used, the threshold actively shows the boundaries of the cells. The images below show a too low threshold which includes too much background (left), a too high threshold which excludes too many cells (middle) and a correct threshold setting (right).

When the thresholding is satisfying, click Apply to all frames. The software will then apply the threshold and analyze the gap in all added frames. This might take several minutes, depending on the number of source frames.

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

1. Determine which threshold calculation method is optimal for the current experiment.
2. If needed, use the Threshold Level slider to adjust the threshold setting.
3. If needed, use the Min Object Size slider to ensure that every cell is included in the analysis.
4. Click Apply to All Frames.



### Results

The results include gap width, cell covered area and cell free area and are presented for each added frame in the Source frames list.

Note that sometimes there is a disturbance in the images, which will cause incorrect data. After applying the threshold, look at the Cell-covered area (%) in the source frames list to ensure that the applied threshold is correct for all images. The percentage covered area should not show a big deviation, but instead it should increase steadily. If there is any exception, it indicates an incorrect

threshold for that image. Select the deviating image, and then correct the threshold setting for that image.

NUMBER	GROUP	PROJECT	GAP WIDTH [MM]	CELL COVERED AREA [%]	CELL-COVERED AREA [MM <sup>2</sup> ]	CELL-FREE AREA [%]	CELL-FREE AREA [MM <sup>2</sup> ]
1	DU-145 (Well A6-1)	woundhealing	326	42 %	136080	58 %	184956
11	DU-145 (Well A6-1)	woundhealing	334	41 %	131707	59 %	189328
21	DU-145 (Well A6-1)	woundhealing	321	43 %	138946	57 %	182089
27	DU-145 (Well A6-1)	woundhealing	317	44 %	141211	56 %	179825
31	DU-145 (Well A6-1)	woundhealing	311	45 %	144553	55 %	176442

### Export data to Excel

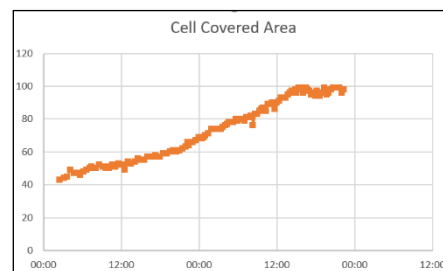
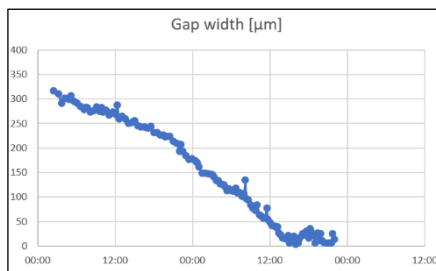
Use the Export button to export the data to Excel. In the resulting xml file, gap width in  $\mu\text{m}$ , as well as cell covered area and cell free area, in % and  $\mu\text{m}^2$ , are given.



As the time of the experiment is given, and not the duration of the experiment in hours, the time needs to be converted before the result can be displayed as diagrams with gap closure, cell covered area or cell free area over time. By using Excel functions, a column with time points from start can be created.

- Add a column to the right of Capture time.
- Format the column as Time.
- Type the formula as shown in the figure.
- Create diagrams to visualize the gap width, cell covered area or cell free area over time.

	A	B	C	D
1	Frame Number	CaptureTime		Group
2		1 2017-08-29 12:01:38		00:00 DU-145 (Well A6-1)
3		3 2017-08-29 12:16:31		00:15 DU-145 (Well A6-1)
4		5 2017-08-29 12:31:19	=B4-B\$2	00:30 DU-145 (Well A6-1)
5		7 2017-08-29 12:46:04		00:45 DU-145 (Well A6-1)
6		9 2017-08-29 13:00:47		00:59 DU-145 (Well A6-1)

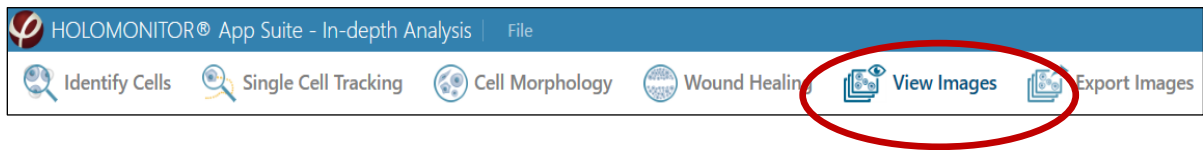


### Cell Front Velocity

Calculate the speed of the cell front by subtracting the last value for gap width in the linear phase from the first value for gap width within the linear phase and divide with the number of hours between the values. In this example:  $(321-25) \mu\text{m}/36 \text{ h} = 8 \mu\text{m}/\text{h}$ . This can also be performed in the Excel data sheet. Note that it is important to use only data from the linear phase of the slope.

## View Images

In View Images, all captured images can be viewed and colored and the software focus can be adjusted. Note that the coloring will only be applied in In Depth.

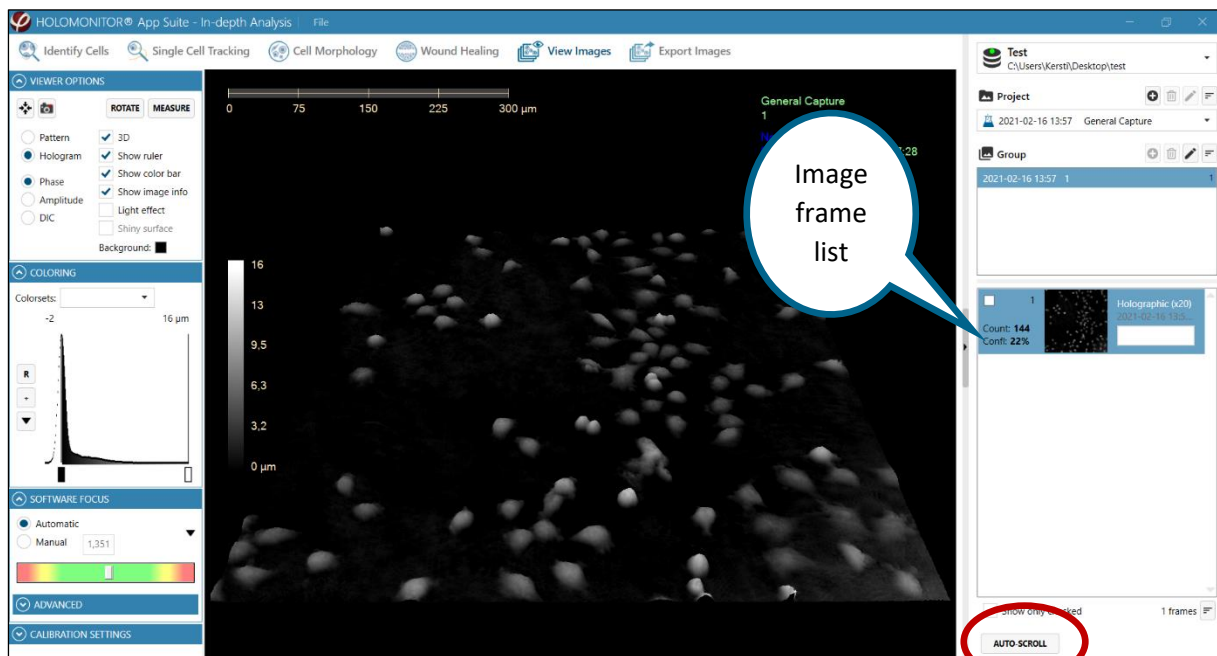


## View an image

Select a Project and a Group in the Image Frame list, found to the right of the Viewing window. An Image Frame list for the selected group will appear. Images, as well as information relating 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.



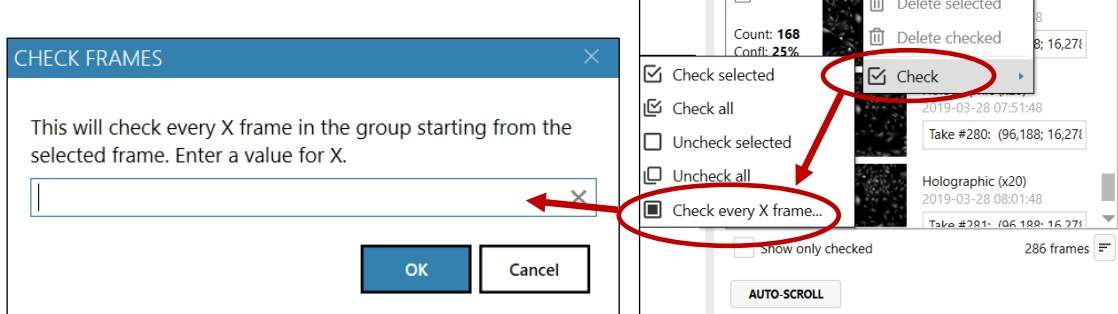
## For Hstudio images, view one position of several in parallel time-lapses

Images captured with the older software Hstudio are often saved in the same Group although they are captured at different positions. 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 captured, 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.





For Hstudio images, view all positions in a group as sequential time-lapses

Use the sorting button, which is found below the image frame list, to sort the images after position. The images will then be displayed as consecutive time lapses when Auto-scroll is active.

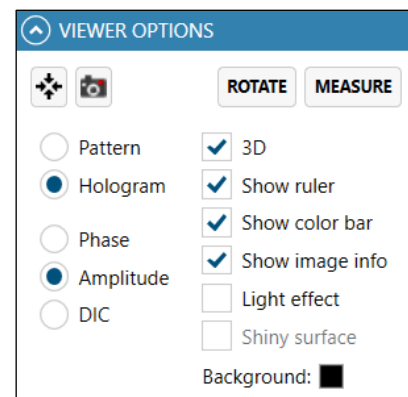
Use Viewer Options to change image display

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

Click  to center the image in the View area.

Click  to save the currently viewed image.

Click Rotate to automatically rotate the image.



Checking the boxes will activate different options. Most boxes can be checked in combination.

*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 image, the bright-field image or a DIC image can be displayed:

*Phase* displays the phase image.

*Amplitude* displays bright-field image.

*DIC* displays a differential interference contrast (DIC) image, also known as Nomarski interference contrast image, created from the interference pattern. Adjust the Image histogram in the Coloring panel to display the image with optimal image dynamics.

*3D* displays the holographic image in 3D.

*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 active when Light effect is checked.


*Background* changes the background color of the image.

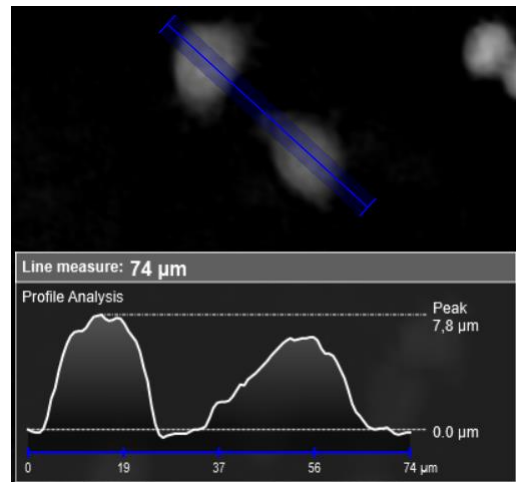
### The Measure function

The Measure button activates the line measuring function, a simple tool for interactively measuring distances and cells.

Once the tool is activated, 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.

It is possible to move and flip the image while the measuring function is activated.

The data can be saved as an image with the  button.



When the Measure button is clicked again, the measuring function is deactivated.

### Move, flip or zoom the cell image

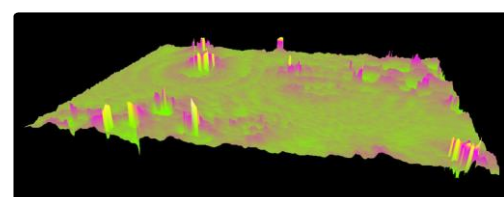
To zoom the cell image, move the mouse cursor to the view area, click and scroll using the scroll button on the mouse.

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 this example, recalculating the software focus may restore focus.



### Recalculate focus manually


Activate Manual in the Software focus side panel.

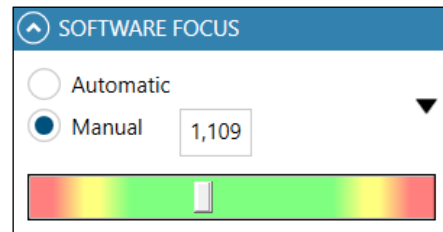
The software focal distance in mm can then be changed either by entering a value manually or by using the slider beneath the text box.

To find a starting value, choose an image that was captured at the same time or immediately before or after and that is well focused. 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 by entering the new focus value from the well-focused image in the text box, or by using the slider to set the value.

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.

Note that the automatic focus will reset the focus to the focus settings that were applied when the image was captured. They may not be the optimal focus settings.

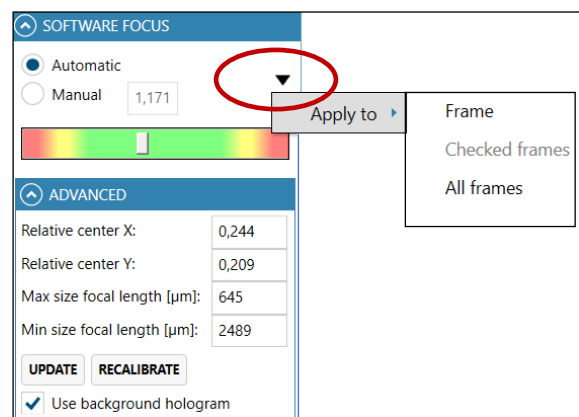
### Advanced

Advanced functions are found below the Software focus slider. The settings are needed for service purposes.

*Update* adjusts the current image display using the set values.

*Recalibrate* calculates new values based on the image.

Note that the new image calculations are not automatically saved. Click the black arrow button to apply the values to the image(s).



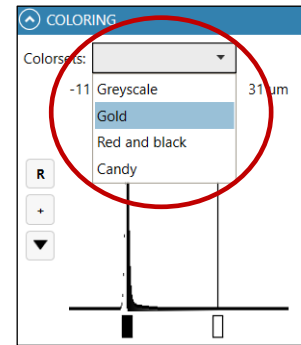
## Change image coloring

All images in In Depth 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.

Saved *Colorsets* can be used with the current image by selecting a colorset in the drop-down list which is found at the top of the Coloring panel.

Additional functions are found by clicking the buttons in the Coloring panel.



*The R button* rescales the coloring to better utilize the dynamic range of the image. This button needs to be operated every time the image coloring is mis-adjusted.

*The + button* adds new color to a Colorset. A colored triangle representing the new color which 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.

*Save a new Colorset* with the current color setting by left-clicking the button and choose Save as.

*Save the current color settings to a previously saved Colorset* by left clicking the button and choose Save. Note that this will overwrite the settings previously saved.

*Delete a Colorset* by selecting it in the drop-down Colorset list. Then left click the button and select Delete colorset.



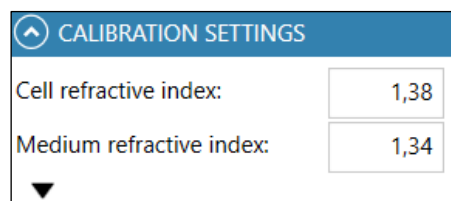
Note that colorsets are stored with the databases. Thus, if a colorset is created in one database, it is not found in a different database, even on the same computer.



Note that no colorsets carry over from the App Suite assays to In Depth and vice versa.

## Change default cell and medium refractive index

As described in Analysis Features in the back of the manual, App Suite uses the difference in refractive index between the cells and the medium to calculate cell thickness in  $\mu\text{m}$ . The default refractive index values can be changed in the Calibration side panel.

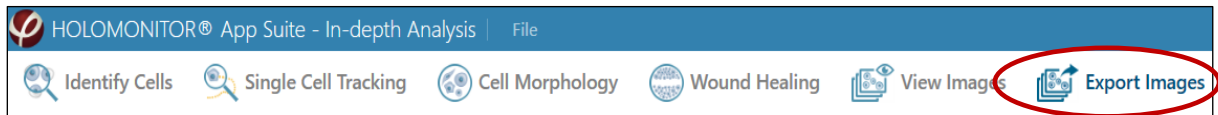


Standard cell culture medium has a refractive index of 1.34, while a normal cultured cell has an average refractive index of 1.38. Use the black arrowhead to apply the refractive index changes to the images, otherwise the changes in refractive index will not be saved.



## Export Images

In the Export images tab, both individual image frames and sequences can be exported as opposed to the image export function in the guided assays, which only allows all images in a position to be exported together. The image display can be edited and images can be exported either as individual images or as movies in several standard formats.



Images intended for export must first be added to the viewing area. Then colors and view angles must be adjusted, and thereafter the images and movies can be exported.

### Add and remove image frames

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.

#### Add selected

By clicking the Add selected button, found below the Image Frame list, a single selected image frame or several selected frames can be added.

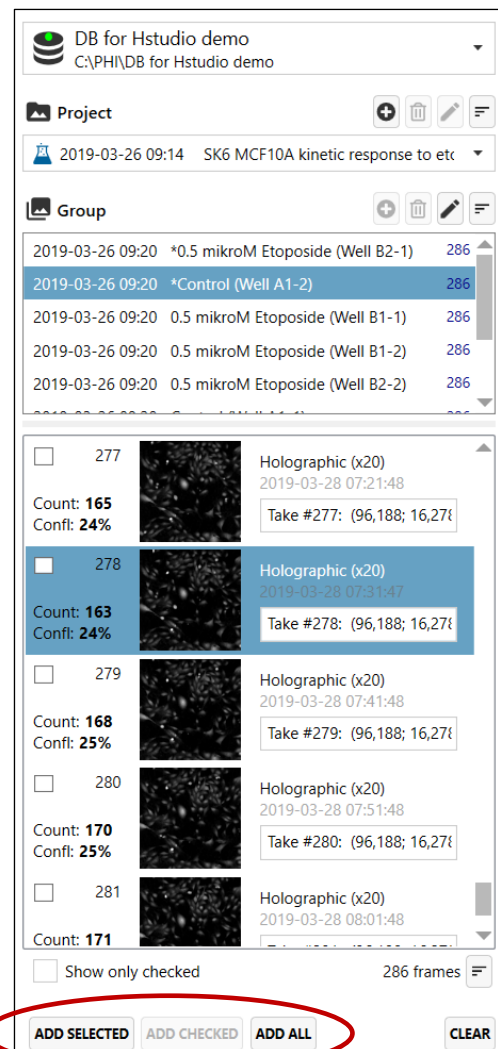
The shift-key can be used to select several consecutive images and the Ctrl-key to select non-consecutive images.

#### Add checked

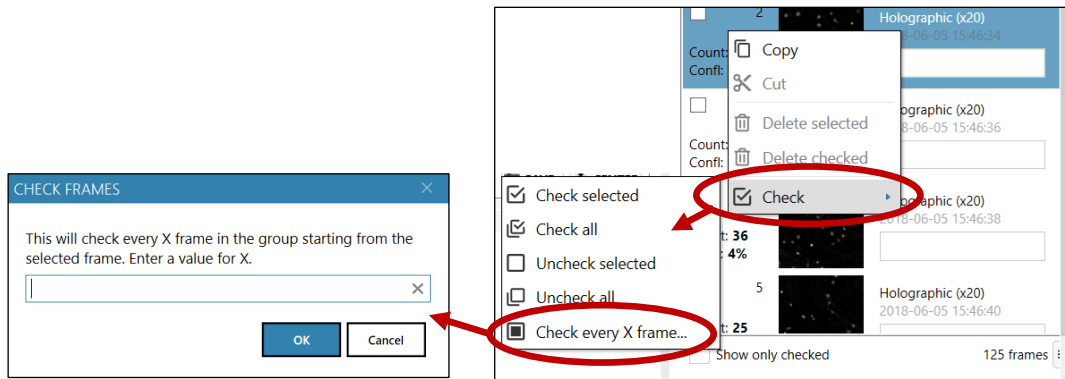
Images can also be selected by checking the box to the left of each image, and then clicking the Add checked button.

Often not every image is required. The check-functions can be used to select images if for example images have been captured every five minutes, but only every 30 minutes are needed.

- Select the first of the images.
- Right click in the Image Frame list, select Check and thereafter Check every X frame. In the example above, 6 would then be entered in the Check frames window (see image below).
- Use the Add Checked function to add the checked images to the analysis.







### Add all

All image frames can be added by clicking Add All.

### Drag and drop

As an alternative, images can be dragged and dropped into the Source Frames window.

### Source frames

The images that are included in the analysis are shown in the Source frames tab, below the View Area.

Use the Remove buttons to remove either the selected or all the added images.

### View area

The View Area contains a view of the currently active added image as well as thumbnails of all the added images. By clicking an image thumbnail, it will be displayed in the View area.



## Adjust image display

### Viewer options

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

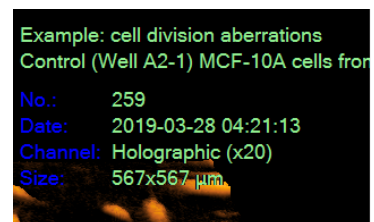
*3D* displays the image as a 3D representation.

*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:

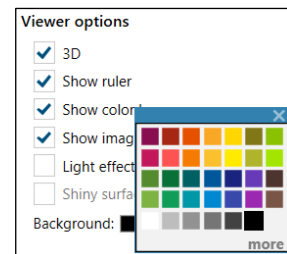
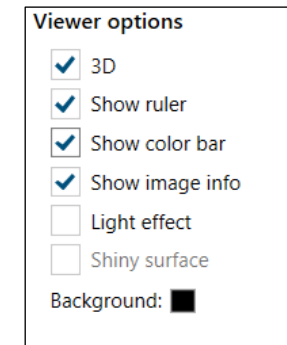
- First row specifies in which Project the image is located.
- Second row specifies in which Group the image is located.
- No. specifies which number the image has in the group.
- Date specifies the capture date and time of the image.
- Channel specifies the image type (holographic) and objective magnification.
- Size specifies image width and height in  $\mu\text{m}$ .



*Light Effect* applies an artificial light source to the image which may sometimes render an improved image, showing more details.

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

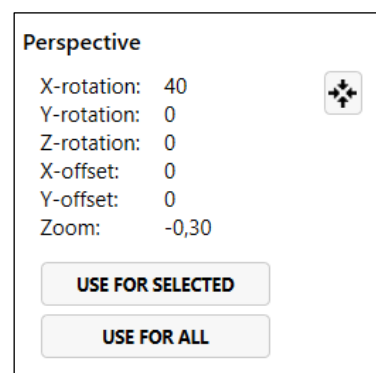
*Background* color is by default set to black. Left-click the Background color box to select a new color.



### Zoom, move or flip the image

The Perspective side panel shows how the image has been moved, flipped or zoomed. In order to use the same perspective for other exports, the values have to be identical.

- 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.

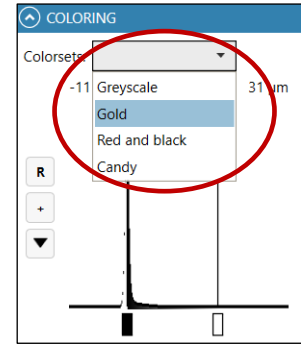


### Change image coloring

All images in In Depth originally appear in gray scale. 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.

A set of colors that are saved together is called a Colorset.

Saved *Colorsets* can be used with the current image by selecting a colorset in the drop-down list which is found at the top of the Coloring panel.



Additional functions are found by clicking the buttons in the Coloring panel.

*The R button* rescales the coloring to better utilize the dynamic range of the image. This button needs to be operated every time the image coloring is mis-adjusted.

*The + button* adds new color to a Colorset. 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.

*Save a new Colorset* with the current color setting by left-clicking the button and choose Save as.

*Save the current color settings to a previously saved Colorset* by left-clicking the button and choose Save. Note that this will overwrite the settings previously saved.

*Delete a Colorset* by selecting it in the drop-down Colorset list. Then left click the button and select Delete colorset.



Note that colorsets are stored with the databases. Thus, if a colorset is created in one database, it is not found in a different database, even on the same computer.

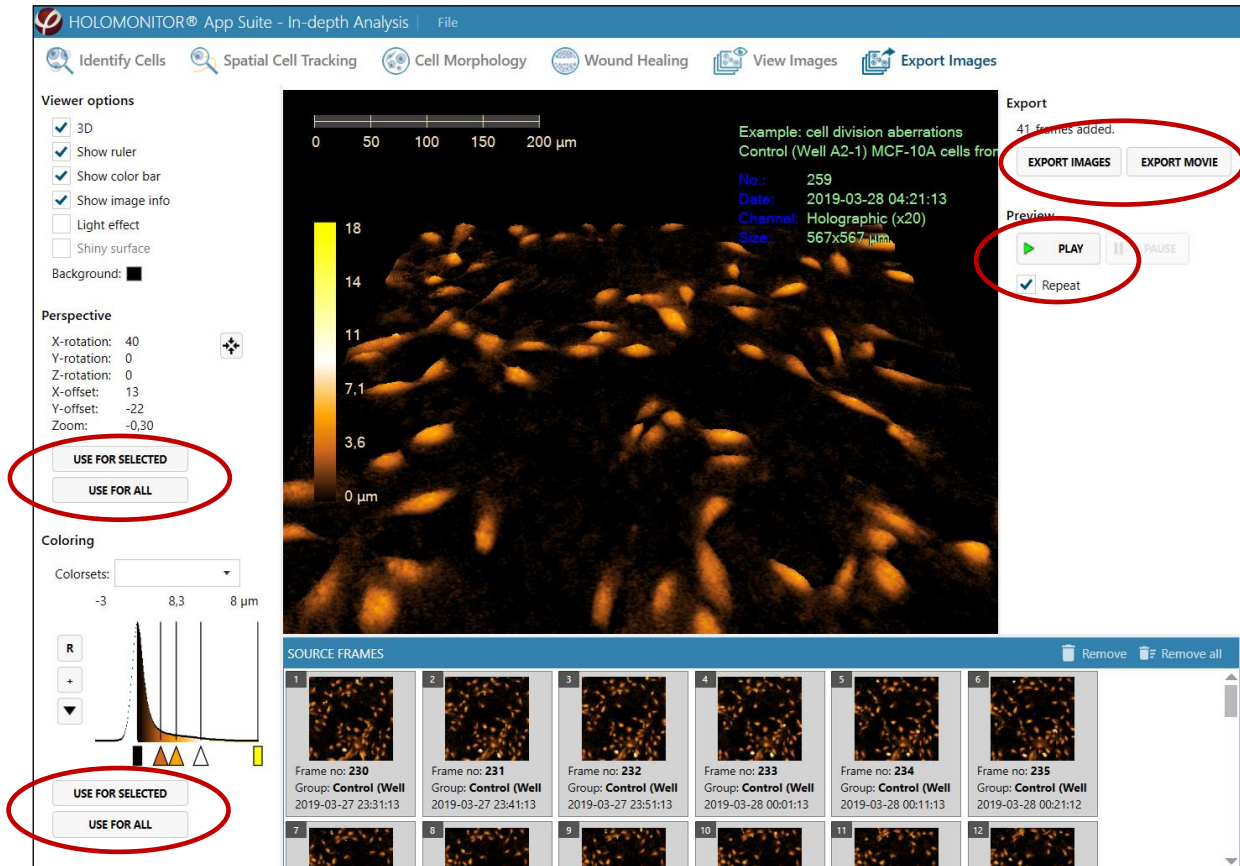


Note that no colorsets carry over from the App Suite assays to In Depth and vice versa.

### Create a movie

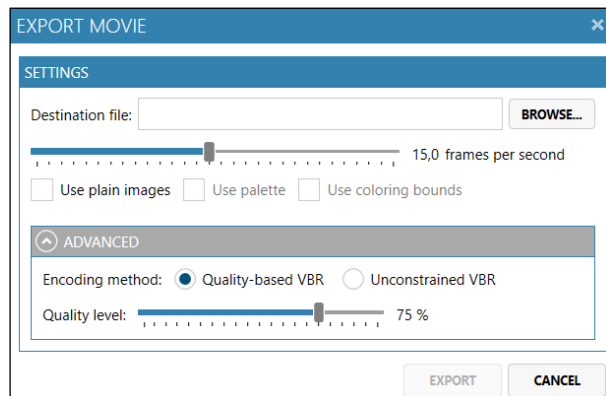
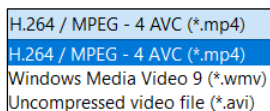
When image display, perspective and color look good, click the Use for all buttons in both the Perspective panel and the Coloring panel.

Preview the movie by clicking the preview Play button. Note that the preview speed of the movie is not the same as for the saved movie, as the speed of the movie can be adjusted before export.



If the preview is satisfactory, click the Export Movie button to open the Export Movie window.

- Select the destination folder by browsing.
- Select video format.

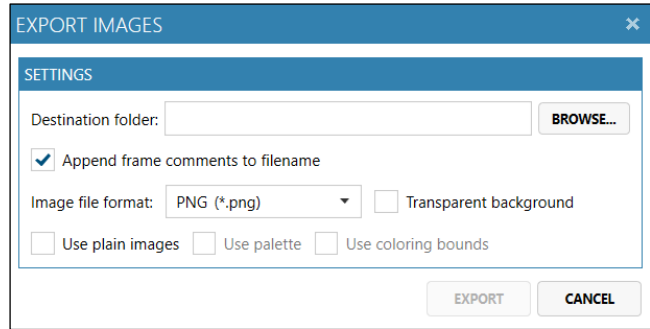


- Move the slide bar to set the number of frames per second that will be shown in the movie.
- If desired, use Advanced settings to adjust the image quality specifications.
  - Quality based VBR: The quality and size of the exported movie file depends on a user-set target quality level for the movie,
  - Unconstrained VBR: The quality and size of the exported movie file depends on a target bitrate (amount of data per second) set by the user.
- When ready, click Export.

## Export images

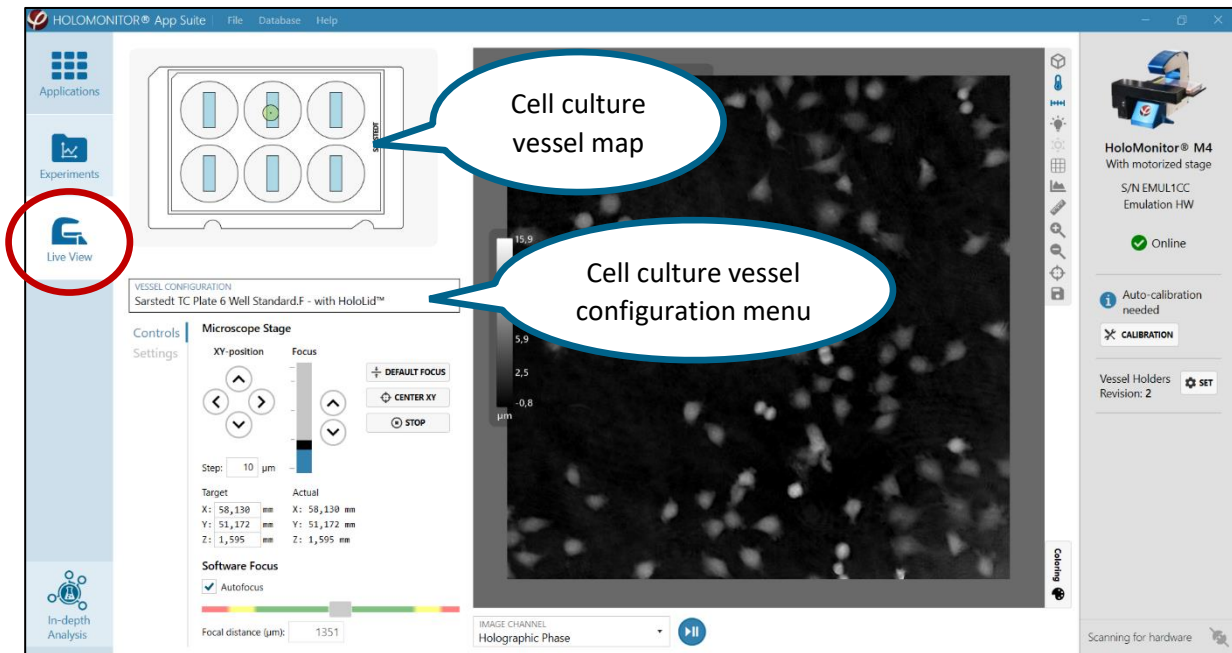
When image display, perspective and color look good, click the Use for all buttons in both the Perspective panel and the Coloring panel. Then click the Export Images button to open the Export Images window.

- Select the destination folder by browsing.
- Check the box to add frame comments to the file name.
- Select the Image file format in the drop-down menu.
- Check Use plain Images to remove any coloring or 3D displayed in the exported image.
- Check Transparent background to export TIFF-images with a transparent background.
- When ready, click Export.



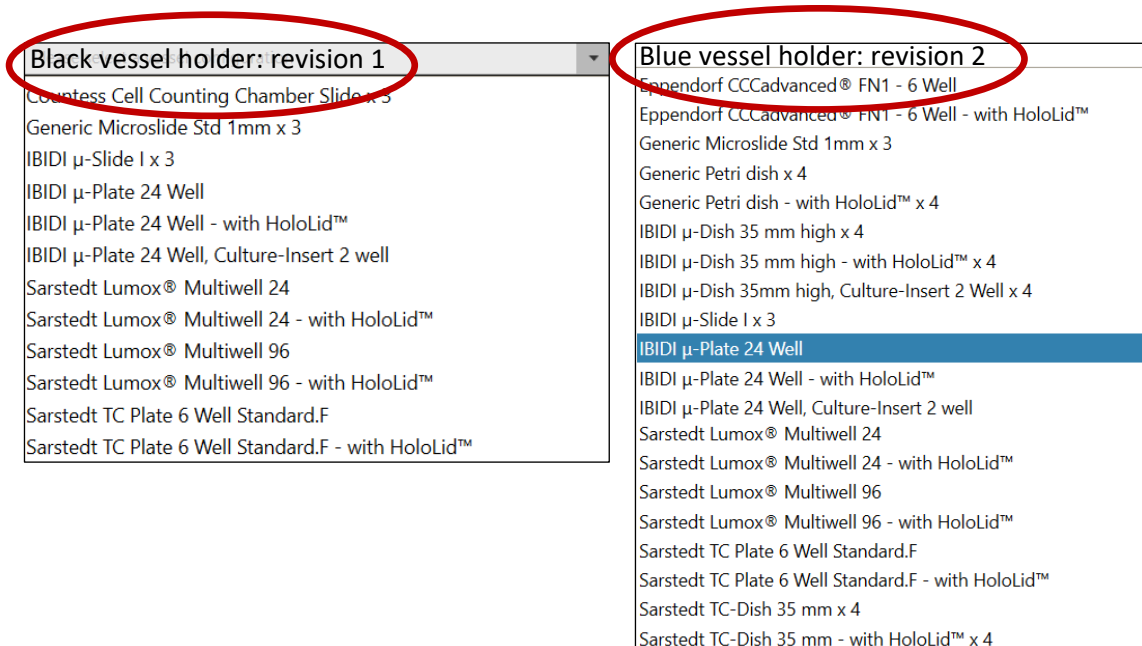
## Live View

Live View allows the user to study cell samples without setting up an experiment, thus allowing the HoloMonitor to function as a microscope. For this function to be active, an instrument needs to be connected and an active license is needed.



## Cell culture vessels

In the upper left corner of the Live View Window, a cell culture vessel map is displayed. Select the correct map using the Vessel Configuration dropdown menu. The following vessels, depending on the vessel holder, are supported:

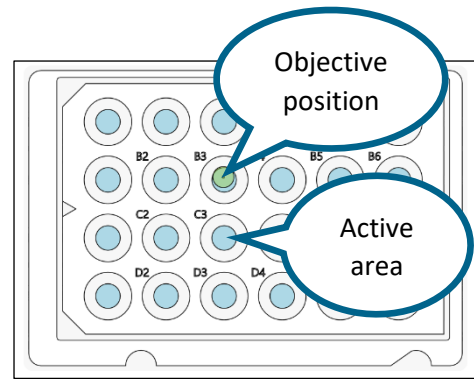




### Moving the motorized stage

The motorized stage can be moved to another position by left-clicking any active area in the vessel map. The blue regions in the pattern indicate the active areas in each well. The green marker corresponds to the objective position.

The stage can be moved in very small steps by using the XY-position arrow buttons in the Microscope Stage control. Adjust the step length in the Step text box.



**Note that the step length applies to all directions of stage movement: X, Y and Z. Too big steps can crash the objective into the sample.**

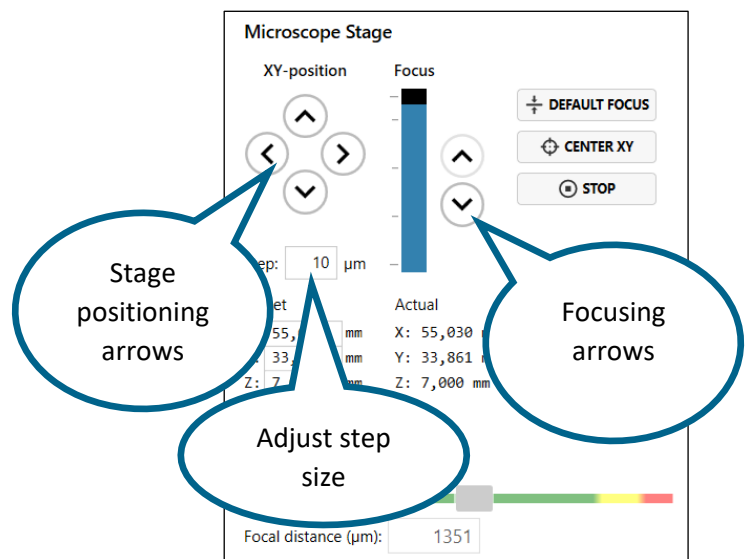
Coordinates of the current position as well as target coordinates are given below the arrow buttons. The target coordinates can be entered manually.

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

Clicking the Center XY button will move the stage to the center position of the vessel.

Clicking the Stop button will immediately stop all stage movement.

Click the Default Focus button to replace the current focus with the pre-set focus settings



### Focus

#### Focusing in theory

The software has pre-set focus settings for each vessel type which are activated with the Default Focus button. This was calibrated when the HoloMonitor was installed, but sometimes focus needs to be adjusted by the user.

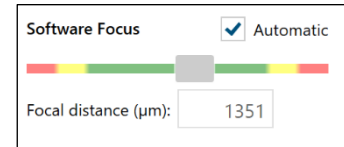
HoloMonitor has both a coarse mechanical and a fine software focus. They need to cooperate in order for the image to be in focus. With the mechanical focus, *i.e.* when moving the motorized stage, the user moves the cells to the approximately correct distance from the objective. The software focus then finds and calculates the cell images. The Software Focus indicator shows how well the software is able to focus an image at the current mechanical focus. The software can only find focus when the cell culture vessel is positioned at approximately the correct distance from the objective.

### Focusing in practice

The black box in the Focus bar represents the motorized stage. When that black box is moved up or down, the motorized stage moves up and down. Clicking the two arrows results in smaller stage movements up or down. The step size is set by entering the step length in the Step text box.



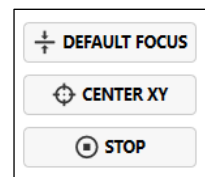
For good focus, the grey marker in the Software Focus indicator should be positioned within the green area, preferably in the middle of the green area. If the grey marker is too far to either the left or the right, move the motorized stage as described above in order to shift the marker to the middle.



Click the Default Focus button to replace the current focus with the pre-set focus settings.

Click the Center XY button to reset the image view.

Click the Stop button to immediately stop stage movement.

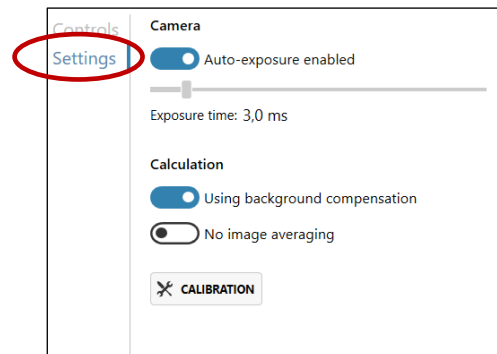


If the focusing does not work as intended, contact [support@phiab.se](mailto:support@phiab.se).

### Settings

In the settings tab, the exposure time of the camera as well as the background compensation for images can be handled. Usually there is no need to adjust these parameters.

Note that the Calibration should be performed before the start of each experiment.



### Auto-exposure

The camera exposure time is set automatically, but it is possible to set the exposure time manually.

### Calculation

Phase images are calculated based on the holograms. In order to reduce noise, a background compensation is used for the calculations.

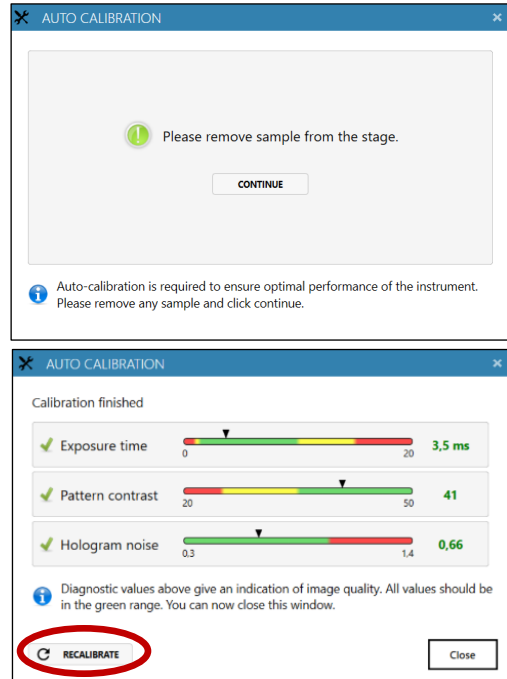


## Calibration

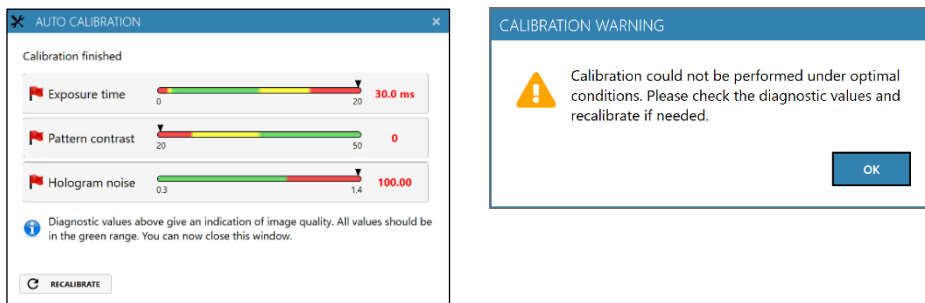
HoloMonitor should be re-calibrated before each use. Auto-calibration gives a background image that is used by App Suite to lower background noise and increase image quality. The calibration procedure takes approximately one minute.

Start the calibration procedure by clicking Calibration and follow the instructions. The first instruction is to remove sample from stage. The calibration is performed automatically.

When the calibration is finished, the calibration wizard will display three diagnostic values measuring the image quality. All values (indicated by black arrow heads) should be in the green range.




If App Suite fails to perform a calibration, a warning will appear. In that case, follow the Cleaning instructions in this manual. After cleaning, click Recalibrate to refresh the Diagnostics values. If any of the Diagnostic values remain outside the green range after several cleaning attempts, contact technical support at [support@phiab.se](mailto:support@phiab.se) for further assistance.



## Live Image

### Pan and zoom

The Live view window shows the current cell sample position. The image can be moved by click and drag, and it can be zoomed in and out with the mouse scroll wheel. The Reset View button  will return the image view to the original. The button is found to the right of the image.

Note that moving the image will not move the motorized stage to a new position.

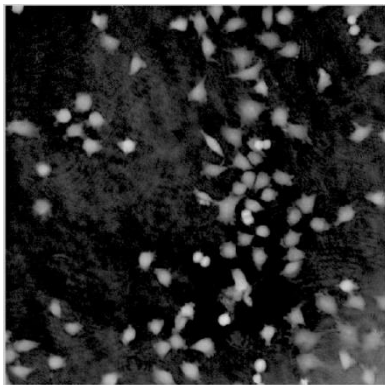
### Image formats

The Image Channel drop down menu can display the image in three different formats (see images below).

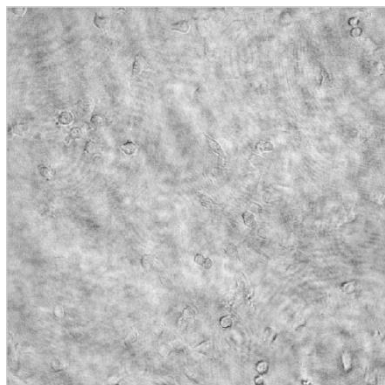
The *Holographic Phase Image* is a reconstructed image based on the light phase information in the hologram.

The *Holographic Amplitude Image* is a reconstructed image based on the amplitude information in the hologram.

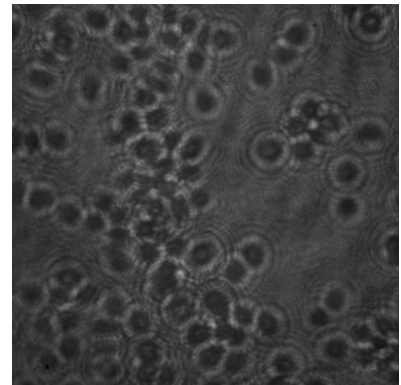
The *Hologram image* is captured by the camera and is the result of interfering light waves. The hologram is the basis for the reconstructed holographic cell images.



Holographic phase image



Holographic amplitude image



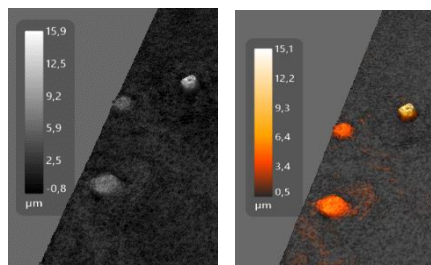
Hologram image

### Adjusting image presentation

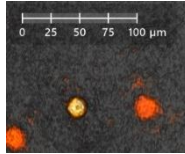
To the right of the Live image view window there are several functions that adjust the presentation of the captured image (see image to the right). Blue functions are active while gray functions are inactive. All adjustments will be present in exported movies and images.

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

*Show color bar* adds a scale that correlates the image coloring with cell thickness (see images below).

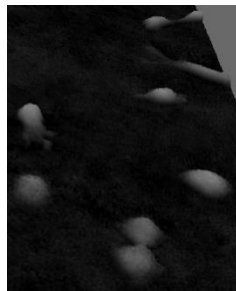


*Show XY scale* displays a horizontal scale bar representative of the distance in X and Y (See image to the right).

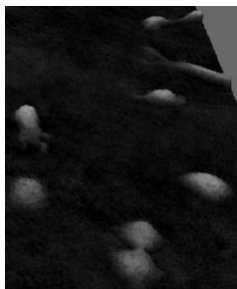


*Enable Lighting* applies an artificial light source to the image which may sometimes render an improved image (See image below).

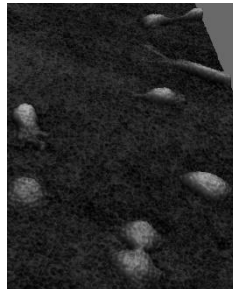
*Enable Shiny surface* shows the rendered surface with a reflective visual effect. Shiny surface is only active when Lighting is active.















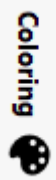
Standard image



Enabled Lighting

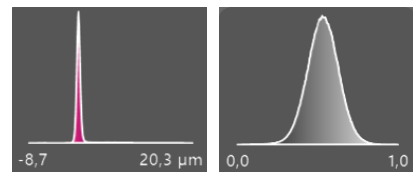


Enabled Lighting and Shiny surface


	View in 3D
	Show color bar
	Show XY scale
	Enable lighting
	Enable shiny surface
	Show background grid
	Show image histogram
	Line measure tool
	Zoom in
	Zoom out
	Reset image view
	Save view as image file
	Show color controls

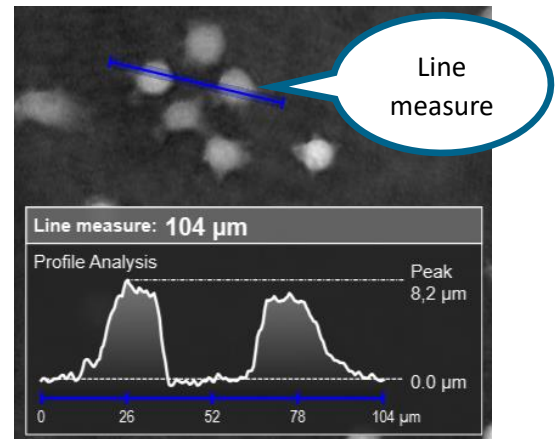
*Show background grid* adds a grid in the background.

*Show image histogram* shows a histogram overlay of the pixel values in the image. For a phase image this equals a histogram of the optical thickness values (left image) and for other image channels such as camera it shows a histogram of the pixel intensity values (right image).



*Line measure tool* interactively measures distances and cells. Once the tool is activated, left click in the image at the starting point of the measurement, then release and left click again at the end point of the measurement.

It is possible to move and flip the image while the measuring function is activated. The data can be saved as an image with the Save View  button. When the line measure tool is clicked again, the measuring function is deactivated.



*Zoom in and Zoom out* will increase or decrease image size.

*Reset image view* will return the image view to default size and center position.

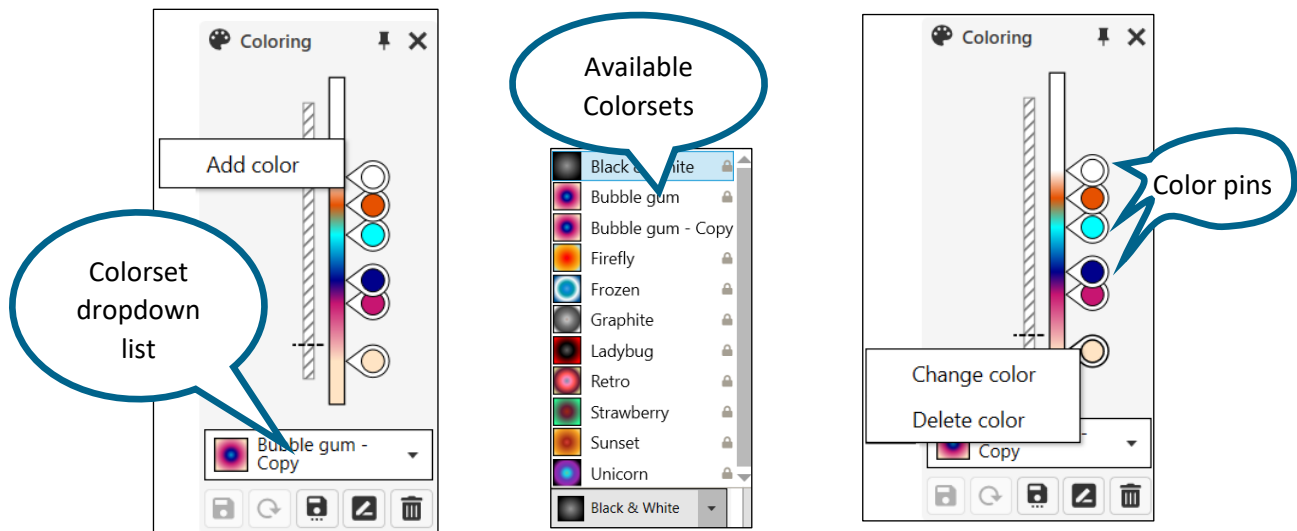
*Save view as image file* saves the image in the Live window exactly as it is shown as bmp, gif, jpg or png files.

*Show color controls* opens the Coloring window (see image below) which is used to adjust the color balance of the image and to color the image relatively to the thickness of the cells. A set of colors that are saved together is called a Colorset. The default colorset for any image is Black & White. There are a number of pre-set colorsets to choose from and it is easy to create new, personal colorsets. Once a colorset has been selected, it will automatically be applied to all captured images in that experiment.






- Switch to a pre-existing colorset by using the Colorsets dropdown list (see center image below).
- Click-and-drag to move the color pins to a suitable position in the color set.
- Create new color sets by right clicking in the Coloring window and then click Add Color (See left image below).
- Change or delete colors by right clicking the color pin and select Change or Delete (see right image below).



Note that no colorsets carry over from the App Suite assays to In Depth and vice versa.



The buttons below the Coloring window are used to

-  Save a new Colorset
-  Go back to the last saved settings
-  Save the coloring preset to a new name
-  Rename the coloring preset
-  Delete the coloring preset

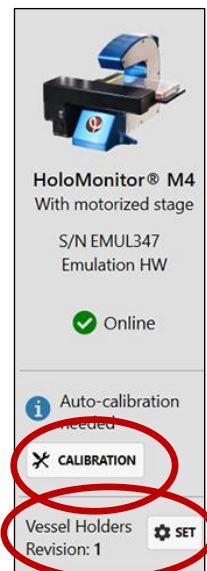
### Connected instruments

Connected instruments will be presented in the right-side panel. The software first initializes the connection to HoloMonitor M4. A green check mark symbol will appear when the connection is established.

### Working with two instruments

The software can connect with two instruments simultaneously. To work with one of the instruments, click the corresponding icon and setup the experiment.

To work with the other instrument, click that instrument icon, then setup the experiment for that instrument. A more powerful computer is recommended for working with two instruments.



Note that the same vessel holder revision must be used with the two M4:s, and that both experiments must be saved to the same database.

#### Auto-calibration needed

The software will often recommend calibrating the connected instrument. Click the Calibration button and follow the instructions (see the paragraph on Calibration). The HoloMonitor M4 should be calibrated before each experiment.

#### Vessel Holders revision

There are two lines of vessel holders for the HoloMonitor. They are black (Revision 1) or blue (Revision 2). It is necessary to have the correct revision selected, as the Vessel Maps are different for the different revisions. Use the Set button to set the correct revision of vessel holders. All new HoloMonitors are delivered with the blue holders.

## Database Handling

The Database Handler organizes HoloMonitor M4 data. Acquired images are stored in databases that can be copied, exported, moved to other databases, or deleted. It makes data backup easy and straight forward. Databases that are already known to the software are displayed as a list in My Databases window. Databases stored both on the computer and on external units can be accessed.



### *Access the Database Handler*

Access the database handler through Database in the top menu.



Note that during an experiment, data needs to be stored on the computer running App Suite. Storing data onto an external hard drive or a server (local or cloud based) during an experiment may cause all data to be lost due to erratic USB connections or weak internet signal.

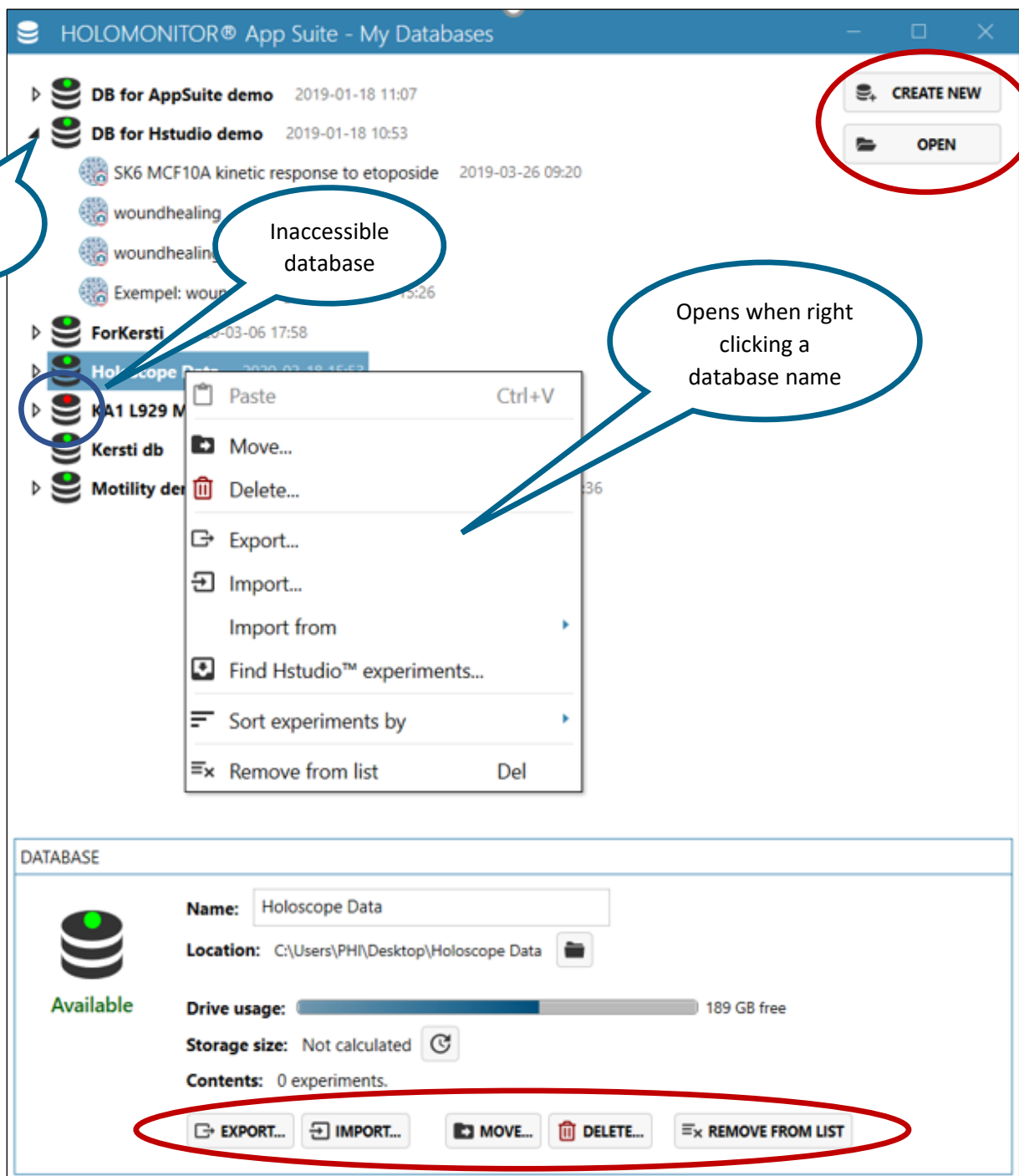


Note that experiment data will be stored in the currently selected database. Before running an experiment, during setup, ensure that the currently selected database is stored on the computer connected to the HoloMonitor M4.

Click the arrow beside a database to display the accessible experiments. If a database has been moved and cannot be accessed anymore, the symbol will have a red dot.

In the Database Handler, there are buttons for Creating New or Open already existing databases, as well as Exporting, Importing, Moving and Deleting databases and for removing databases from the list. Removing a database from the list will not delete it. Many of the functions can also be accessed by right clicking the databases or the experiments.





### Create a new database

1. Click Create New
2. Browse to the desired location.
3. Create a new folder and name the new database.
4. Click Create.

The new database will show up in the database list.

### *Browse to access existing databases*

In order to add an existing database to the list, click Browse. Locate the desired database and click OK. The added database will show up in the database list.

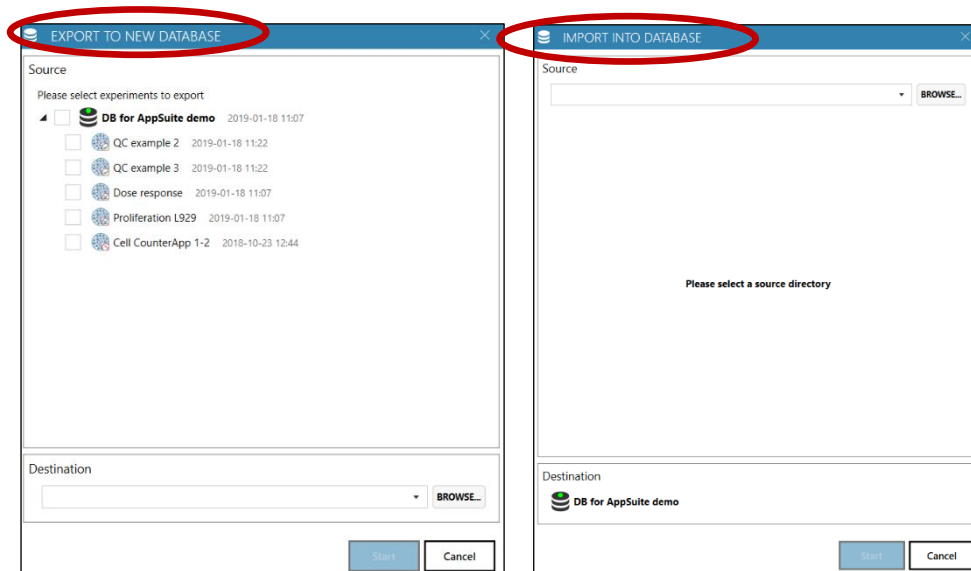
### *Export and import databases*

#### Export

Select a database and then click Export to open the window Export to new database (see image below). Follow the instructions to create a new database and export the data to that new database. As a copy will be exported, the original database will still be present.

#### Import

Select a database and then click Import to open the window Import into database (see image below). Follow the instructions to find and import a database into the present database.



### *Move a database*

Select a database and then click Move to open the Move database window. Browse to the new location and then click Start. The entire database will be moved to the new location.

### *Delete data*

Select a database or an experiment and click Delete to remove it from the database list. A security window will open to confirm, click Yes. Then another security window will open, repeat confirmation. This will permanently delete all data.

### *Remove database from list*

Removing the database from the list will not delete it.

### Find Hstudio experiments

Experiments recorded using Hstudio can be analyzed in App Suite. To make an Hstudio experiment available in App Suite it must first be added to the list of available databases. Click Open and browse to the location of the Hstudio database and add it to the list. Then select the database in the Database Handler list of databases. Right click the selected database and select Find Hstudio experiments. Any available Hstudio experiments will be shown in a list. Select an experiment and click Add. This will convert the Hstudio data to App Suite data.

If the Hstudio experiment is accessed through the In-Depth analysis, it does not need to be converted to App Suite format. The data only needs to be converted if Guided Assays will be applied.



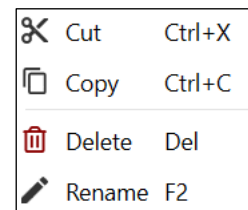
Note that conversions do not duplicate the images. Instead, the Hstudio data are made available for App Suite. Thus, if the experiment is later deleted in App Suite it will be deleted for Hstudio as well.



Note that not all older Hstudio experiments are possible to convert to AppSuite experiments. If that is the case, experiments are still available for analysis in AppSuite through the In-Depth Analysis tab. Use the database drop list to access the database.

### Copy and transfer individual experiments

Click the little arrow beside a database in the Database Handler to show the Experiments included in the database. Right-click the Experiment and select Copy. Right click the desired database and paste the copied Experiment into the database.



### Backup/restore data

#### Create a database backup

There are two ways to create a backup. One way is to first create a new database in the back up storage device. Then right-click on the Experiment you wish to backup and select Copy. Browse to find the backup database and paste the copied Experiment into the backup database.

Alternatively, follow the instructions above to export the entire database to a new database.

#### Move data from a backup to the computer

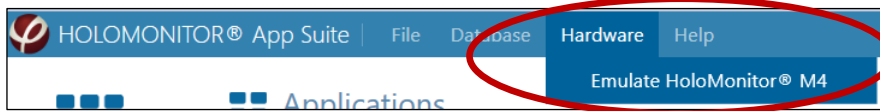
There are two ways to move data back to the computer. One way is to first create a new database on the computer. Browse and find the backup database. Copy and paste the backup data into the new database on the computer. The database data are now on the computer and can be accessed by App Suite.

Alternatively, follow the instructions above to import the entire database to a new database.

### Analyzing data on a remote database

Note that analyzing data on an external hard drive or server (local or cloud based) works well if the USB or internet connections are stable.

## HOLOMONITOR EMULATOR



The HoloMonitor Emulator is a virtual HoloMonitor M4. When activated it gives access to all live features of the software as if a HoloMonitor was connected to the computer. It can be accessed through Hardware in the top menu. Once activated, it will show up in the instrument panel to the right.



Note that the Emulator needs an active license to access the software, similar to an actual HoloMonitor.

**HoloMonitor® M4**  
✓ Motorized stage  
S/N EMUL0  
Emulation HW  
✓ Online  
i Auto-calibration needed  
✗ CALIBRATION  
Vessel Holders  
Revision: 2 SET

# TROUBLESHOOTING

## Guided Assays

### *Live Imaging*

#### The software cannot find the HoloMonitor

*The USB- and power cables are not properly connected*

Ensure that all cables are properly connected. Disconnecting and then reconnecting the USB-cable may restore the connection.

*App Suite needs to be restarted.*

Restart the software.

If the software still is unable to find the HoloMonitor M4, contact [support@phiab.se](mailto:support@phiab.se).

#### The Auto-calibration does not pass

*All calibrations values are in the red*

If you cannot get the Calibration wizard to pass a calibration step, and all values (Exposure time, Pattern contrast and Hologram noise) are in the red ranges, this might indicate that the laser is not able to produce enough light.

If all values are in the red, this could be caused by:

- Laser not properly attached to HoloMonitor M4
- Electricity cable is not connected to the laser hub
- Dirty optics
- Dirty laser tip or fiber connector
- Laser requires periodic recalibration
- Dirt/condensation inside HoloMonitor M4

*Check the laser*

- Is the red light shining on the objective?
- If not, is there power to the laser and the green light on the laser module is blinking?
- Does the image in the App Suite live capture tab look normal?
- Is the optical fiber cable damaged or crushed?
- Is the laser connection to the back of the HoloMonitor M4 positioned correctly and tightened?
- Inspect the Laser tip. Unscrew the Laser connection from the back of HoloMonitor and see if the tip is scratched or dirty.
- When the laser connection is detached from the HoloMonitor M4, check if the laser light shines in a strong and perfect red circle when shining on a white paper. If not, it is either dirty, or the tip could be damaged or cracked.

*If the laser tip needs cleaning*

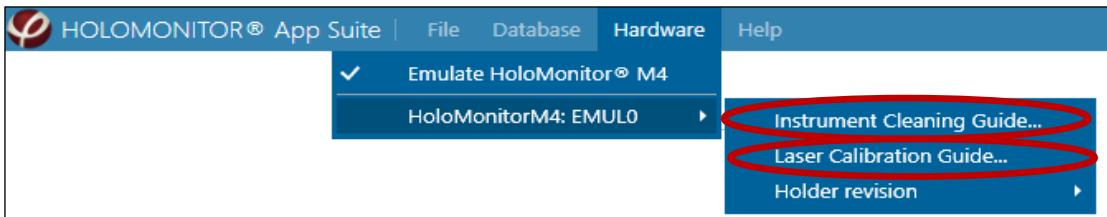
- Wipe the tip with a dry cotton swab in **one** direction.
- Take a new cotton tip and clean the fiber connector.
- Carefully reconnect the laser, paying attention that it is **keyed**. The guiding metal ridge on the laser connection must slip into the guiding notch in the connector socket at the back of the HoloMonitor M4. Connections must be finger tight.

*After checking the laser, recalibrate*

After testing all above, re-run the Calibration wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range.

*Pattern contrast is in the red range or unstable*

An unstable Pattern contrast value in the green range or a value in the yellow/red range could indicate that the laser needs periodic recalibration. Go to the top menu, select Hardware and then the current HoloMonitor M4. Activate the Laser Calibration Guide and follow the instructions. Afterwards, re-run the Auto-calibration wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range.



*Hologram Noise is in the red range*

A Hologram noise value in the red range could indicate that there is dirt on the optics or dirt inside HoloMonitor M4. Go to the top menu, select Hardware and then the current HoloMonitor M4. Activate the Instrument Cleaning Guide and follow the instructions. You may need to repeat the cleaning 2-4 times depending on how dirty the optics are. Afterwards, re-run the Auto-calibration wizard. After successful auto-calibration all values (black arrow heads) should be in the green range.

*If nothing works*

If any of the Diagnostic values remain outside the green range after several cleaning attempts and/or laser calibration, contact the technical support at [support@phiab.se](mailto:support@phiab.se) for further assistance.

**There is no vessel map for petri dishes**

*The HoloMonitor M4 has black cell culture vessel holders.*

HoloMonitor M4 with motorized stage is delivered with a set of cell culture vessel holders. At present there are two revisions of the holders which can be distinguished by their color. Revision 1 is black, and Revision 2 is blue. In App Suite, there are Vessel Maps to match the holders. All new HoloMonitors are delivered with the blue holders.

For Revision 1 there are Vessel Maps matching the microscope slide holder and the standard multi-well plate holder. For Revision 2 there are Vessel maps matching the microscope slide holder, the standard multi-well plate holder and the standard Ø 35 mm Petri dish holder. The precision of the Revision 1 holders is not sufficient for petri dishes, and thus they cannot be used with App Suite.

### The default focus is not working

*The motorized stage may need to be calibrated*

Please contact your distributor or [service@phiab.se](mailto:service@phiab.se).

*The cell culture vessels used do not follow the recommended specifications*

Switch to the recommended cell culture vessels.

### It is impossible to focus the live image

*The cell culture vessel is scratched or smudged*

Even wiping the cell culture vessel clean with ethanol will probably not help much as traces of dirt and tissue fiber 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 damaged.

*There is condensation on the inside of the cell culture vessel*

The PHI HoloLids™ are designed to handle condensation. It is recommended to use HoloLids™ whenever possible. If the cell culture vessel is room temperature, allow the vessel to reach incubator temperature before starting the experiment.

*There is condensation on the outside of the cell culture vessel*

Allow the cell culture vessel to reach the same temperature as the surroundings before capturing images. Use pre-warmed media to avoid condensation.

*The objective and the laser window need cleaning*

Clean the instrument according to the Cleaning instructions in this manual.

*The instrument has not been installed properly in the incubator*

Allow the instrument to warm up in the incubator for at least three hours, preferably over night, after installation.

*The cells may be very thin or very sparsely distributed in the vessel.*

Ensure that all calibrations are optimal.

If the cells are very thin, use cell culture vessels with glass bottom to improve image quality.

If the cells are sparsely seeded, use a cell culture with higher seeding density.

*Floating cells or debris disturbed the automatic software focus*

Replace the old medium with new, fresh medium.

*Something causes the instrument to vibrate*

Check that the cables connected to HoloMonitor M4 are not loose and that they do not touch anything that vibrates. Ensure that no vibrations come from sources like centrifuges or hard-working air vents. Make sure that the incubator is leveled and stands without disturbances.

### The position of the live image in the vessel map does not correspond to the actual position in the vessel

*The cell culture vessels are not the recommended versions.*

All standard well plates are not the same. They are all slightly different. If a different vessel than the recommended is used, the Vessel Maps will not correlate with the actual wells.

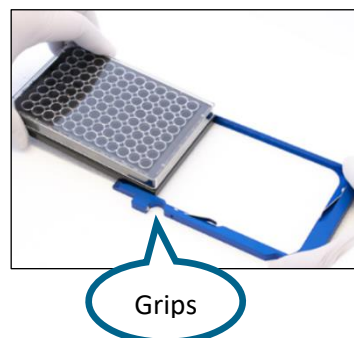


*The revision of the vessel holders is not correctly set in the software.*

There are two revisions of cell culture vessel holders, the Black (Revision 1) and the Blue (Revision 2) holders. Their versions are slightly different, and unless the correct version is set, the Vessel Maps will not correlate with the actual wells.

*The multiwell vessel is accidentally placed the wrong way in the vessel holder.*

Ensure that well A1 (Sarstedt) or well 1 (Eppendorf) of the plate is placed to the left when the grips of the holder face towards the operator.



Vessel	Vendor cat. number	HoloLid™	Final working volume	Growth area, cm <sup>2</sup> /well	Vessel cut in a holder
Sarstedt TC 6-well plate	83.3920.005	71120	3.0 mL/well	8.80	top left
Sarstedt lumox® 24-multiwell plate	94.6000.014	71130	1.9 mL/well	1.90	top left
Sarstedt lumox® 96-multiwell plate	94.6000.024	71140	170 µL/well	0.34	top left
Eppendorf CCCadvanced® FN1 - 6 well	0038110010	71150	3.0 mL/well	9.40	bottom right

The live image focus was OK, but it slowly turned bad and now it cannot be set again

*There is condensation on the underside of the vessel bottom*

Ensure that the medium has the same temperature as the surrounding. If a treatment is to be added, preheat the treatment solution in the incubator.

*A drop of water is hanging from the top/lid of the cell culture vessel.*

As the drop grows larger it will increasingly disturb the image focus. Use HoloLids™ to avoid drop formation.

The image looks garbled

*Dirt, smudges, scratches, condensation, fingerprints on the vessel, top laser window or objective and wrong focus will affect the images negatively.*

Try capturing at different positions in the cell culture vessel. There might be clear areas.

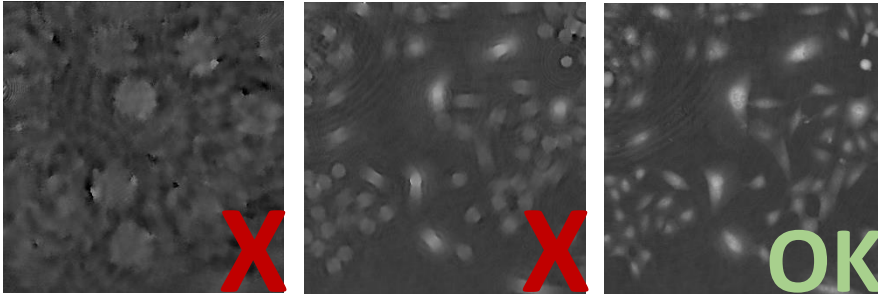
Clean the instrument as described in the Cleaning instructions.

There may be condensation in the ceiling or on the roof of the vessel. Use vessels which are condensation free, e.g. ibidi-µ channel slides, or use the recommended vessels with the PHI HoloLids™.

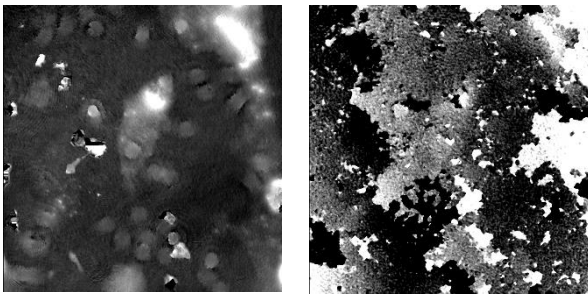
Re-calibrate according to the Calibration procedure.

Vibrations may cause image disturbances. Check that cables connected to HoloMonitor M4 are not loose and not touching anything creating tension or vibrations.

Wrong focus



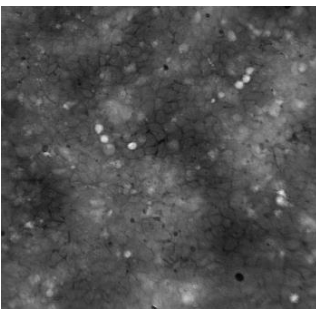
Condensation



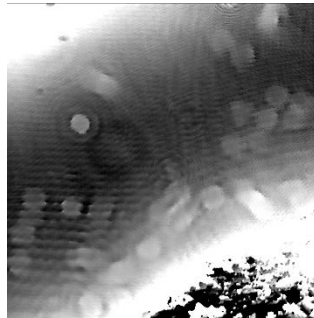
Some condensation

Full condensation

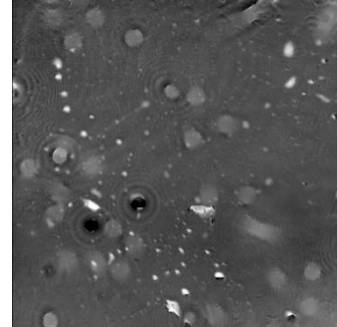
Cells in many layers



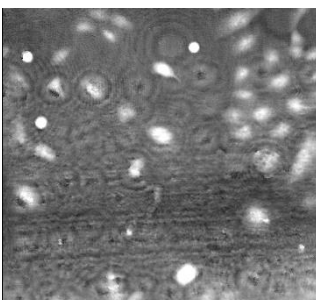
Air bubble



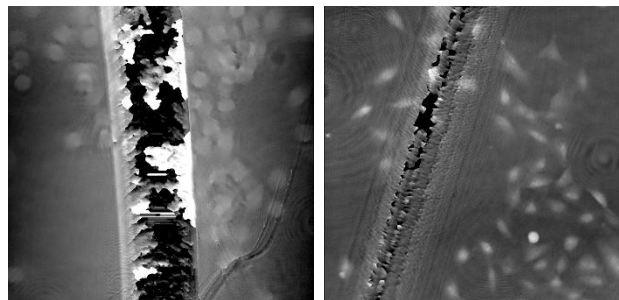
Dirt & Particles



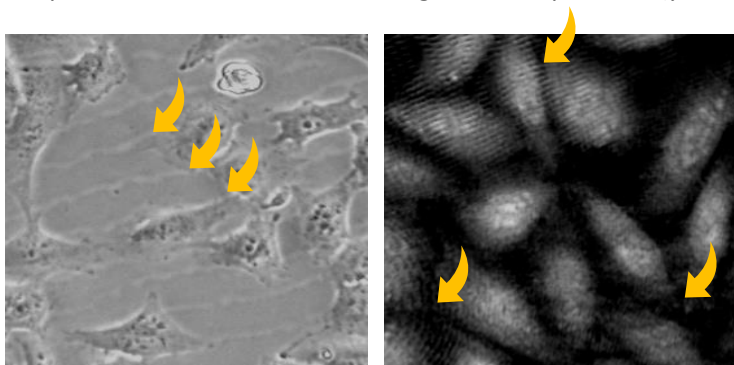
Smear



Small and big scratches



Bad plastic, which creates disturbing lines and patterns (yellow arrows) to various extents.



*The laser light is not optimal*

Make sure that the laser beam intensity is adequate. The exposure time for an image without a sample should be less than 5 ms and never more than 10 ms. If that is not the case, contact the local distributor or [support@phiab.se](mailto:support@phiab.se) for advice.

*Condensation has formed inside HoloMonitor M4*

The HoloMonitor M4 may suffer from internal condensation. This can occur if the incubator is excessively humid, if the HoloDry unit is not changed frequently enough, or if the instrument power cord has been unplugged.

Remove the instrument from the incubator and let it dry for three days at room temperature. After drying, calibrate HoloMonitor M4 according to the [Calibration procedure](#) to ensure that the Diagnostic Values are in the green range before proceeding to incubator acclimatization. Change to a new HoloDry unit.

If drying out does not improve the image sufficiently, clean the objective. If the image quality is still bad, contact the local distributor or [support@phiab.se](mailto:support@phiab.se) for advice.

**The live cell image is black**

*The laser unit may not be connected properly.*

Start by checking that the laser unit is connected properly both to power and to the instrument. Restart computer and software. If the laser is connected properly and the image still is black, please contact [support@phiab.se](mailto:support@phiab.se)


**The cells in the images are very large and fuzzy**

*The image has been zoomed in too much.*

Use the Reset button  to the right of the image, use the mouse scroll wheel to zoom out or double-click left mouse button.

**The live cell image is very small**

*The image has been zoomed out too much.*

Use the Reset button  to the right of the image, use the mouse scroll wheel to zoom in or double-click left mouse button.

## Experiment Set up and Capture

### There are no databases in the Database Handler

*All databases have been removed from the Database Handler.*

Browse to locate and open databases.

*There are no databases on the computer.*

Create a new database for the experiment.

### The databases in the Database Handler cannot be accessed.

*If a database has been moved and cannot be accessed anymore, the name will be inactive and there will be a red dot.*



Remove the inactive database from the list, and then browse to access the database at the new location.

### The Proceed to Capture button does not work

*The button is inactive until a treatment has been added to the experiment setup.*

Add treatments.

### The time-lapse sequence position is unstable

*The time-lapse is drifting in X, Y or Z*

Sometimes there will be a slight drift in the beginning of the run. The stage needs to warm up.

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

The cell culture vessel may not be 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 your local distributor or [support@phiab.se](mailto:support@phiab.se) for further assistance.

*The motorized stage needs to be serviced*

Contact your local distributor or [support@phiab.se](mailto:support@phiab.se) for further assistance.

### The capture was interrupted

*The computer screen sleep and power settings are not set to "never".*

Ensure that the computer power is set to never go to sleep.

*There was a power failure.*

The instrument will not automatically restart an interrupted experiment.

*The data was stored on an external hard drive.*

USB and internet connections are not stable over time, causing an interruption in image storage. Set up experiments using the computer storage instead of an external hard drive or server (cloud based or local).

*The computer updated software automatically and then restarted.*

The instrument will not automatically restart an interrupted experiment. Please ensure that all Windows auto-updates are turned off, and that the computer is not connected to internet. If available, use flight mode.

## Results

### There are no databases in the Database Handler

*All databases have been removed from the Database Handler*

Browse to locate and open databases.

### The databases in the Database Handler cannot be accessed

*If a database has been moved and cannot be accessed anymore, the name will be inactive and there will be a red dot*

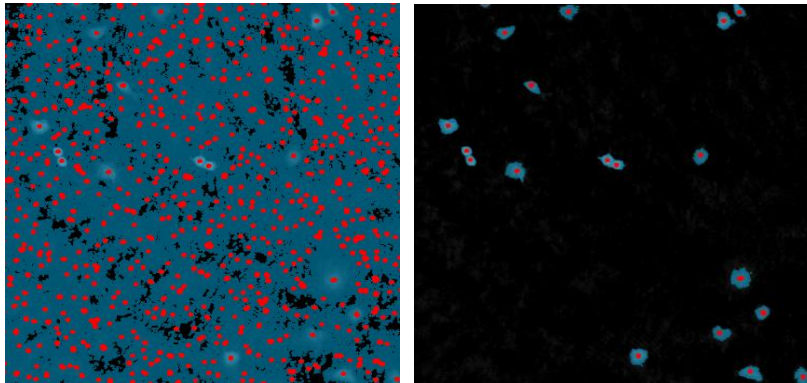


Remove the database from the Database Handler and then browse to access the database at the new location.

### Image analysis is not optimal

*The automatic cell identification looks strange*

The threshold setting may not suit the cells. Use the Sensitivity parameter in the Cell Detection Controls to adjust the threshold. The examples show an image before (left) and after (right) threshold adjustment.



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. Use the Sensitivity parameter in the Cell Detection Controls to adjust the threshold.

### Cell count is not accurate

*Cells have grown to high density or have clustered together without having distinct outlines*

The software cannot properly identify individual cells, and the cell count will be either too high or too low. Use confluence instead.

### No image frames are visible in the Positions Image list in Image Analysis

*When editing Treatments or Conditions and Doses in Experiments Setup, all treatments were deleted*

Go to Setup in the result view and redefine the treatments or conditions and doses. The result will be updated, and all images will again be visible in the Positions Image list.

### Images are not optimal

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

There are different reasons for bad images, such as a scratch or a smudge on the vessel or there may have been a passing disturbance, e.g. floating cell debris or air bubbles, which caused the software autofocus to miscalculate.

In App Suite, bad images are filtered out and are not included for the analyses, however some bad images may remain. Uncheck those manually in the Images list and click Apply changes.

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

Ensure sure that auto-calibration has been performed with acceptable results prior to the experiment.

## In-Depth Analysis

### Identify cells

No image frames are visible in the Image Frame list

*No project and group have been selected.*

Select a Project and a Group.

*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 be suitable for the cell type.*

Try a different threshold method.

*The cells grow at a very high density.*

The threshold setting methods cannot identify individual cells if the cells grow very close to or on top of each other. Adjust the Background threshold in Adjustment tab. Make sure that only the background and not any cells are defined as background. Then use the confluence to determine the amount of cells instead of the cell number.

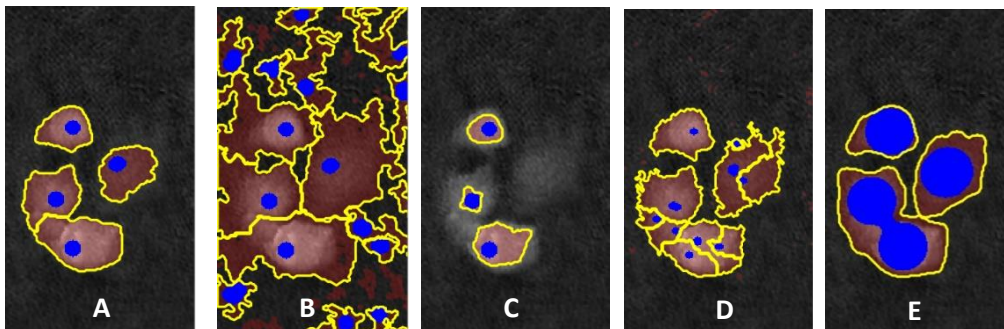
Seed the cells at a lower density.

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

Ensure sure that auto-calibration has been performed with acceptable results prior to the experiment.

*The background and the cell size identifications look strange*

Adjusting the threshold between cell and background with the slider will adjust what is considered cell covered area in the image.



- A. Threshold is OK, Object Definition is OK
- B. Threshold is set too low, too much of the background is counted as cell area
- C. Threshold is set too high, too little of the cell is included in the cell area
- D. Threshold is OK, Object Definition set too low, the cells are segmented into smaller units
- E. Threshold is OK, Object Definition set too high, the cells are merged into larger units



*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 confluence is very high, and the cells are difficult to separate out. Separate the two cells by using the Add/remove cell marker button under the Manual changes tab in the Identify cells tab. Note that manual changes are only applied to that specific cell in that specific frame, they cannot be applied to the other frames in a time-lapse.

### *Single Cell Tracking*

The tracks are very irregular

*The cell segmentation is not properly done.*

Use the Identify Cells tab in the Single Cell Tracking and make sure that the segmentation is properly performed.

A larger movement than average is seen as a long line between time points.

*The tracking function finds the wrong cell after the cell has left the image field or merged with another cell.*

Correct the tracking error for the individual cell manually.

There is a very high number of warnings

*The cell segmentation is not properly done.*

Use the Identify Cells tab in the Single Cell Tracking and make sure that the segmentation is properly performed.

### *Cell Morphology*

No image frames are visible in the Image Frame list

*No project and group have been selected.*

Select a Project and a Group.

*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

*Changes in settings and display have caused the dots to be outside the range of the scatter plot values.*

Set the plot area to Auto-scale.



## Woundhealing

There are no positions where both wound edges fit into the image frame

*The gap is too wide*

Add two positions which are parallel to each other and have one edge with cells. Use XY position arrow buttons to move stage in steps and when satisfied, press Add Current Location button. For the analysis, calculate cell front velocity for each edge with cells and assess cell covered area (%) change in the field of view to evaluate gap closure kinetics.

## View images

No image frames are visible in the Image Frame list

*No project and group have been selected.*

Select a Project and a Group.

*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.

## 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.

*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.

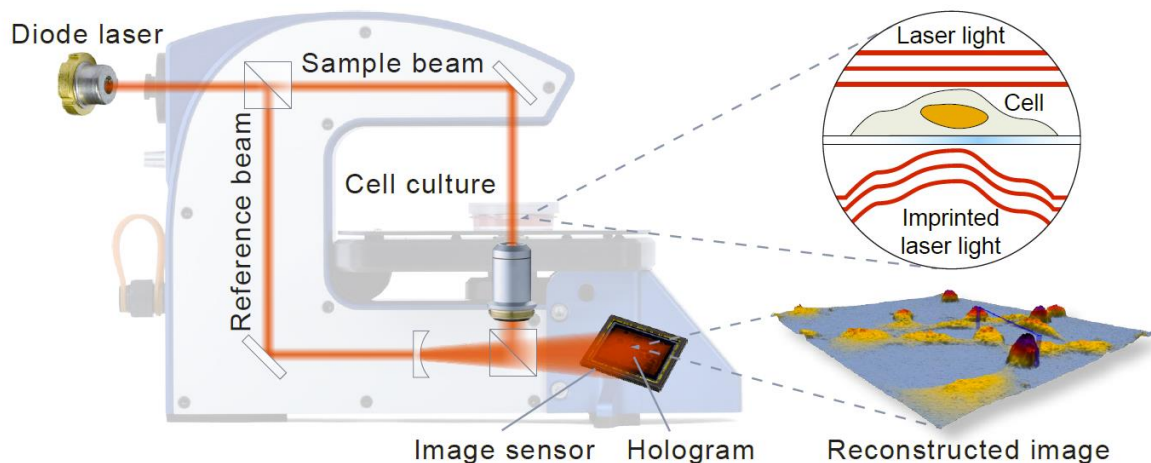
## ANALYSIS FEATURES

### Holographic Microscopy

To properly understand the output variables that App Suite presents, it is important to understand digital holography. Below is a crash course. For more information, see Alm et al. 2011, 2013; Kim 2010.

#### *In short*

HoloMonitor M4 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, the light is slowed down and a phase delay is imprinted on the light beam. By subsequently merging the sample and the reference beam, an interference pattern is created. This 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). In other words, all calculations are based on how much the light slows down when it passes through the cells.



*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.*

#### *Calculations*

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. Below described cell morphology features are calculated by the App Suite software, based on the phase image. The estimated cell thickness ( $T$ ) of a cell in pixel  $i$  is obtained from the phase shift ( $\varphi$ ), the wavelength of the laser light ( $\lambda$ ), the refractive index of the cells ( $n_c$ ) and the refractive index of the surrounding medium ( $n_m$ ):

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

#### *Refractive index*

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 and Richards-Kortum 1996; Farinas and Verkman 1996;

Kemper et al. 2010; Rappaz et al. 2005). 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 (less than +/- 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.

In short, the delay of the light caused by the cells when the light passes through them is called the phase shift and is the original feature that is measured with digital holography. The different ways to calculate features based on the phase shift reveals different effects of cell treatments.

## Output Variables for Guided Assays

### Output variable statistics

In App Suite, output variables are typically presented as an average value, sometimes including a variation measure, such as Coefficient of Variation. These statistical output values are calculated in a hierarchical manner:

- first averages are calculated as a frame average of all cells in one frame
- then averages are calculated as a well average of all frame averages in that well
- finally, averages are calculated as a treatment and/or condition average of all well averages in that treatment/condition

The mean and standard deviations of all output features at all hierarchical levels (e.g. frame, well, treatment) are presented in the Excel exports. Note that it is important to verify that the variation satisfies the needs of the current application at all levels.

### Mean Cell Area ( $\mu\text{m}^2$ )

The mean cell area ( $\bar{A}$ ) is calculated across all single cells in one frame. Single cell area ( $A$ ) is calculated in the same way as when using conventional microscopy:

$$\bar{A} = \frac{\sum A}{k} \quad \text{where} \quad A = N \cdot s_{xy}^2$$

where  $k$  is the number of single cells in the frame,  $s_{xy}^2$  is the calibrated size of the area imaged by a single pixel and  $N$  is the number of pixels used to image the cell area.

### Cell Count (cells/cm<sup>2</sup>)

The cell count is based on the automatic cell identification and results in the number of cells per area.

### Confluence (%)

The confluence is based on the automatic cell identification and describes the total area of a frame that is covered by cells.

### Accumulated Mean Cell Distance ( $\mu\text{m}$ )

The mean cell distance is based on the average cell motility, which is the average distance traveled by all cells between two frames in a time-lapse. The distance is calculated using the displacement of the cell center of mass for each cell, compared to the previous image. The center of mass is the arithmetic mean of all points weighted by the thickness of the cell. The accumulated mean cell distance is simply the mean cell distance accumulated over time. Using the cell center of mass enables consistent measurements despite variations of cell shape over time.

Note that individual tracking is not performed throughout the time-lapse, as the cell displacement is only calculated frame by frame. The Accumulated Mean Cell Distance is a cell population measurement.

### Mean Cell Speed ( $\mu\text{m}/\text{h}$ )

The motility speed is based on the mean cell distance from one frame to the next, divided by the time between frames. The Mean Cell Speed is based on all cells in the frame. Although this feature is highly variable it is an interesting variable over time.

### Mean Cell Diameter ( $\mu\text{m}$ )

The cell diameter ( $\bar{\phi}$ ) is calculated using the standard area to diameter formula for a circle, that is

$$\bar{\phi} = \frac{\sum \phi}{k} \quad \text{where} \quad \phi = 2 \cdot \sqrt{\frac{A}{\pi}}$$

where  $k$  is the number of single cells in the frame,  $A$  is the cell area. Note that this assumes that the cell is circular and is therefore just an approximation of the diameter for non-circular cells. This assumption allows consistent diameter measurements despite variations of cell shape over time.

### Mean Cell Volume ( $\mu\text{m}^3$ )

The estimated volume of a cell ( $V$ ) is calculated from the phase shift (Kemper et al. 2010):

$$\bar{V} = \frac{\sum V}{k} \quad \text{where} \quad V = \frac{\lambda \cdot S_{xy}^2}{n_c - n_m} \cdot \varphi_{sum} \quad \text{and} \quad \varphi_{sum} = \sum_{i=1}^N \varphi_i$$

where  $k$  is the number of single cells in the frame. Note that the cell volume is independent from the cell shape.

## Cell morphology features for In-Depth Analysis

### 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):

$$V = \frac{\lambda \cdot s_{xy}^2}{n_c - n_m} \cdot \varphi_{sum} \quad \text{and} \quad \varphi_{sum} = \sum_{i=1}^N \varphi_i$$

### 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:

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

### 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:

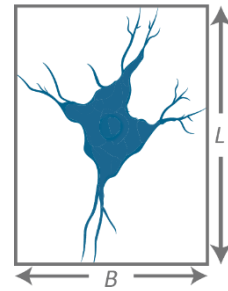
$$OPD_{avg} = T_{avg} \cdot (n_c - n_m) = \lambda \cdot \frac{\varphi_{sum}}{N}$$

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

$OPD$  is expressed in  $\mu\text{m}$ . 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 feature 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

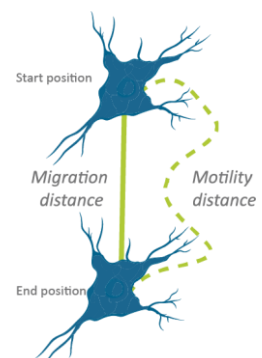
A measure of how much the circumference of the cell deviates from the circumference of a perfect circle. A value of 0 means the cell is circular and higher values mean a longer, more irregular outline. The calculations are based on a threshold setting that distinguishes background from cell.

### Migration distance

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

### Motility distance

The motility distance is the actual distance traveled by the cell between the starting point and the end point of the cell path.



### Motility speed

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 endpoint 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 feature 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  $2\pi$ .

### 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 (RMS) 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.
- 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 features 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.

- Texture cluster shade** 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 cluster tendency** Indicates into how many clusters the gray levels present in the image can be classified.
- 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.

<b>Texture correlation info1</b>	A different way of calculating the texture correlation.
<b>Texture correlation info2</b>	A different way of calculating the texture correlation.
<b>Texture energy</b>	Provides the sum of squared elements in the gray-level co-occurrence matrix. Also known as uniformity or the angular second moment.
<b>Texture entropy</b>	Entropy is a statistical measure of randomness that can be used to characterize the texture of the cell image.
<b>Texture homogeneity</b>	Measures how many different gray levels the image holds.
<b>Texture maxprob</b>	High values occur if one combination of pixels dominates the pixel pairs in the window.

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