HoloMonitor M4

HoloMonitor M4FL

App Suite 4.0

Setup and Operation Manual

Revision 1

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Content

THIS MANUAL

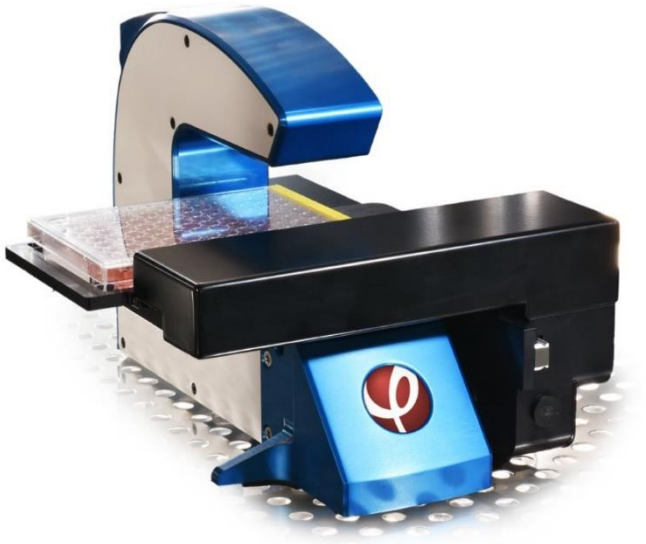
This manual is meant to describe the HoloMonitor platform and its software, App Suite, as well as be a guide to users of this platform. As laid out in the table of contents, this manual provides details on instrument safety, instrument handling, suitable culture vessels including HoloLids, guidance for the App Suite software including fluorescence use, and instrument troubleshooting and maintenance. At the back of the manual, holographic and fluorescence technology and cell morphology parameters are described.

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

INTRODUCTION TO HOLOMONITOR® M4

HoloMonitor® M4 is a live cell imaging time-lapse cytometer. It can image and quantify unstained living adherent cells directly in their culture vessels inside a cell culture incubator. It can be used for a wide range of applications, including but not limited to:

- Cell count
- Cell culture quality assessment
- Cell proliferation
- Cell motility
- Cell migration
- Cell morphology
- 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. This shift in the light is used by the software to reconstruct topographic images of the cells. These topographic images contain a wide variety of cell data. The technology is described more in depth in the Technology 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.

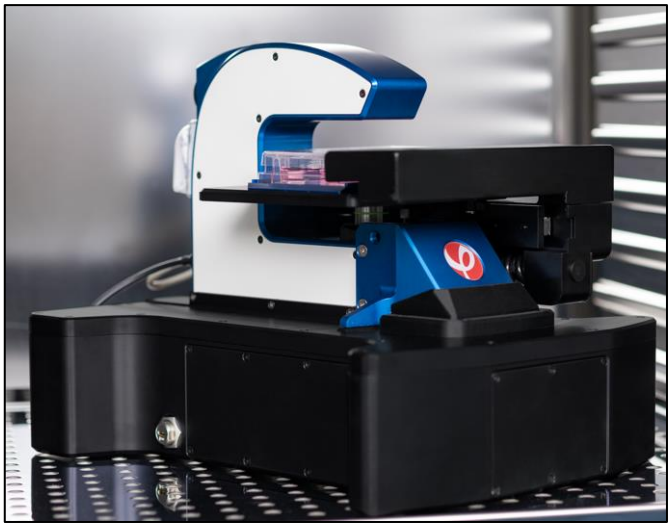
HoloMonitor M4 is equipped with a motorized XYZ-stage that accepts several types of vessel holders, allowing for accurate and automatic tracking of specified locations within the vessels.

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

INTRODUCTION TO HOLOMONITOR® M4 WITH FLUORESCENCE

HoloMonitor® M4FL is an automated, live cell, quantitative holographic microscope combined with green fluorescence imaging. It can image and quantify unstained and fluorescent living adherent cells directly in their culture vessels inside a cell culture incubator. The holographic images of each experiment can be analyzed in the same way as any other HoloMonitor experiment. Analyzing the fluorescence images is only possible using the In-Depth capabilities of App Suite.

HoloMonitor M4FL is incubator compatible, allowing analyses 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 M4FL is equipped with a motorized XYZ-stage that accepts several types of vessel holders, allowing for accurate and automatic tracking of specified locations within the vessels.

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

INSTRUMENT SAFETY

CE-labels

HoloMonitor 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)

Warning symbols

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

Phase Holographic Imaging (the “Manufacturer”) guarantees that HoloMonitor (the “Product”) 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 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 the 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 and for accessories sold separately or included in the sales pack of the main device. No guarantee is provided for defects caused by natural wear (wearing parts in particular) or improper use.

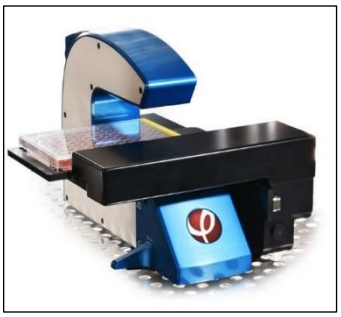
During the warranty period, Manufacturer will, in a reasonable time, remedy the Defect free of charge by either repairing or replacing the defective Product or the defective part of it at its discretion, provided that you have informed the Manufacturer of the Defect before the warranty period expires. 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.

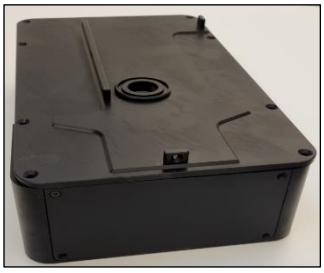
TECHNICAL SPECIFICATIONS

Technical specifications for HoloMonitor M4 with motorized stage



- Light source: External laser unit
- Sample illumination: 635 nm, 0.2 mW/cm2
- Objective: 20x
- Lateral resolution: 1 µm
- Field of view: 567 µm × 567µm
- Working distance: 0.5 – 2 mm
- Autofocusing range: 1.5 mm
- Image capture rate: 1 image/s
- Image size: 1024 × 1024 pixels
- Stage travel range: 100×70×10 mm (X×Y×Z)
- Stage repeatability: 5 µm
- HoloMonitor® M4 with stage, dimensions: 290×200×190 mm (W × D × H)
- Space required in incubator: 400×270×190 mm (W × D × H)
- Weight: 5.15 kg

Technical specifications for the fluorescence unit



- 1-channel fluorescence: Optimized for EGFP (FITC-Cy2)
- Excitation/Emission: 470/525 ± 20 nm
- Exposure time: 1 – 1000 ms
- Field of view: 567 µm × 567µm, 0.25 mm²
- Gain: 1 – 4
- Magnification: 10x, matched with mounted HoloMonitor M4
- Image Resolution: 0.5 µm
- Image size: 1024 × 1024 pixels
- Dimensions: 310 x 180 x 85 mm (W × D × H)
- Weight: 4.15 kg
- Power: < 5 W
- Interface: USB-C connector

COMPUTER REQUIREMENTS

HoloMonitor is controlled by the proprietary App Suite software (App Suite) running on an external computer. App Suite needs to be installed prior to use of HoloMonitor. Two HoloMonitors can be connected to each computer with App Suite.

Recommended minimum 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 A ports

For HoloMonitor M4FL it is important to use the USB C to USB A cable that comes with HoloMonitor. If the computer has only USB C ports, use the USB A to USB C adaptor that comes with HoloMonitor. Do not use a USB C to USB C cable. HoloMonitor will not connect properly with the computer.

Internet access

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

INCUBATOR REQUIREMENTS

HoloMonitor is designed to work in an incubator with 95% humidity and 5% CO_2 at 37°C but will also work from 10-40°C, at ambient humidity and in any combination of the ambient gases (CO_2, O_2, N_2 etc.).

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

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:	HoloMonitor M4: 40×27×19 cm (W×D×H)
	HoloMonitor M4FL: 40×27×28 cm (W×D×H)

SAMPLE REQUIREMENTS

Cells

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 any other single use hemocytometer without grid, or a cell culture vessel similar to IBIDI slides. For the Cell Count App, Countess grid-less cell count chamber which can be ordered from www.thermofisher.com.

Fluorescence labels

- For fluorescence labeling used with HoloMonitor M4FL, the excitation wavelength for the fluorophore needs to be 470±20 nm and the emission wavelength 525± 25 nm.

Cell culture vessels

For the best imaging quality, we recommend using any of the following:

- 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

Recommended vessels	Vendor cat. number	Growth area, cm2/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	0.27
ibidi® µ-slide	80106	2.50
Eppendorf CCCadvanced® FN1 - 6 well	0038110010	9.40

Keep the cell culture vessels clean and free from damage

For the Product to perform as intended, it is critical to keep the cell culture vessels perfectly clean, unscratched, and free from damage. 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. Avoid wiping the surface as this may leave particulate or residues. Cell culture plastic is very easily scratched.

Smudges and scratches will affect image quality.

Non-recommended vessels may cause problems such as

- HoloLids not fitting
- Recommended volumes for HoloLid use are not correct
- Vessel pattern does not fit
- Image quality may be affected

SOFTWARE

Software installation

App Suite is installed through a single installer executable with guidance 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/). Open the installer and follow the instructions to install the software.

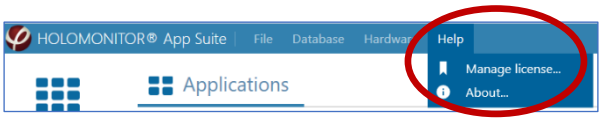
Software activation

After successful installation, the software needs to be activated. A request for activation will appear automatically the first time someone uses the software. The activation step usually requires internet access but may be performed without internet access by using another, internet-connected, computer.

One software license can be activated on two computers, but a trial license can only be activated once on one computer.

Your sales representative will supply you with license and password at the time of installation.

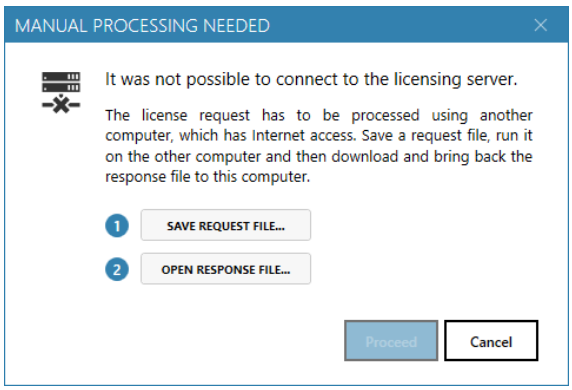
Activating license online



After installation, go to Help in the Top menu and select Manage license. Then enter the License ID and Activation Password which have been provided. Once App Suite is activated, the purchased applications become available.

Activating license offline

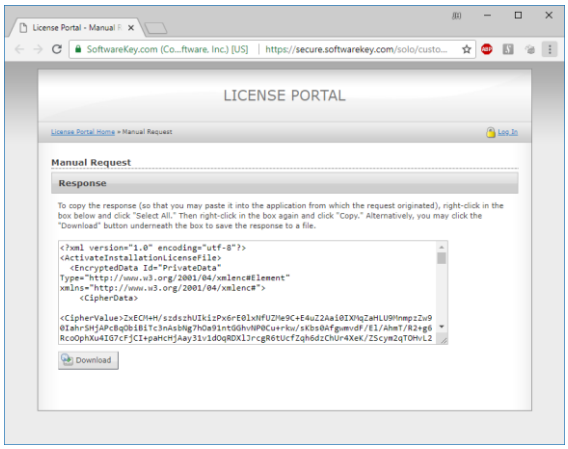
When Internet is not available on the computer it is still possible to activate, refresh and return a license using another computer with Internet access.



The entry to all actions is the same as when Internet is available, i.e., through buttons on the license dialog. The Manual Processing Needed dialog is shown to the user when license activation is attempted without internet connection.

The user must save a file, transfer it to another computer via a USB-drive, run it there and bring back a response file.

- Start by clicking on Save request file. This brings up a standard file browser dialog, prompting the user to save a file called request.html.
- Save request.html onto a USB-drive. The file is active for 30 minutes only.
- Transfer the USB-drive to a computer with Internet access.
- Clicking on request.html will open a browser and contact the user-part of the license server. The request will be processed and then it is possible to download a response as a file by clicking Download. A new file called Response.xml is created.



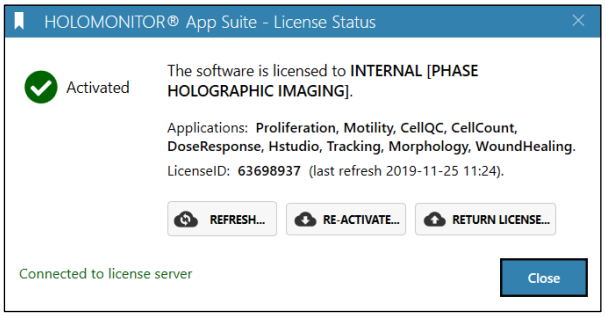
- Save the Response.xml file to the USB-drive and take it back to the first computer where the Manual Processing dialog waits for it.
- Click Open response file and browse to the Response.xml file on the USB-drive and the Manual Processing dialog becomes ready and Proceed button is activated.
- Click Proceed and the response is processed just as if Internet had been available.

License status

The current license status can always be accessed via the Help tab in the top menu bar. Here it is possible to refresh, re-activate or return the current license.

Refresh license

Refresh will allow access to newly acquired functions after an update of the license. This may be necessary for newer versions of the software or if new applications have been purchased.



Re-activate license

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

Return License is used to deactivate the software on the current computer. The license can then be activated on another computer. Each license can be active on two computers, but a trial license can only be activated once on one computer. For issues with returns, please contact support@phiab.com.

Internet access

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

Windows Update may restart the computer, thus disrupting any ongoing experiments. Therefore, it is recommended to schedule updates to a time when the computer is not used and to disable internet connection or enable Flight mode 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

Hardware installation

HoloMonitor should be delivered and assembled under guidance of trained personnel, either on-site or remotely, to ensure that the instrument is correctly assembled and calibrated. The installation movie shows installation step by step https://phiab.com/video-gallery/holomonitor-how-to-tutorials/.

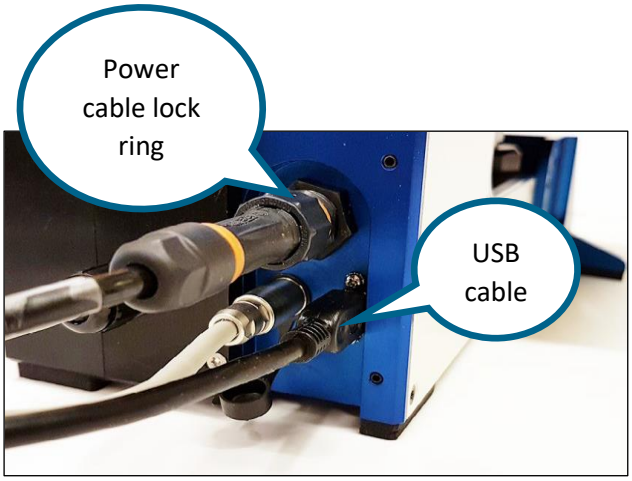
Two HoloMonitor can be connected to each computer with App Suite.

Instrument assembly inside incubator

1. Use a paper towel damped 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 computer power cable to the wall outlet.



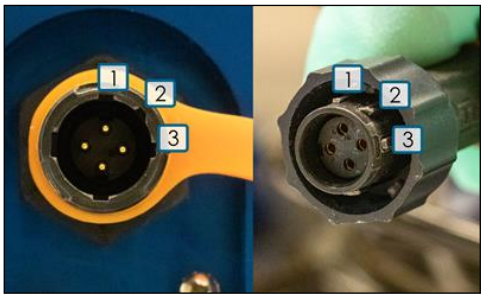
4. Clean the instrument exterior. The instrument can be wiped down with a paper towel damped with 70 % ethanol. Cleaning must be performed so that no liquid enters the interior of the instrument.

5. Attach a HoloDry.

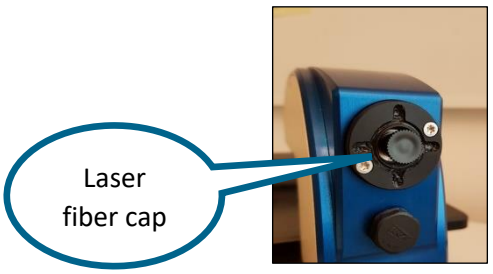
6. Place HoloMonitor inside the cell culture incubator. Make sure that there is space for the motorized stage to move.

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

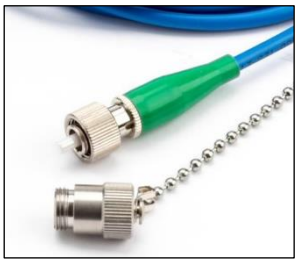
7. Wipe down the power cable with ethanol and connect it to the instrument. Ensure that it is properly aligned before tightening the lock ring. The contact has ridges in a pattern that will fit slots in the connector. When the connector is properly aligned, the lock ring will be guided into place when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



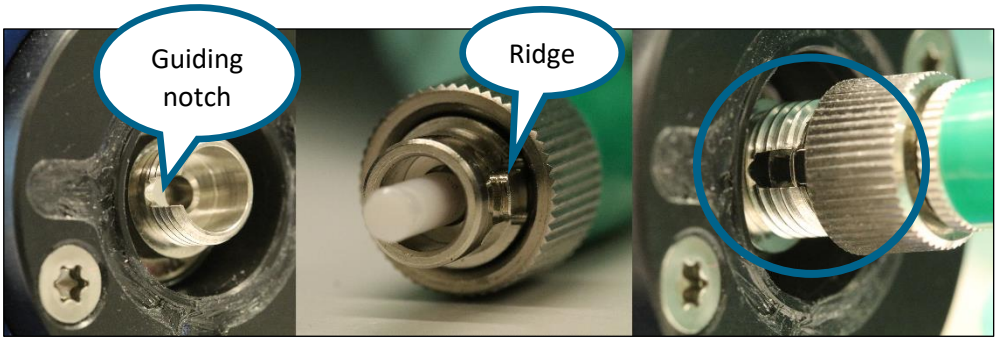
8. Remove the black cap covering the laser fiber connection point. Save the protective cap for future use.



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



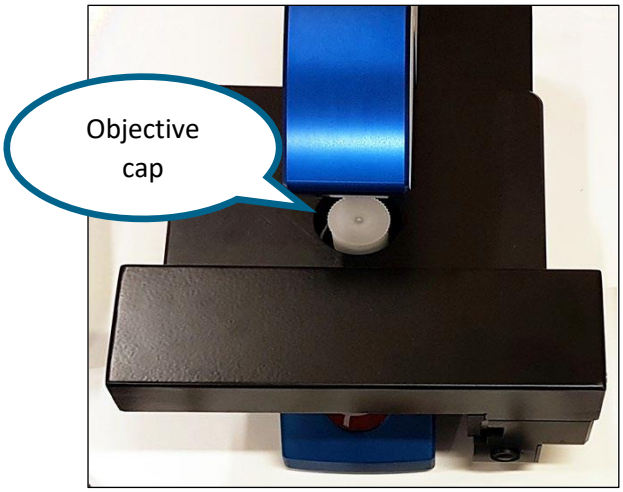
10. Gently insert the optical fiber tip into the fiber connector. Make sure that the metal ridge on the fiber tip is aligned with the guiding notch in the connector socket before tightening the lock ring (see image below).



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 has been acclimatized, the objective protective white cap can be removed. Save the protective cap for future use.



14. Run the Auto-calibration procedure.

To avoid build-up of internal condensation, the instrument power must always be switched on and have a HoloDry attached 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.

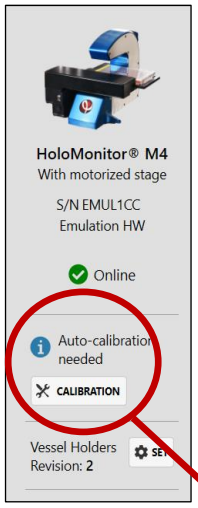
The laser needs approximately 30 seconds to warm up before the laser light is visible.

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

To ensure optimal laser function, the surrounding temperature for the laser unit must be maximum 24 C. Do not place the laser unit in direct sun light.

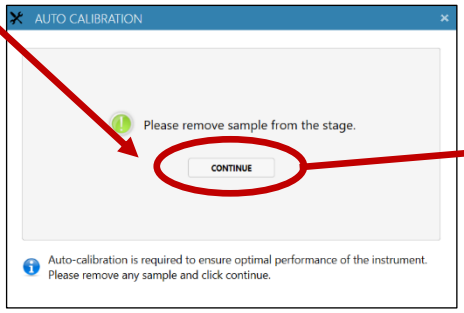
The HoloMonitor laser unit is a Class 2M laser. Avoid laser light exposure to eyes when handling the disconnected laser fiber.

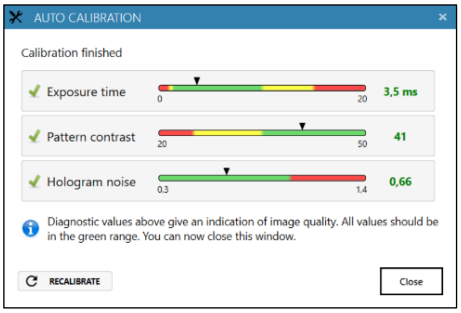
AUTO CALIBRATION



Perform Auto-calibration before each use, especially when setting up an experiment. Auto-calibration gives a holographic background image that is used by App Suite to lower background noise and increase image quality. The calibration procedure takes approximately one minute. A Calibration button can also be found in the Live View tab under Settings.

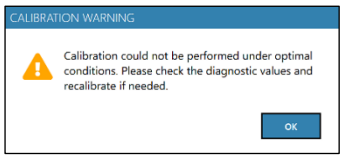
1. Make sure that the laser has been switched on for a couple of minutes.
2. Start App Suite and wait for HoloMonitor to initialize.
3. When HoloMonitor is ready, click the Calibration button below the instrument information.
4. Follow the instructions and ensure that there is no sample on the stage.
5. Click continue, and the calibration is performed automatically.

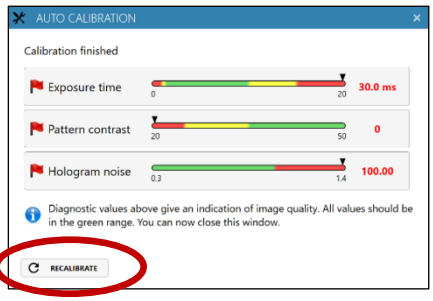




When the calibration is finished, the calibration wizard will display three diagnostic values measuring the image quality. All values (indicated by black arrow heads) should be in the green range.

If App Suite fails to perform a successful calibration, a Calibration warning dialog box will appear. The calibration wizard will show which values that are not acceptable.





Try cleaning the instrument by following the Instrument Cleaning Guide (see Cleaning Instruction). After verifying that HoloMonitor is properly installed and cleaned, click Recalibrate.

For more information regarding auto-calibration troubleshooting see Troubleshooting chapter.

HOLODRY™

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

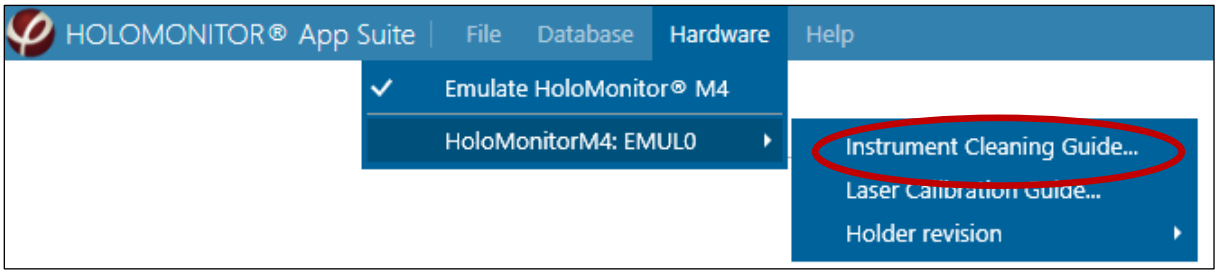
For further information, see the HoloDry protocol (https://phiab.com/resources/manuals-protocols/).

New systems are adapted for HoloDry, but older microscopes need a simple upgrade, where the spout replaces the previous filter plug.

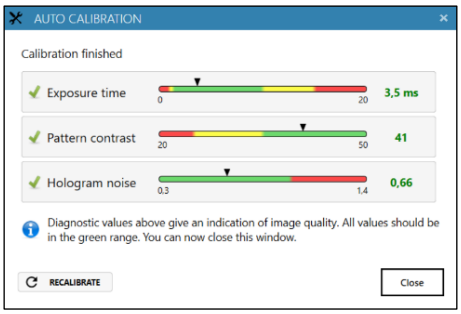
CLEANING INSTRUCTIONS

Cleaning optical surfaces

Go to the title bar, select Hardware menu, current HoloMonitor and activate the Instrument Cleaning Guide. Follow the instructions in the Instrument Cleaning Guide window.



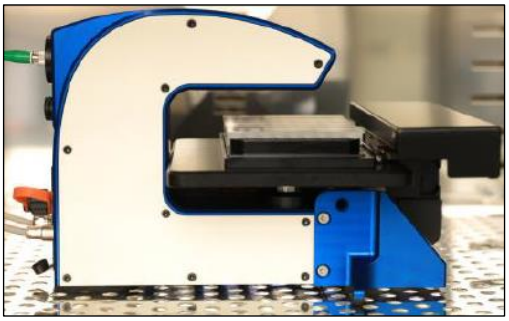
You might have to repeat the cleaning several times depending on how dirty the optics are. Afterwards, re-run the Auto-calibration. After successful auto-calibration all values (indicated by black arrow heads) should be in the green range.



If any of the Diagnostic values remain outside the green range after several cleaning attempts, contact the technical support at support@phiab.com or your local distributor for further assistance.

Cleaning instrument exterior

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

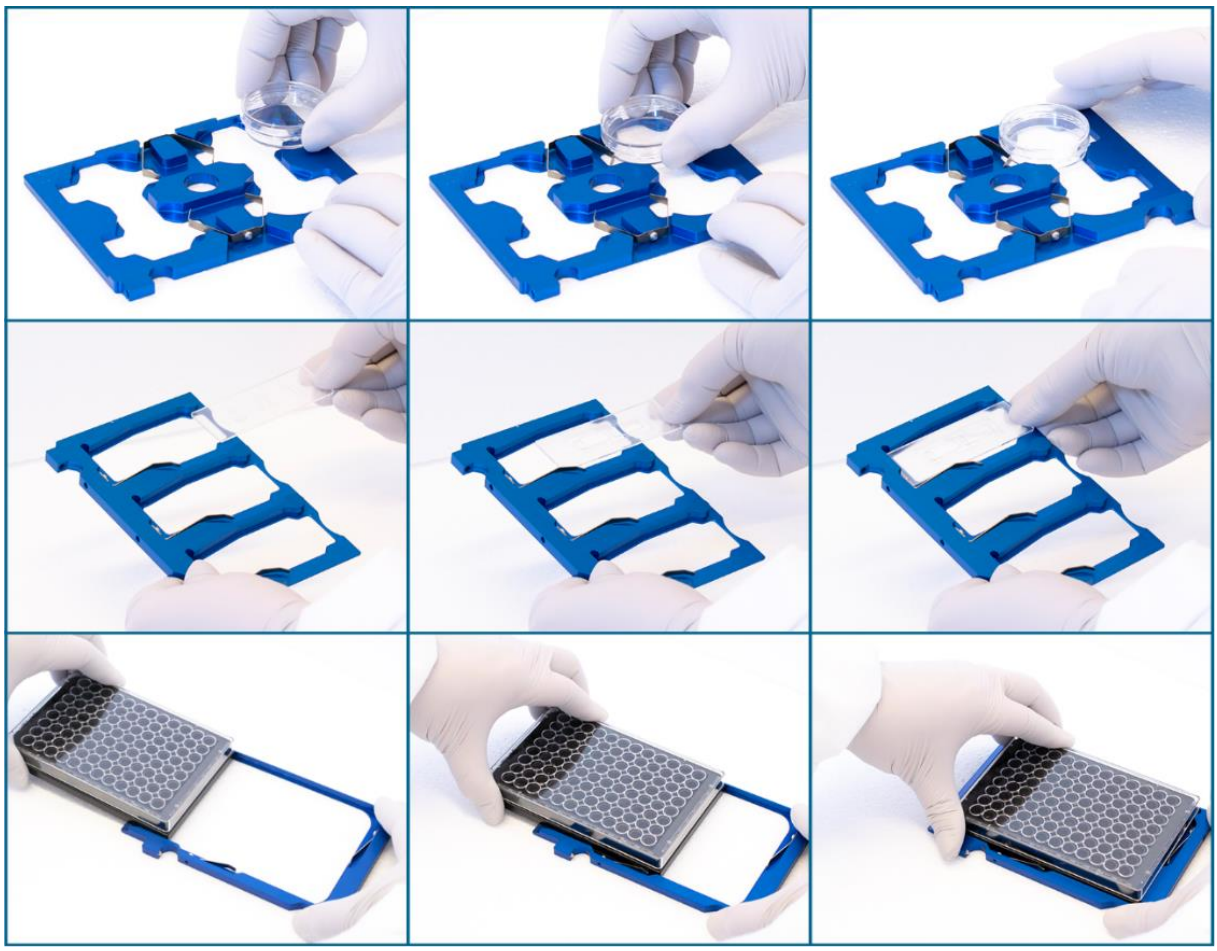


Avoid having any liquid enter the interior of the instrument.

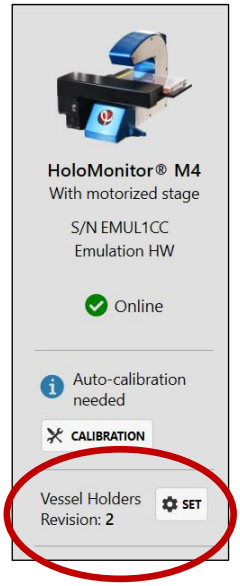
On a regular basis, once every week, wipe down the cables connecting the instrument as well as the body and stage of HoloMonitor inside the incubator using 70% ethanol to minimize any microbial build-up.

VESSEL HOLDERS

HoloMonitor is equipped with a set of blue cell culture vessel holders called *revision 2*. There is a standard Ø 35 mm Petri dish holder, a microscope slide holder, and a standard multiwell plate holder. In App Suite, there are vessel maps to match the holders.



There is an older black version of holders (*revision 1*) with a microscope slide holder, a standard multiwell 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 and attaching the vessel holder

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

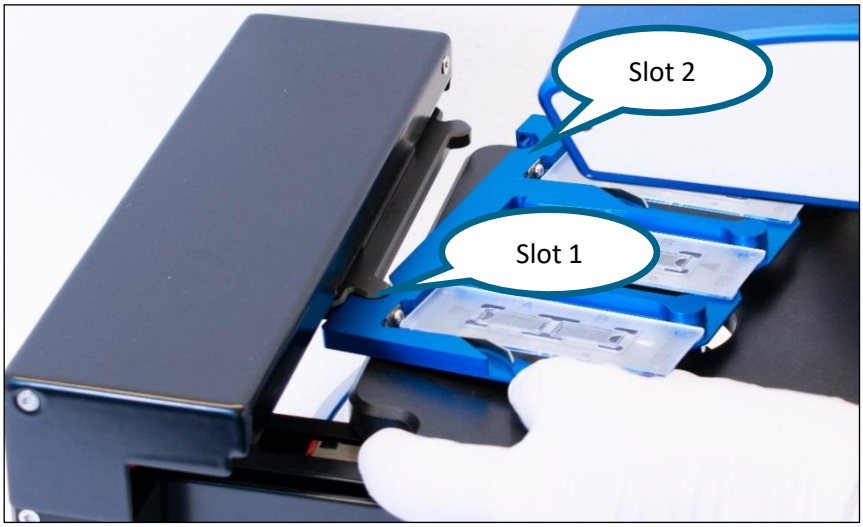
1. Place the culture vessels on to the holder. Place the vessel as shown above and ensure that they are correctly fitted on to the holder. For multiwell plates, slide them sideways along the tracks on to the vessel holder with the bevel/s (=cut off corners) according to the table below. There is a spring that holds the vessel in place.

Vessel	Bevel orientation
Sarstedt TC 6-well plate	Top left
Sarstedt lumox® 24-multiwell plate	Top left
Sarstedt lumox® 96-multiwell plate	Top left
ibidi® µ-plate 24 Well Black	Top left
Eppendorf CCCadvanced® FN1 - 6 well	Bottom right

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

2. 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.

3. Place the vessel holder on the HoloMonitor stage, click slot 1 in place, and thereafter slot 2. Slot 2 has a ball detent (the little knob in slot 2), that allows the holder to slide into place.



LONG-TERM STORAGE

If HoloMonitor 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 be taken out of and placed back into the incubator repeatedly, as temperature fluctuations can cause disturbances. Once the HoloMonitor is removed for long term storage, preferably, leave at room temperature at least two days.

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

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

To extend laser unit working hours, it is recommended to disconnect the laser from AC power when HoloMonitor is not in use.

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

HOLOLIDS™

HoloLids™ have been specifically designed to eliminate image disturbances caused by surface vibrations of liquids 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 occurs inside cell culture vessels. For the lids to fulfill their function, an appropriate amount of cell culture medium needs to be added to each well.

As all vessel brands are slightly different, there are no standard HoloLids. Instead, they are fitted to specific brands and well sizes.

Vessels	HoloLid™ PHI cat. #	Final volume mL/well	Growth area cm2/well
Sarstedt TC-dish 35 (cat. # 83.3900)	71110	3.00	8.00
Sarstedt TC 6-well plate (cat. # 83.3920.005)	71120	3.00	8.80
Sarstedt lumox® 24-multiwell plate (cat. # 94.6000.014)	71130	1.90	1.90
Sarstedt lumox® 96-multiwell plate (cat. # 94.6000.024)	71140	0.18	0.34
ibidi® µ-dish 35 mm, high (cat. # 81156)	71111	2.50	3.50
ibidi® µ-plate 24 Well Black (cat. # 80241)	71131	1.70	1.54
Eppendorf 6-well plate (cat. # 0038110010)	71150	3.00	9.40

HOLOMONITOR® APP SUITE SOFTWARE

Introduction

HoloMonitor App Suite™ software is specially designed for capture and analysis of holographic microscopy images using HoloMonitor. It enables the user to capture both single images and time-lapses. Based on images captured at regular intervals at the same position, time-lapses can be used to study slow cell processes.

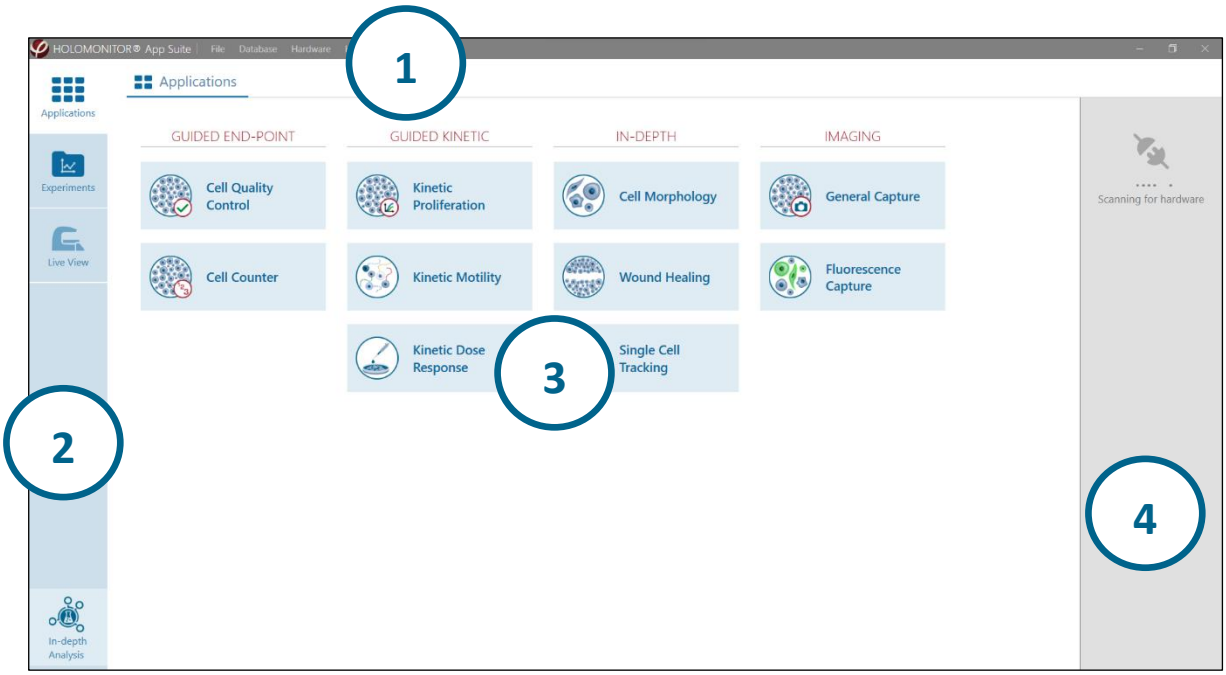
The imaging procedure is simple and does not affect the cells in any measurable way. To simply count cells takes less than a minute. Confluence measurements and morphology measurements are performed simultaneously. To further analyze the cell morphology, either the built-in analysis functions can be used, or data can be exported as .xlsx or .csv files for further work in Excel or similar software. Using App Suite, 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 Cell Counting, Cell Quality Control, Kinetic Proliferation, Kinetic Motility, Kinetic Dose Response, Cell Morphology, Wound Healing, and Single Cell Tracking. 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. All protocols can be found on https://phiab.com/resources/manuals-protocols/.

In order to activate applications, specific licenses are needed. For more information, please visit www.phiab.com or contact your distributor or support@phiab.com.

App Suite works with all general Windows commands, such as ctrl A and shift-ctrl-click.

Overview



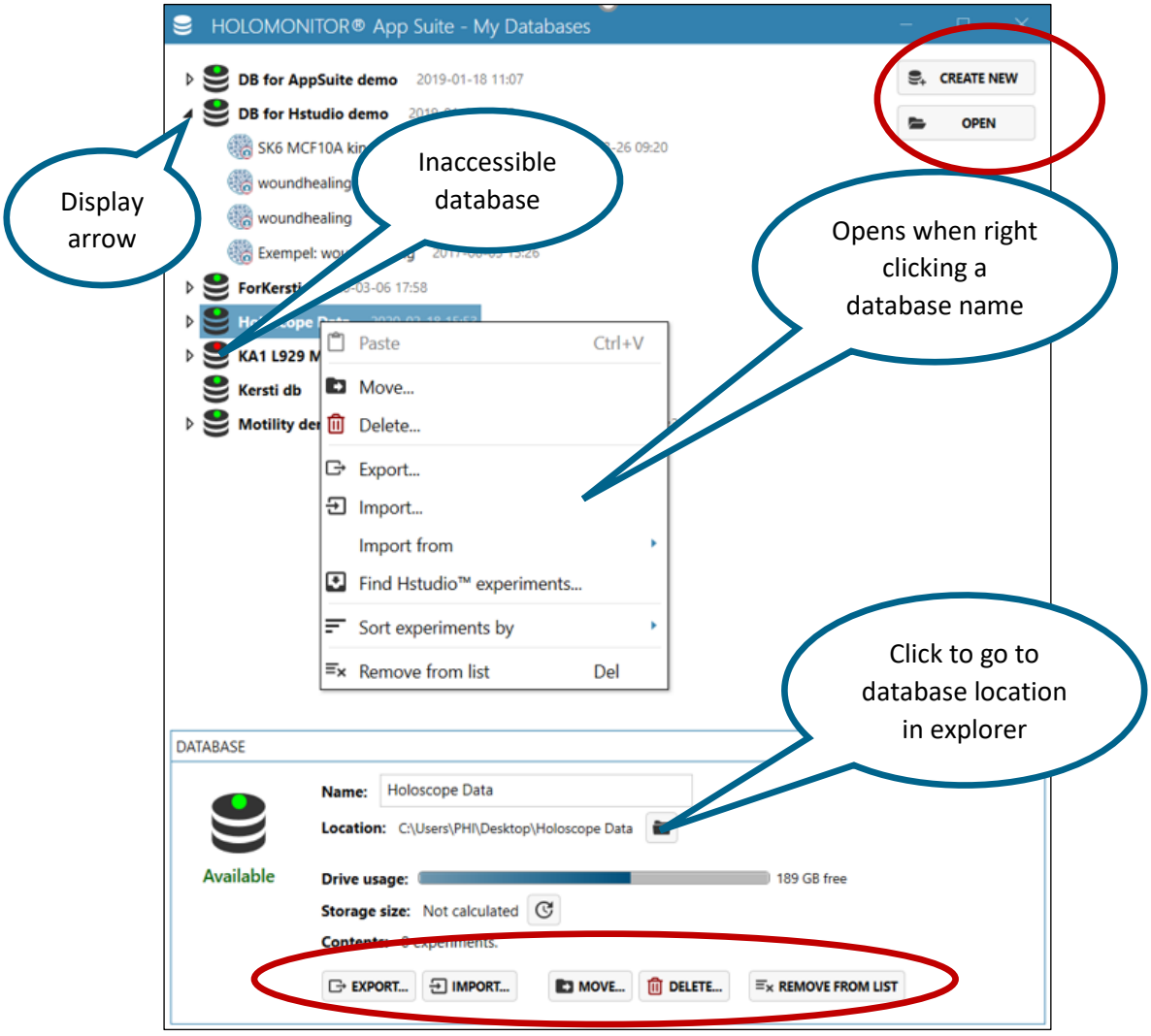
1. In the top menu bar, the My Databases, the Instrument Cleaning Guide, the Laser Calibration Guide and the HoloMonitor Emulator can be accessed. My Databases is a database handler that allows for copying, editing, transferring, and deleting data. The HoloMonitor Emulator is a virtual HoloMonitor. When 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, Experiments, Live View and In-Depth Analysis. The Applications tab contains the applications library and is shown by default.
3. In the center panel, details and functions regarding the selected App Suite component tab to the left are displayed.
   - By default, the Applications library is displayed. Click an application name to access a short description, as well as a button to start setting up the application. 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. It is only for viewing, not for image capture.
   -In-depth analysis accesses software for detailed studies of individual cells and for wound healing analysis.
4. The right-side panel presents connected instruments, calibration status and vessel holder specification. Two instruments can be connected simultaneously.

Database handling and data backup

My Databases is a database handler where HoloMonitor data can be organized and managed. All acquired images are stored in databases that can be copied, exported, moved to other databases, or deleted. Databases that are already known to the software are displayed in a list in My Databases. Databases stored both on the computer and on external units can be accessed.

Access the databases

Access My Databases, which is a database handler, through Database in the top menu bar.



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.

Experiment data will be stored in the currently selected database. During the experiment setup, ensure that the currently selected database is stored on the computer connected to the HoloMonitor.

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.

We recommend creating new databases for every couple of experiments. Database handling will be much more convenient if the databases are not over-loaded.

In My Databases, there are buttons to Create New or Open already existing databases, as well as Export, Import, Move and Delete databases and to Remove 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.

We recommend only keeping actively used databases on the list as overloading the list may result in sluggish data access.

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.

The new folder where the database is going to be located has to be empty.

Browse to access existing databases

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

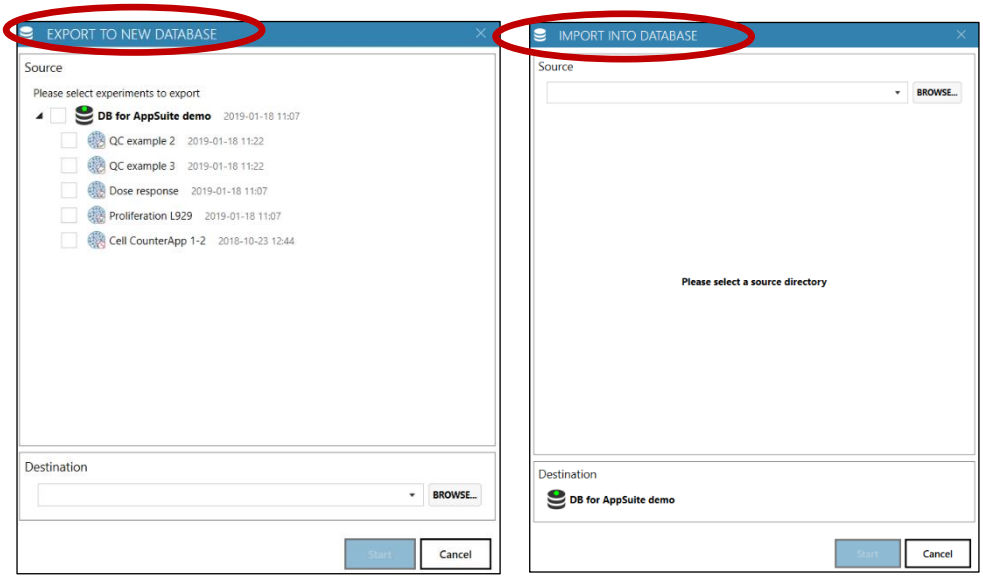
Export and import databases

Export

Select a database and then click Export to open the Export to new database window (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 Import into database window (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 Open database list will not delete it from a storage location.

Find Hstudio experiments

Experiments created 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 list of databases in My 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.

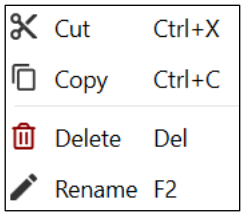
If the Hstudio experiment is accessed through the In-Depth analysis, it does not need to be converted to App Suite format. The data only needs to be converted if results from Guided Assays will be extracted.

Converting an experiment from Hstudio to App Suite does not duplicate the images. Instead, the Hstudio data is made available for App Suite. Thus, if the experiment is later deleted in App Suite it will be deleted for Hstudio as well.

Not all older Hstudio experiments are possible to convert to App Suite experiments. If that is the case, experiments will still be available for analysis in App Suite through the In-Depth Analysis tab. Use the database drop-down list in In Depth to access that database.

Copy and transfer individual experiments

Click the arrowhead beside a database in My Databases 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 HoloMonitor data

Create a database backup

In My Databases, there are two ways to create a backup.

Follow the instructions above to create a new database in the back up storage device. Then right-click the Experiment you wish to backup and select Copy. 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.

Use My Databases for all operations. First create a new database on the computer. Then browse to 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.

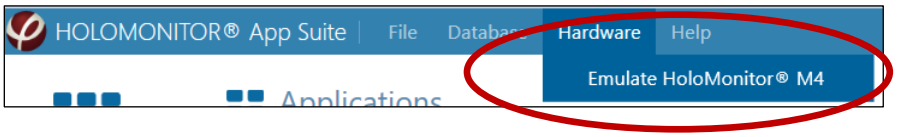
Databases can always be copied and moved using windows explorer, just like any folder or map in the computer. Ensure that all files in the database folder are copied, otherwise the data cannot be accessed. If possible, zip the folder with the database before copying it.

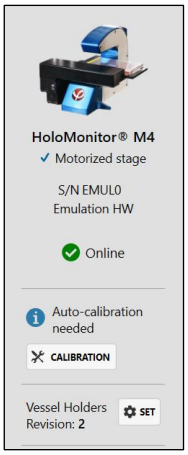
Analyzing data on a remote database

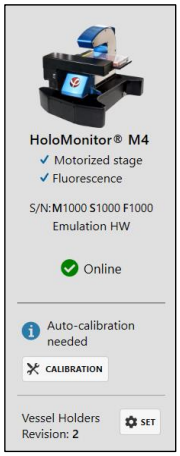
Note that analyzing data on an external hard drive or server (local or cloud based) works well only if the USB or internet connections are stable. If the connections are unstable, data can be lost.

HoloMonitor hardware

HoloMonitor Emulator





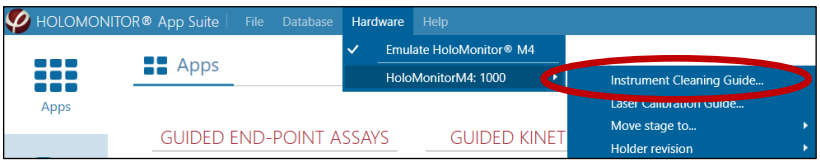


The HoloMonitor Emulator is a virtual HoloMonitor. When activated it gives access to all live features of the software as if a HoloMonitor was connected to the computer. It can be accessed through Hardware in the top menu bar. Once activated, it will show up in the instrument panel to the right.

The emulator needs an active license to access the software, applications, and functions similar to an actual HoloMonitor.

Instrument cleaning guide

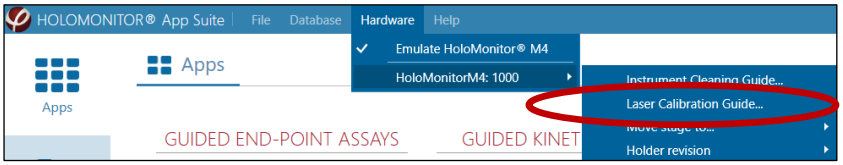
The instrument cleaning guide gives a step-by-step instruction on how to clean the exterior optics of HoloMonitor.



The cleaning guide is only available if HoloMonitor is connected, or the emulator is activated.

Laser calibration guide

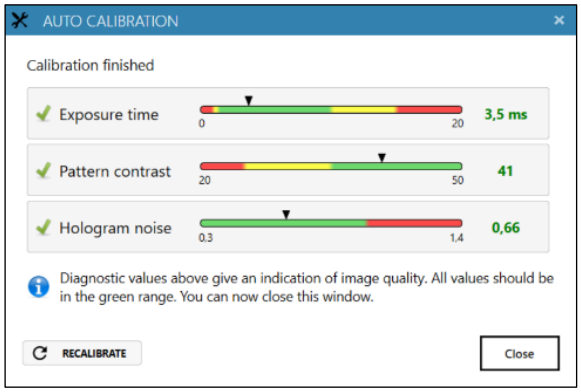
The laser calibration guide gives a step-by-step instruction on how to calibrate the holography laser.



The laser will need periodic recalibration. The need for laser calibration is indicated by an unstable Pattern contrast value in the green range, or a value in the yellow/red range when autocalibrating.

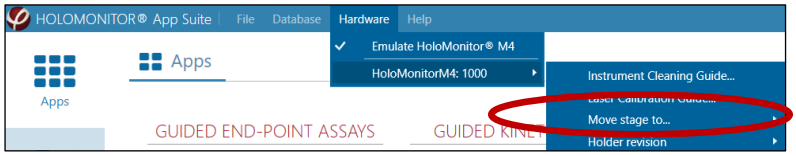
When needed, activate the Laser Calibration Guide and follow the instructions. Afterwards, re-run the Auto-calibration wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range and stable.

If the autocalibration problems persist even after laser calibration and cleaning, please contact support@phiab.com.



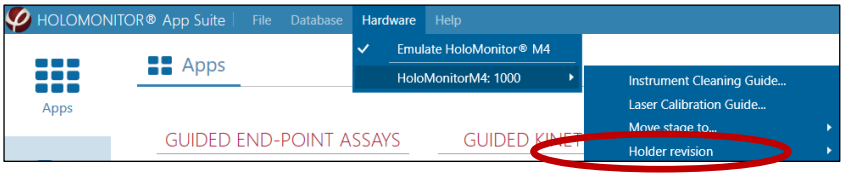
Move stage to

The move stage to function moves the stage to a position where it can be easily removed from the body of HoloMonitor. This is necessary when shipping to service.



Holder revision

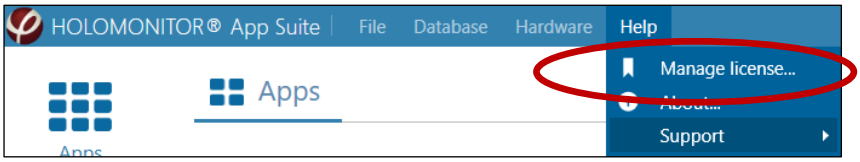
There are two generations of sample holders, the older black (revision 1) and the newer blue (revision 2). If you switch from black to blue holders, please update the revision number.

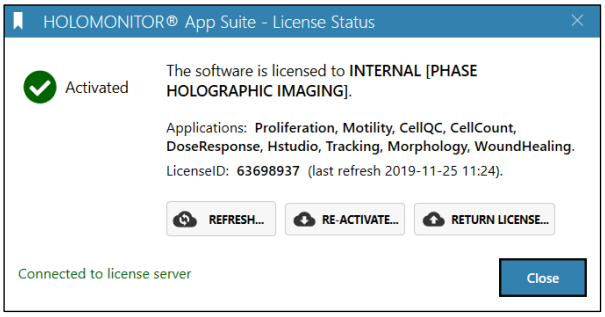


Help

Manage License

The current license status can always be accessed via the Help tab in the top menu bar. Here it is possible to refresh, re-activate or return the current license.





Refresh license

Refresh will allow access to newly acquired functions after an update of the license. This may be necessary for newer versions of the software or if new applications have been purchased.

Re-activate license

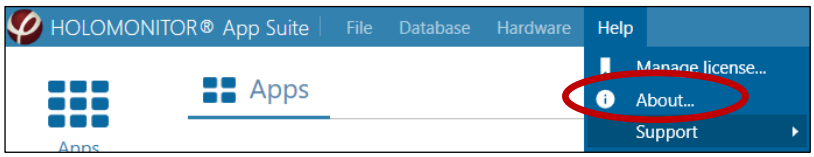
Re-Activate makes it possible to switch to another License ID, *e.g.* switching from a time-limited to a standard license, without reinstalling the software.

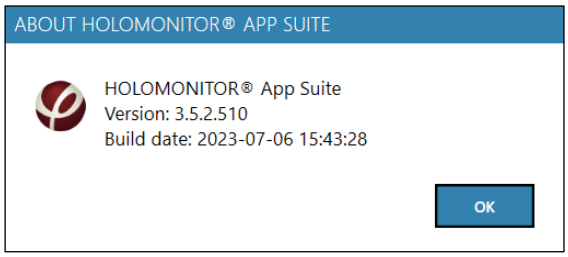
Return license

Return License is used to deactivate the software on the current computer. The license can then be activated on another computer. Each license can be active on two computers, but a trial license can only be activated once on one computer. For issues with returns, please contact support@phiab.com.

About

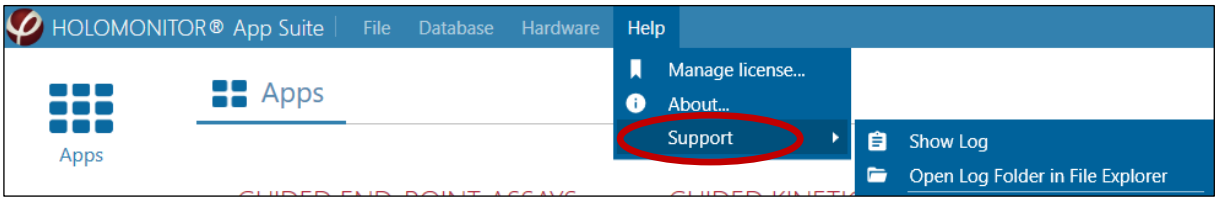
About gives information about the software version.





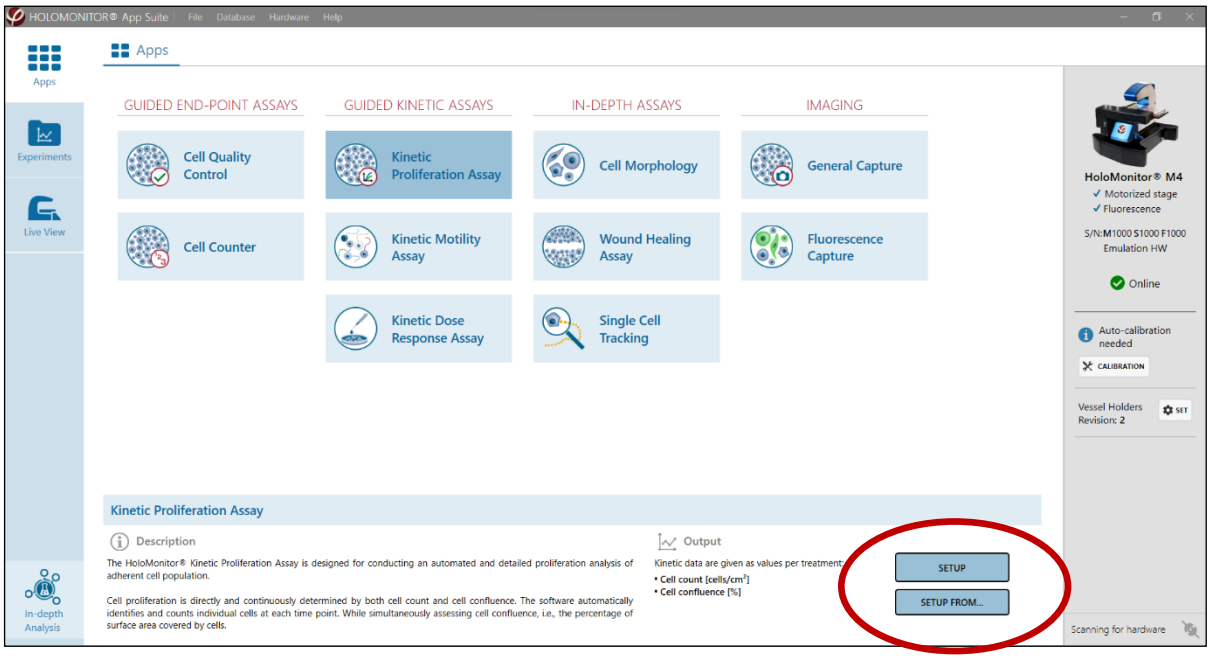
Support

With the Support function, the computer logs for use of HoloMonitor and App Suite can be accessed. This is often necessary for support.



Applications

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



Applications in short

Cell Quality Control

Cell Quality Control can be used to ensure that the cell count and/or confluence for adherent cells is appropriate e.g. at the start of a long term time-lapse 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 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

Kinetic Proliferation is designed for a continuous automated 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

Kinetic Motility is designed to explore the motility of adherent cell populations 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

Kinetic Dose Response is designed for automated 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 can be displayed for any selected time point. The output is cell count, cell confluence and mean cell volume.

Cell Morphology

Using Cell Morphology, a wide range of features for individual adherent cells as well as cell populations can be analyzed. Scatterplots visualize correlations between control 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

Using Wound Healing, cell migration and/or cell proliferation can be studied over time using adherent cells. Kinetic data for gap closure and 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 Single Cell Tracking, individually selected adherent cells can be monitored and analyzed for cell motility, migration, and morphology. 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

General Capture 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.

Fluorescence Capture

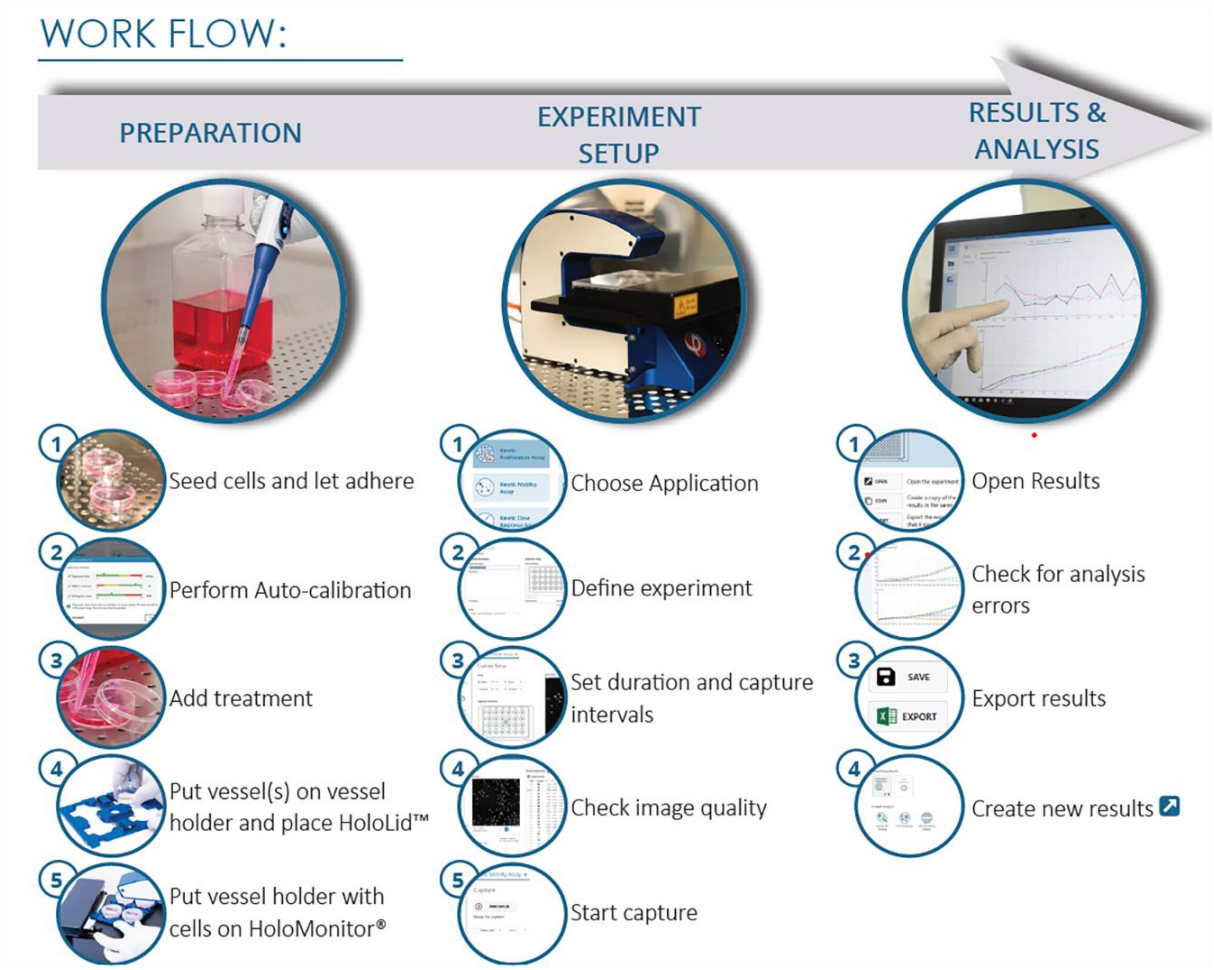
Fluorescence Capture can be used for a flexible setup of an experiment with fluorescently labeled cells. The created image databases can be used for analysis by multiple licensed applications. The Holographic images in the experiment can be analyzed both by the guided applications and the In Depth applications, while the fluorescence images only can be analyzed using the Single Cell Tracking and Cell Morphology 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 at the back of this manual.

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 Assays 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/.



Setting up an experiment

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

The exact procedure for each application is described in the corresponding protocol. The protocols can be found at https://phiab.com/resources/manuals-protocols/. This manual describes the general functions.

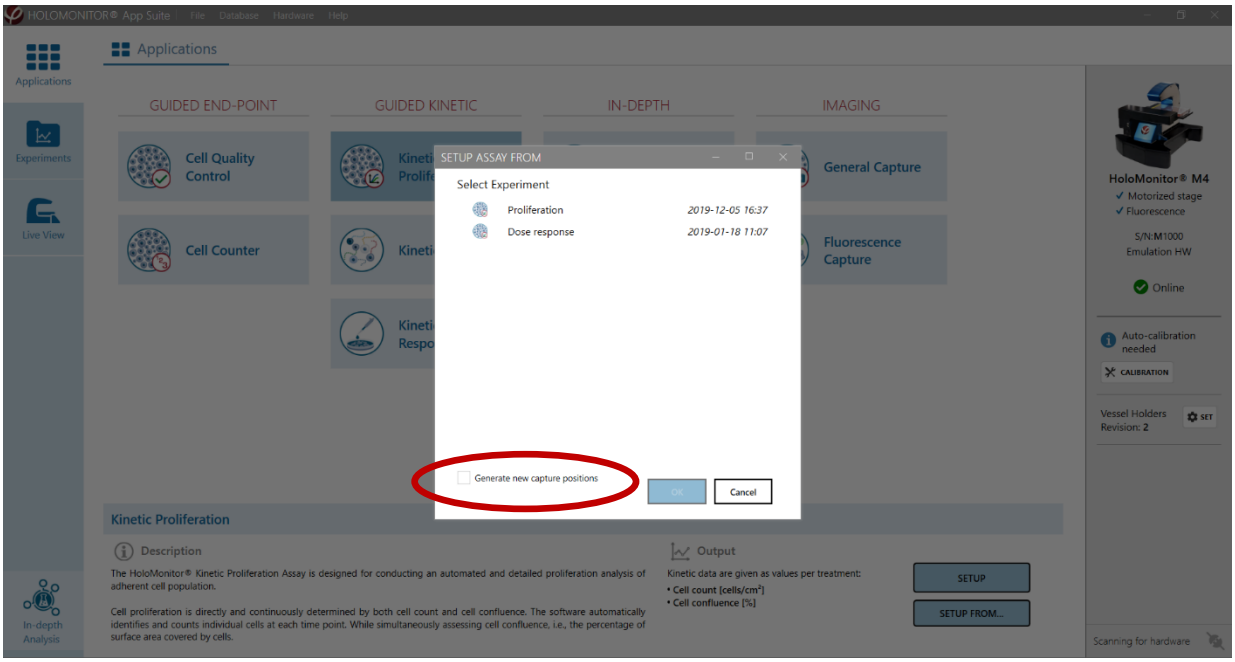
Setup

Click the Setup button to begin experiment set up.

Setup from

Click the Setup From button to set up a new experiment based on a previous experiment. It is possible to set up a new experiment from previous experiments of the same type (see image below).

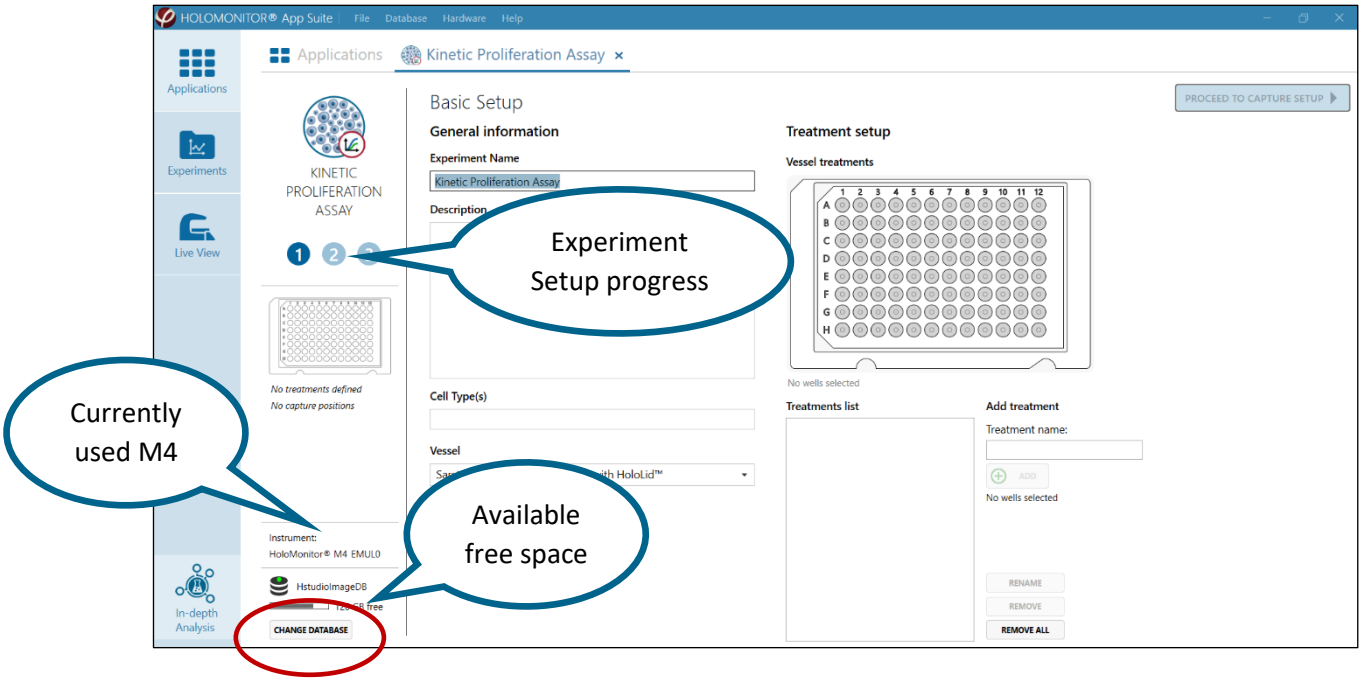
All settings from the selected experiment will be applied by default for the new experimental setup, including the capturing positions. To create new capturing positions for the new experiment, check Generate New Capture Positions.



Experiment progress

Once Setup 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.

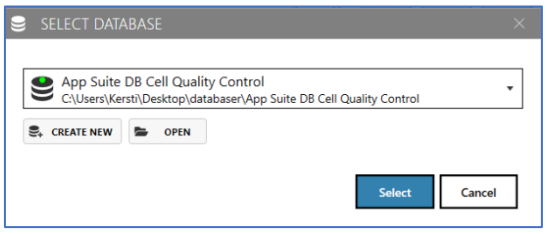
As the experiment setup proceeds, more details will be displayed below the setup progress indicators, such as cell culture vessels used, number of treatments added, the duration of the experiment and the storage space required.



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. Here it is possible to switch to another database or create a new database. The database 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.

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

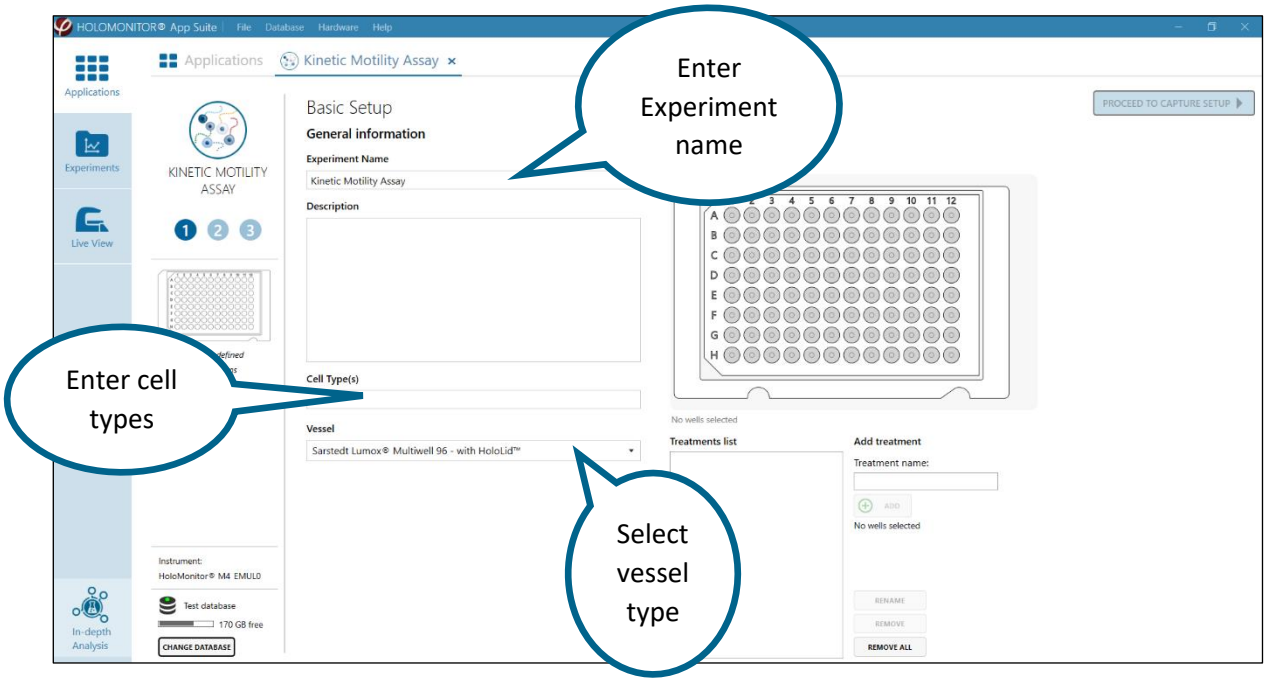


To make data access easier, avoid overfilling the databases. Create new data bases for every couple of exeriments or every set of experiments.

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

Here, users will describe the experiment, select which vessel to use and set which treatments that are added to each well. Other comments regarding the experiment can be entered as well. The information will be saved with the experiment and is 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 list. The following vessels are supported with the 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 I 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™

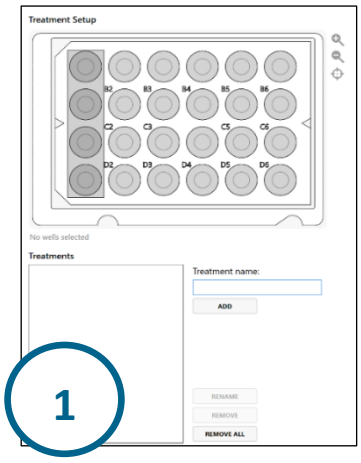
Blue vessel holder: revision 2

- 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 I 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

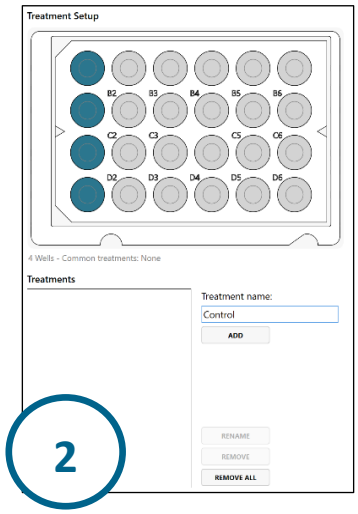
Here, which treatments or conditions that have been added to which wells is defined.

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

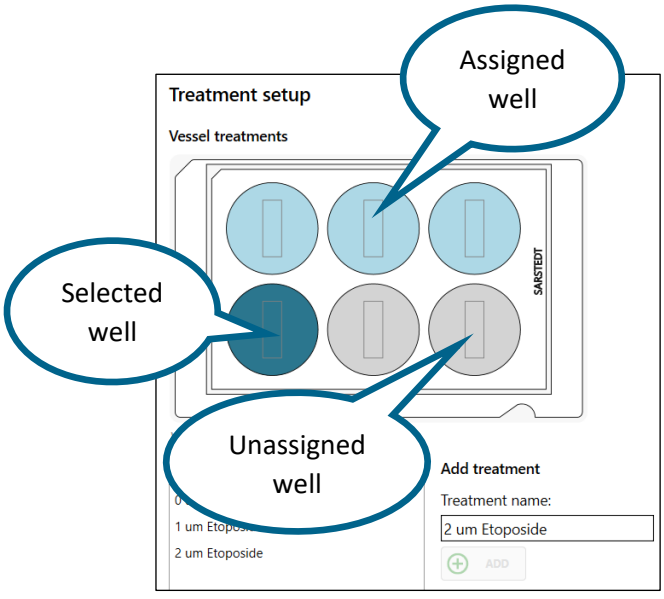
1. To define the first treatment, start by selecting wells in the vessel map. Wells can be selected by left clicking or by drawing a rectangle using the mouse cursor.
2. The selected wells will be dark blue.
3. Enter a treatment name in the text box and then click Add to save the selection with the treatment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

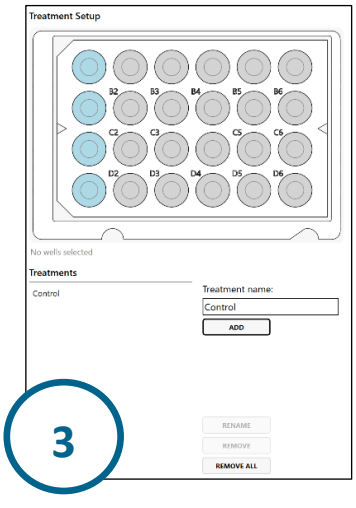


To define another treatment, select the next set of wells, type the treatment name and click Add. Repeat until all wells that are included in the experiment have been labeled.

All wells may also be added as a single treatment.

This page is slightly different for different applications.





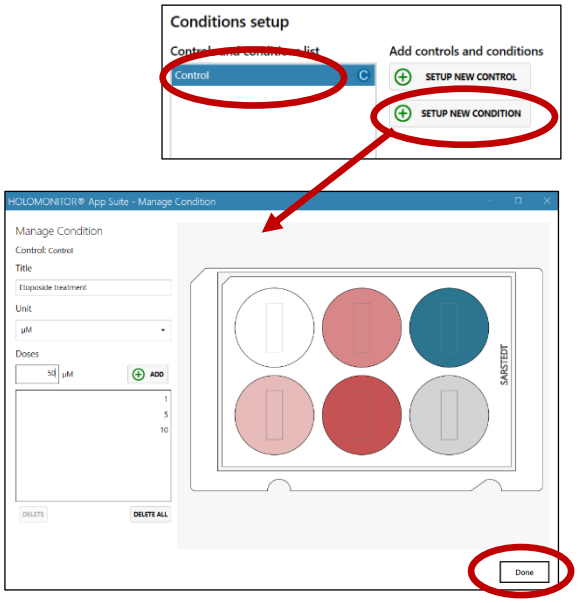
Set dose-response conditions

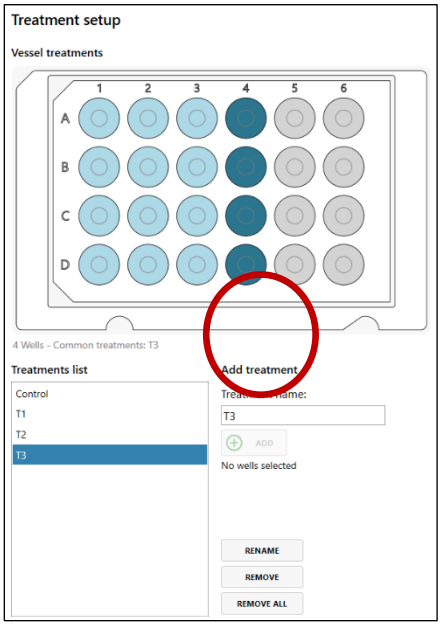
For Dose-Response experiments, a control must be set before other experiment conditions can be added. Clicking Setup New Control will open Manage Control (see image below). Select the well/wells that comprise a control, enter the control name and click done.

An 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

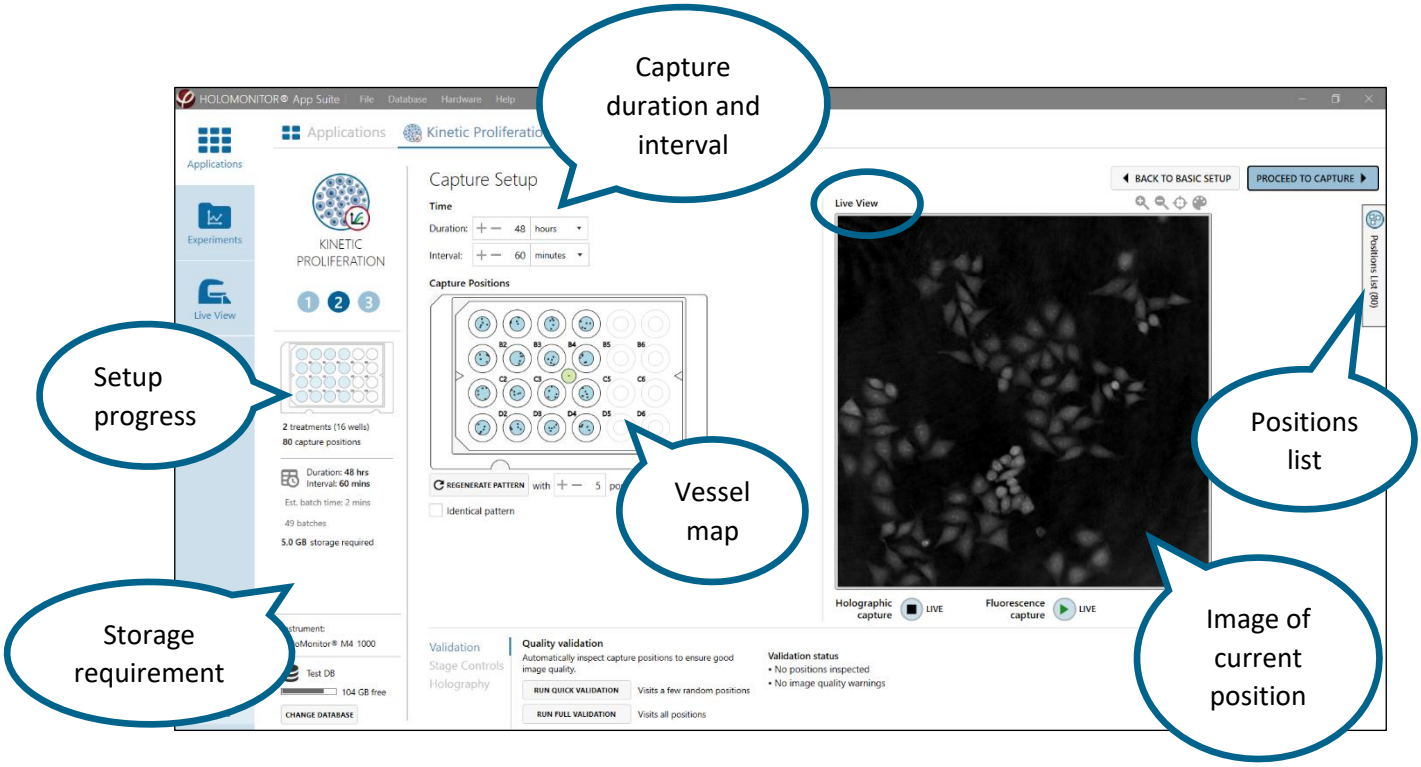
To Rename or Remove Treatments, select the items to be changed and use the buttons next to the treatments list.

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 default settings which can be adjusted as needed.

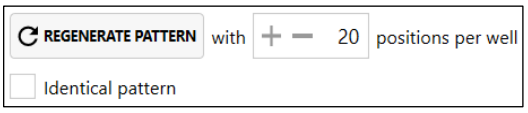


Set capture duration and interval

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

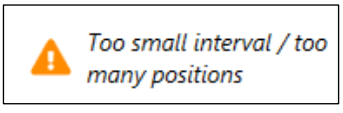
Set capture positions automatically

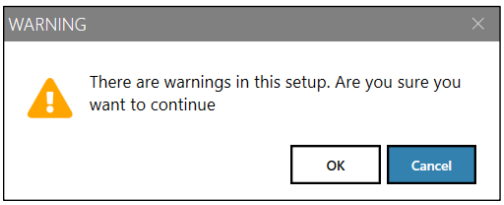
Change the number of capture positions either by clicking the "+" and "-", or by entering numbers in the text box below the vessel map. Then click Regenerate Pattern to generate a new random capture pattern which is unique for each well.



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

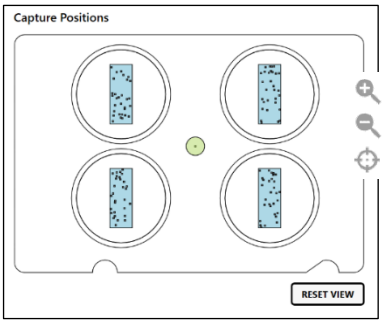
Note that if there are too many capture positions for the set time interval, it will not be possible to capture all positions as scheduled. A warning will appear, and a question if you want to continue the set up.





Moving the stage to the capture positions

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

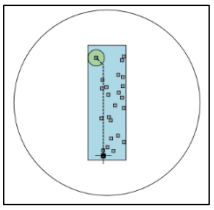


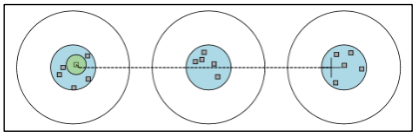
The green circle shows the current objective position. When clicking one of the positions, the motorized stage will move to that position.

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

Moving the stage to any position

Move the sample by clicking any 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).





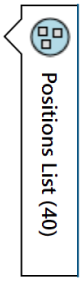
Scan and zoom the Vessel map

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.

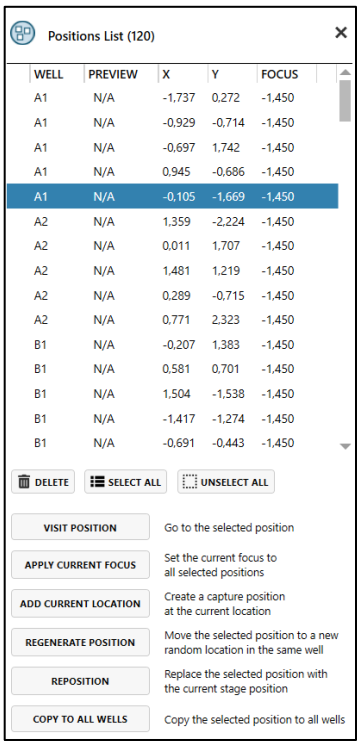
Using the buttons to the right of the vessel map is another way to zoom in and out as well as to reset to original size (see buttons to the right). The vessel map can also be reset by double-clicking the left mouse button.

Positions list

Each capture position that is marked in the Vessel map is also listed in the Positions list. Click the Position list button in the upper right corner of the page (see button to the right) to access the list. The list shows X and Y coordinates and 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:

- Open the Positions list.
- 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
- Inspect the image quality in the Live View window.
- Adjust the focal length as needed until a focus of approximately 1300 is reached.
- Return to the Positions List and click "Apply Current Focus"

- Click the Add Current Location button at the bottom of the Positions list to set the current position.
- Repeat the previous 5 steps until all desired locations have been added.

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.

Unselect All will unselect all selected positions.

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.

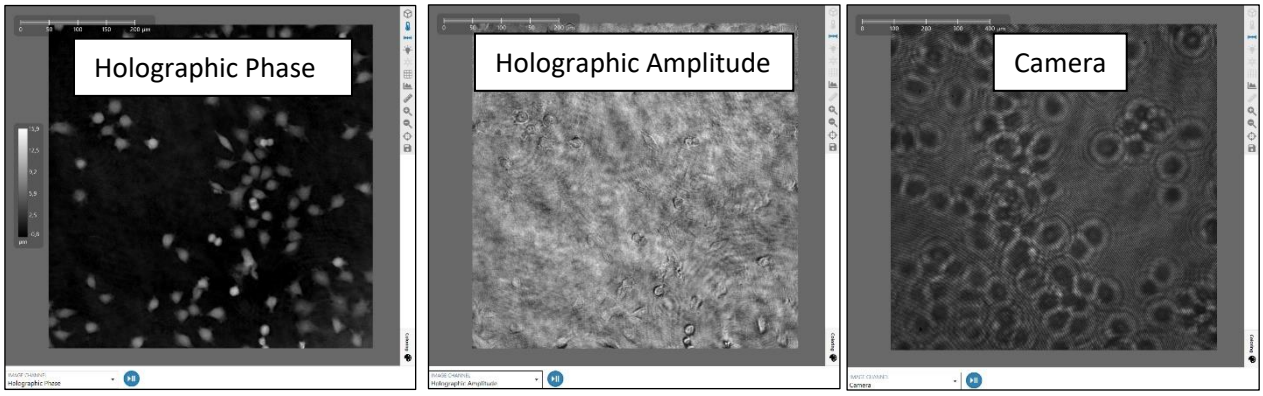
Reposition will replace the selected capture position with the current stage position.

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.

The cell image can be shown in three different channels:



Holographic Phase shows the reconstructed holographic image based on the phase information. This is the default image channel for the HoloMonitor 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 and amplitude images

Pause live imaging

Use the black start button)ause the showing of live images.

Play/pause function does not turn on and off holographic laser illumination

Pan and zoom

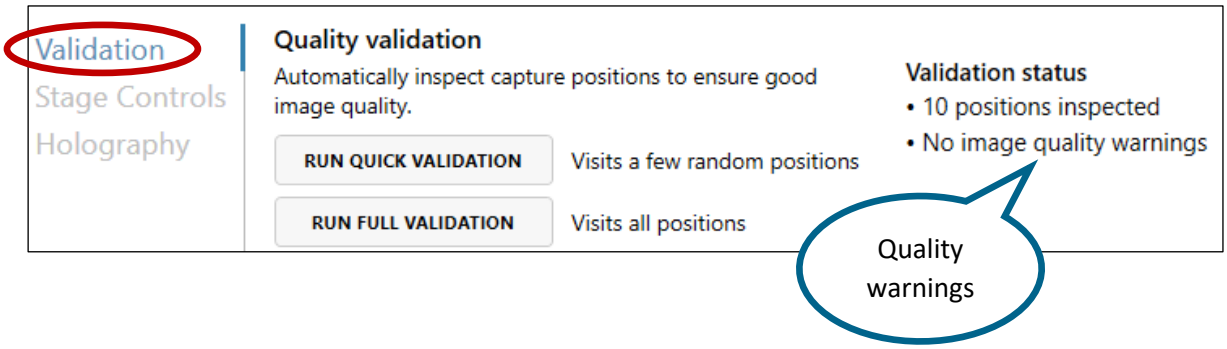
To better see the image, move it with a mouse by click-and-drag, and zoom in or out by click-and-scroll. Use the buttons to the right of the Live View window as another way to zoom in and out as well as to reset to original image size (see image to the right). The image can be also reset by double-clicking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

These actions will only pan and zoom the image. The sample and the motorized stage will not be moved.

Validating Capture Positions

To ensure that the experiment turns out well, the capture positions should be validated by 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.

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 all capture positions, while Full Validation investigates all capture positions.

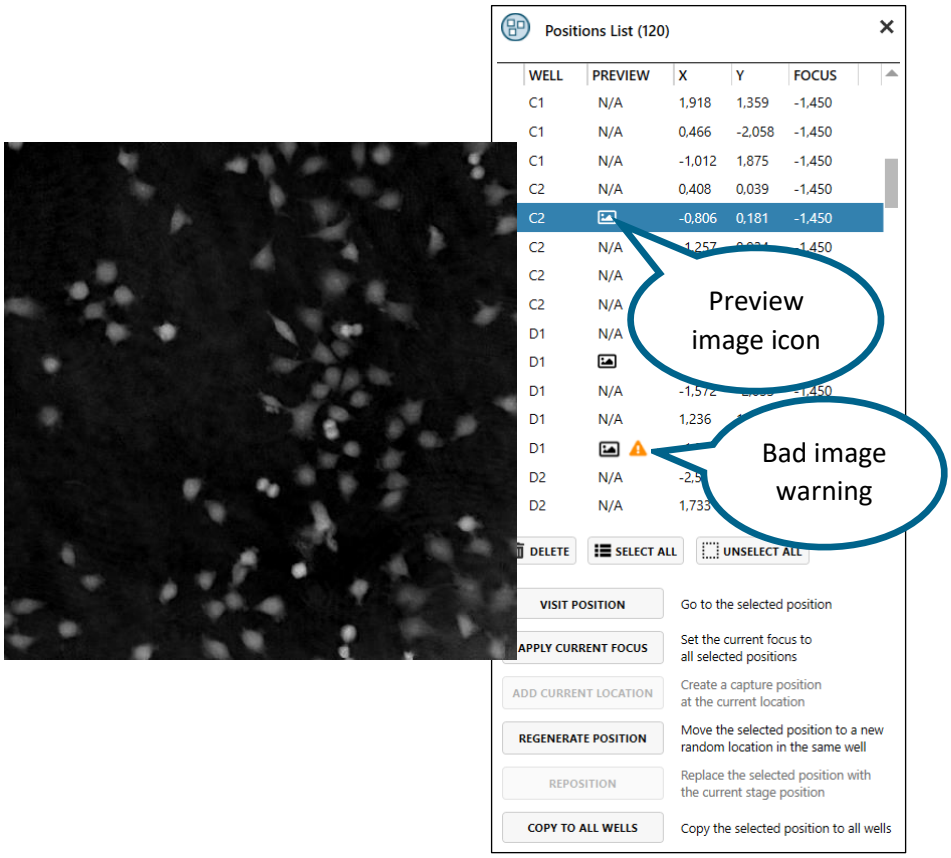
After validation, the software will either indicate that no image quality warnings were found, or that there were warnings.

Viewing validated positions

When a position has been validated, a preview image has been captured. The Previews will appear in the Positions list. Display the Positions list by clicking the list button in the upper right corner (see list button to the right).

Hovering the cursor on top of a preview icon in the Positions list will display the preview image temporarily (see image below).

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.

Unselect All will unselect all selected positions.

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.

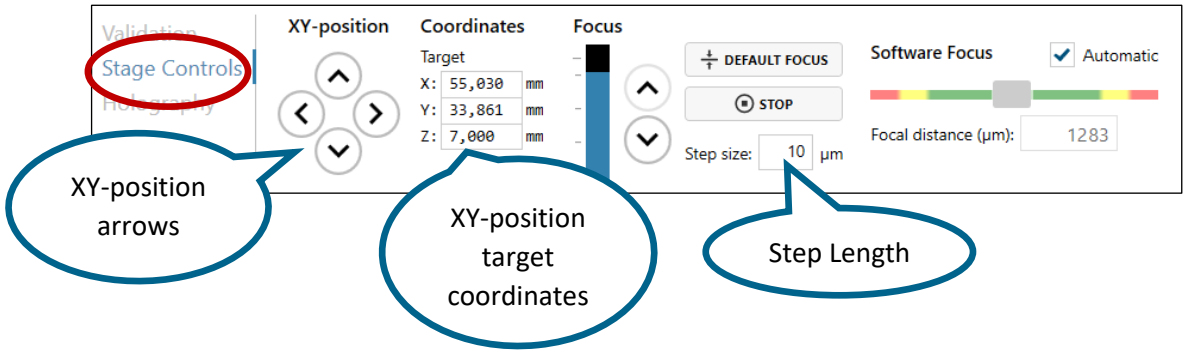
Motorized stage controls

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 Stage Controls tab to find the motorized stage controls.

Stage movements in XY directions can be controlled, and both hardware and software focus can be adjusted.

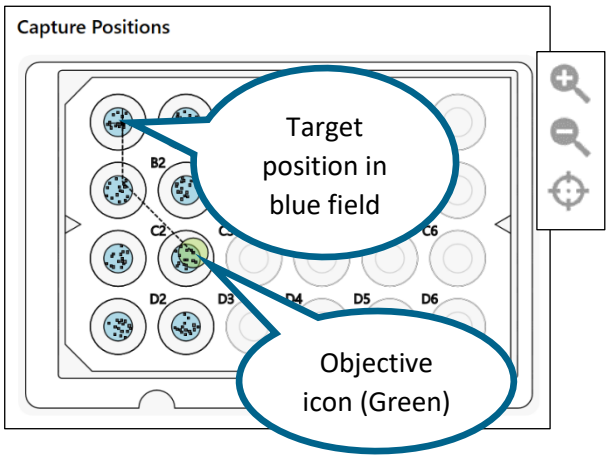
The current field of view will be displayed in the Live View window.



Stage movement

The stage can be repositioned 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.

The green circle indicates the current position of the objective, and the "+" symbolizes the new position.

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

Moving the stage 560 µm in X or Y direction will move the stage by one field of view.

Move the stage to one of the existing capture positions by clicking the position marker in the vessel map.

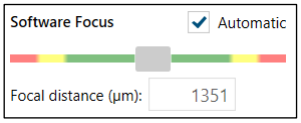
To better see the Vessel map, the window can be moved by click-and-drag, and it can be zoomed in or out by click-and-scroll. The window can also be zoomed in and out as well as reset to original size with the buttons found to the right of the Vessel map or double-clicking left mouse button.

Focusing 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 has both a coarse mechanical and a fine software focus. They need to cooperate for the image to be in focus.

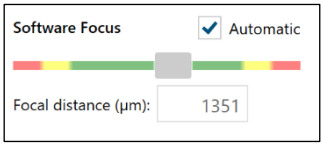
The software can only find focus when the cell culture vessel is at the approximately correct distance from the objective. Use the mechanical focus, *i.e.* move the motorized stage, to move the cells to the approximately correct distance from the objective. The software focus then finds and calculates the cell images. The Software Focus indicator shows how well the software can focus an image at the current mechanical focus setting.



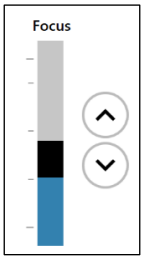
Focusing in practice - semi automatic focusing

By default, Automatic focus is activated.

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

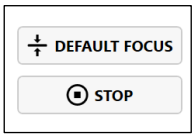


The black box in the Focus bar represents the motorized stage. Move the black box up or down to move the motorized stage up or down. Click the two arrows for controlled stage movements up or down. The arrows move with the step length entered in the Step length text box.



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.

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.

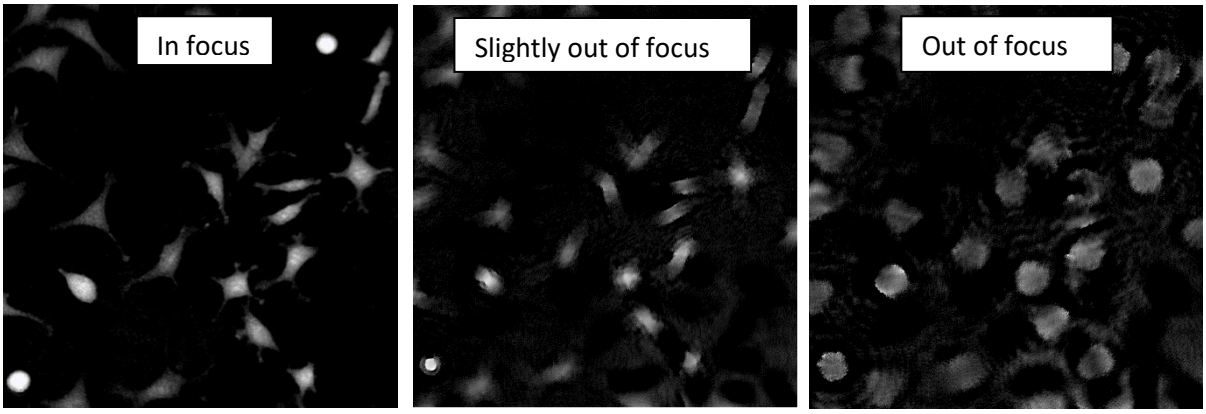
Manual focusing

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

- Uncheck Automatic in the Software focus panel.
- First adjust the software focus settings. Use the mouse cursor to move the grey marker in the Software Focus indicator until it is centered in the green area.
- Thereafter adjust the mechanical focus by moving the stage 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 stage until the cells are in focus.

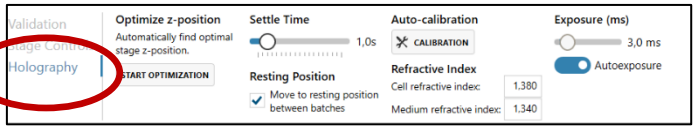
Images in and out of focus

The images below show a holographic phase image that is in focus, slightly out of focus and totally out of focus.



Holography

In the Holography tab, the holographic z-position can be optimized, the stage movement settling time can be set, the resting position can be handled, and the the Exposure time of the camera can be set. The Calibration function can be accessed here. Note that Auto-calibration should be performed before the start of each experiment.



Optimize z-position automatically by clicking the button. The software will aim at focusing at 1300±5 for the holographic software focus.

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.

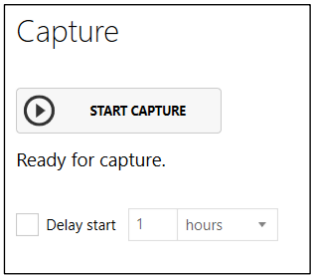
Auto-calibration gives a background image that is used by App Suite to lower background noise and increase image quality. There is a more detailed description in the Image calibration section in the HoloMonitor Installation chapter.

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 features. 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.

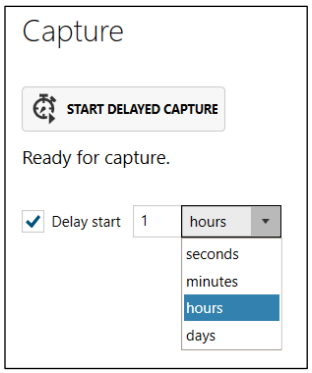
Capture

In this page, Image capture is started and monitored.



Start capture

To start the experiment capture, click the Start Capture button.



Delaying start capture

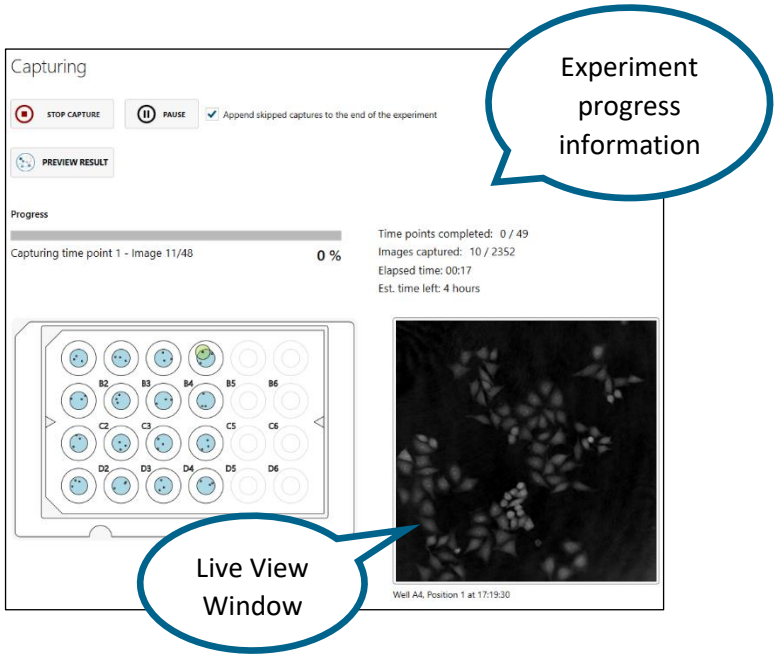
The start of the experiment can be delayed by a timer.

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
- Progress bar showing current experiment status



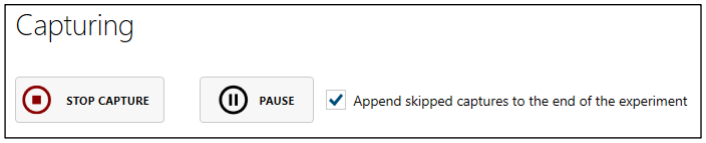
Preview Results

For guided applications, results are calculated during the experiment and can be viewed while the experiment is running. Click the Preview Result button to be directed to the results page (See Results for Guided Assays, next section).

For in depth applications, no automatic results are presented. The user needs to extract results as needed (see In Depth analysis section).

Pause capture

The capture can be paused by clicking the Pause Capture button. The same number of images that are skipped during the pause can be used to prolong the experiment once it is restarted.

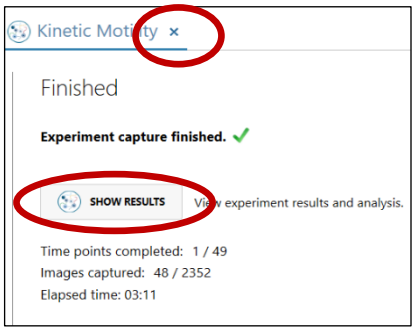


Stop capture

The capture can be interrupted by clicking the Stop Capture button in the upper left corner. The dialog box will appear to confirm the cancellation. The experiment will then be interrupted and closed.

Finished experiments

When the experiment has finished, a message appears stating that the capture is finished.



Results from guided assays can be accessed immediately with the Show Results button.

Close the experiment capture by using the x-button beside the experiment name.

For the results from In-Depth assays, the Show Results button will transfer to Experiments overview page, or results can be accessed through the Experiments tab.

Results for guided applications

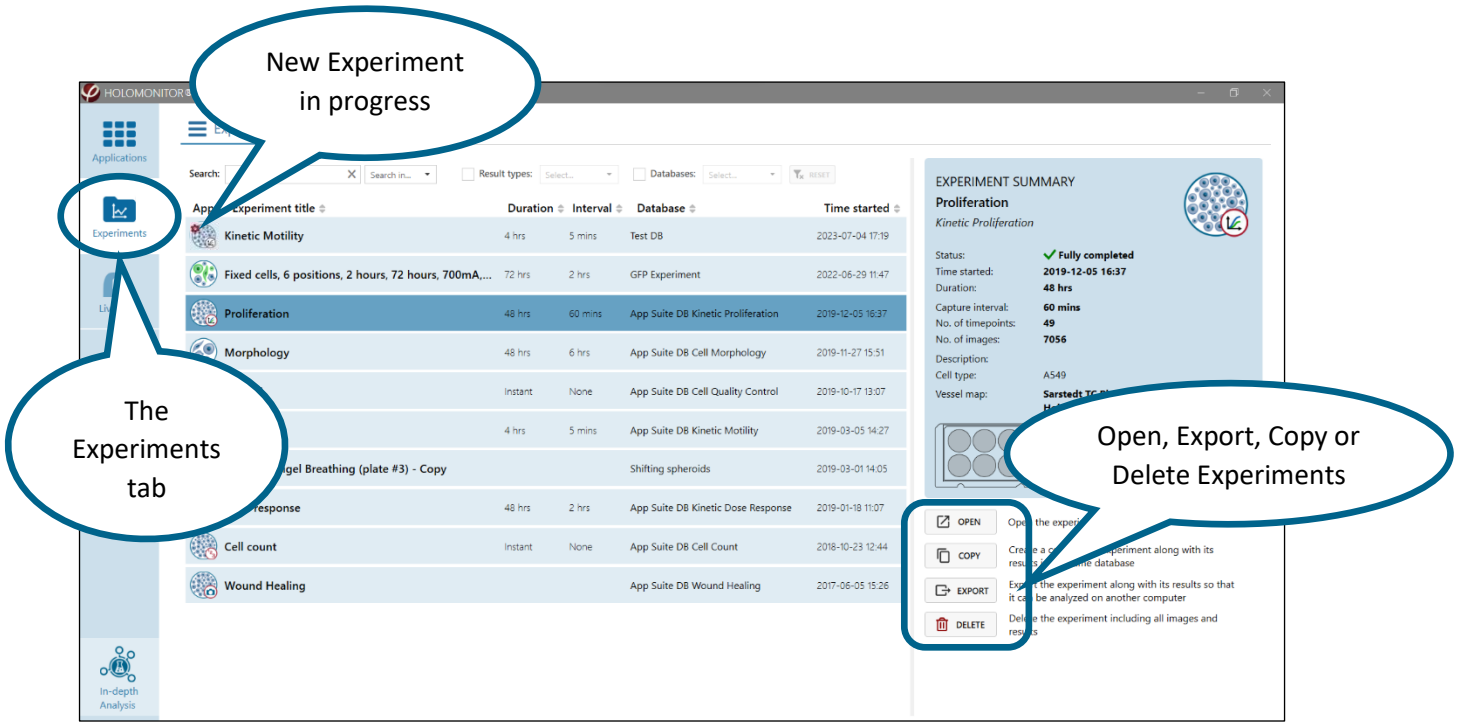
The analysis of a guided experiment is automatic and starts when image capture begins, making the results ready for viewing even during capture. Experiment results are mainly presented as graphs and/or tables, and all images can be accessed. After capture is finished, all results can be exported to Excel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

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 an experiment is ongoing and will only display preliminary results.

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



Open experiment results

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

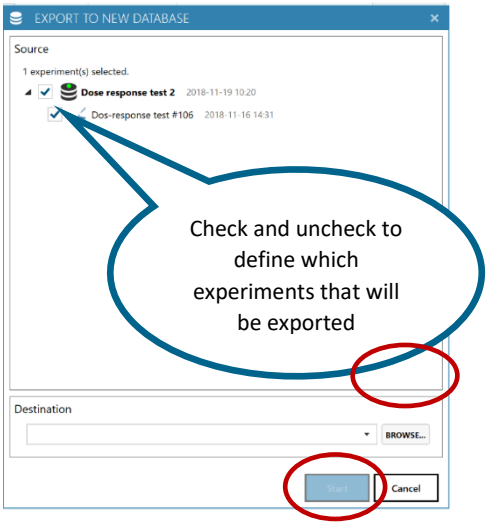
Copy an experiment

Click Copy to make a copy of an experiment, including all settings, treatments or controls and conditions and analyses. The copy will be placed in the same database as the original experiment. This is useful e.g., if the experiments need to be analyzed for two different treatment definitions.

Export an experiment

Click Export in order to create a backup or a copy of the experiment that can be analyzed on a different computer.

1. When the Export to New Database window opens, Browse to find the destination.
2. At the destination, right click to create a new folder that will contain the new database with the exported experiment. Name the folder and then selectit.
3. Click Start to export all database data connected with the experiment, including all image frames, settings, treatments or conditions, and analyses.



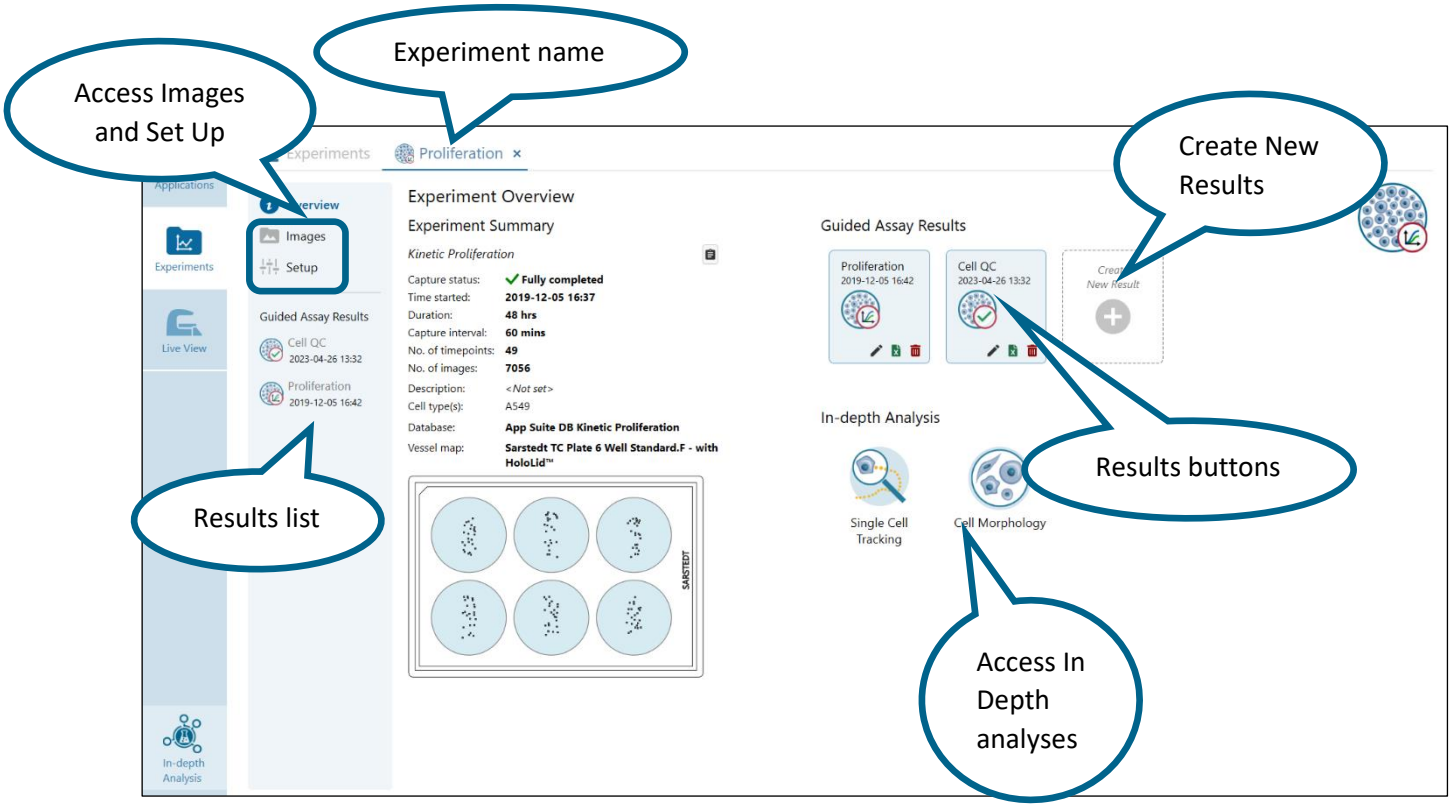
Delete an experiment

Click Delete to delete the entire experiment with results, images and analyses.

Deleting an experiment is a destructive action. There is no way to recover deleted data.

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 center, experiment information is displayed. To the right, new results can be created.

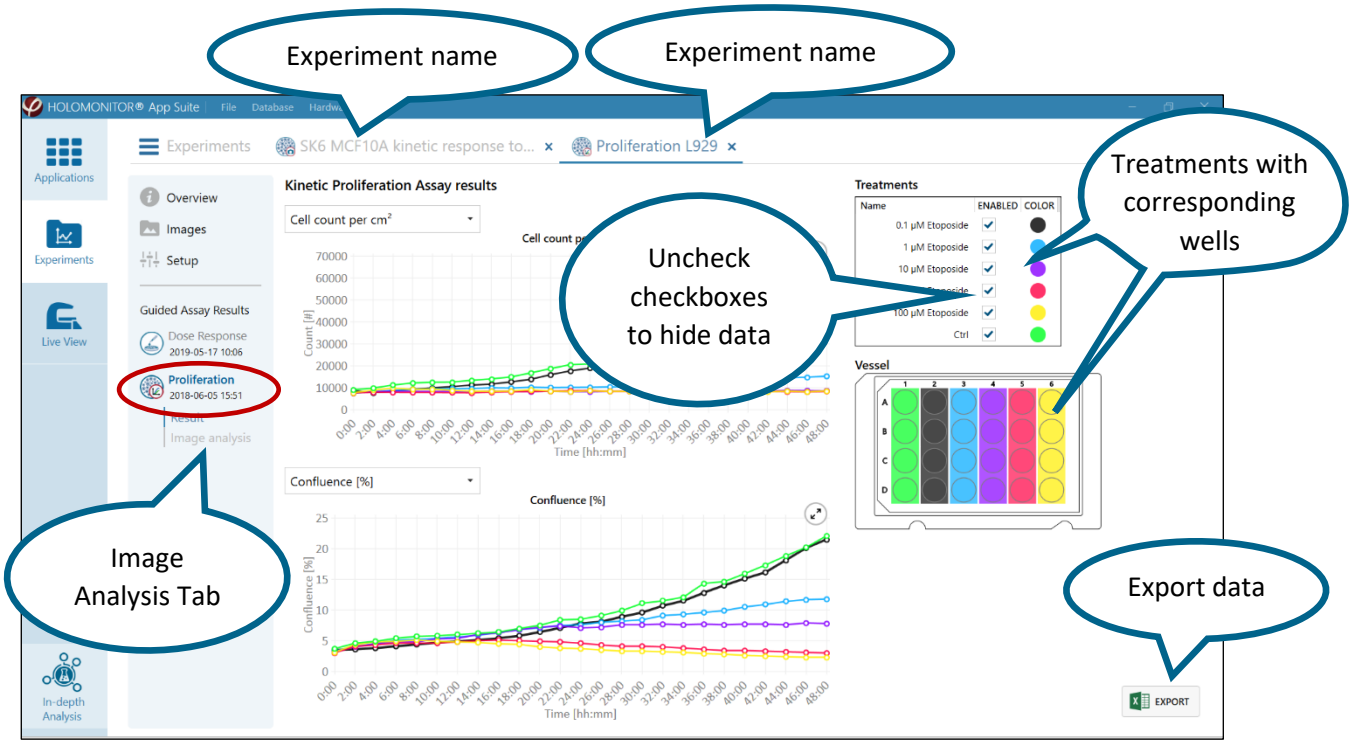


When Guided Results are opened, graphs and tables are displayed, showing the mean of the output variables. All different applications have different outputs. Details pertaining to each specific application are found in protocols (https://phiab.com/resources/manuals-protocols/). A more detailed description of the output variables is found in the Analysis Features section in the back of this manual.

For further analysis, the results can be exported to Excel or similar software.

The Experiment name is shown in the corresponding tab header. The tabs make it easy to switch between different open experiments.

When the results are displayed, the analyses of all frames can be accessed in the Image Analysis tab.



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 vessel map. Uncheck checkboxes to hide data in the graphs.

Export results to excel files

Export the data to excel for further analysis by using the Export button.

Normalized graphs

Graphs for 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, e.g., caused by pipetting errors.

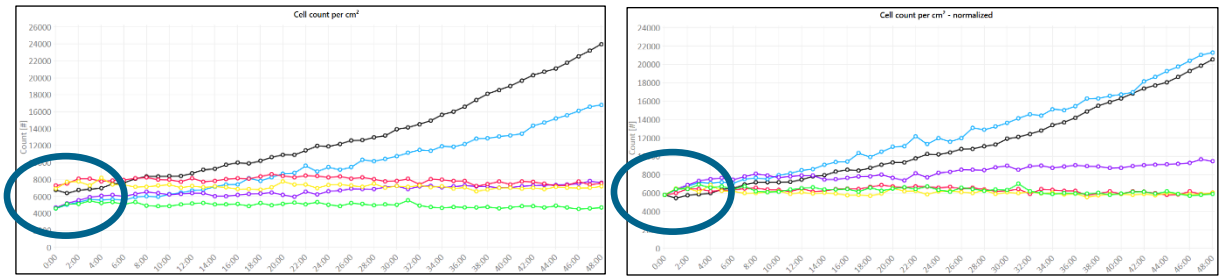


Image analysis

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

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

To ensure high quality results, especially if the graphs look irregular or spiky, it is necessary to examine the analysed images to see if there are anomalous image captures or faulty error analysis of the images. Faulty images can be excluded, either individual images, or all images from one capture position or whole wells.

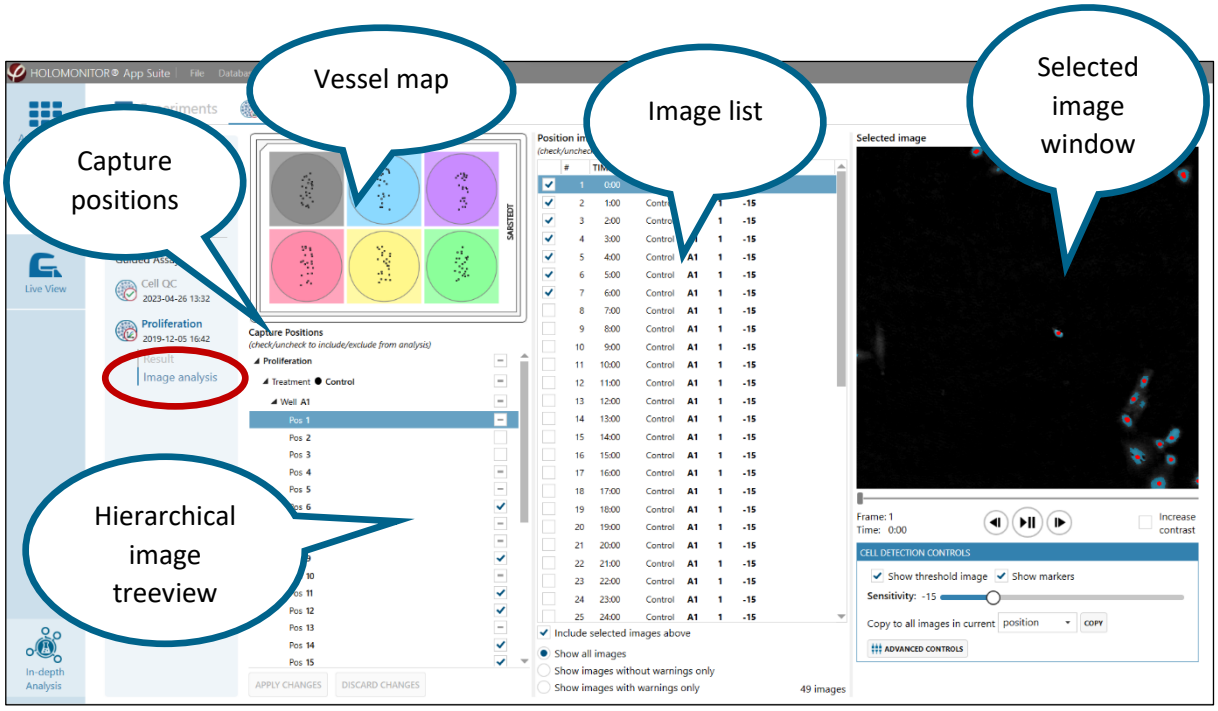
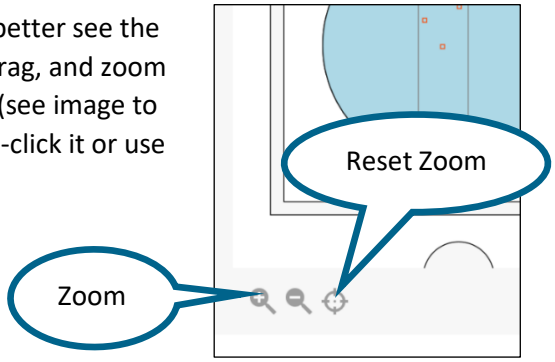
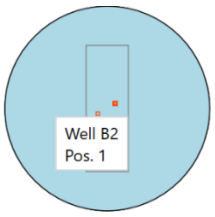


Image positions in the vessel map

All capture positions are displayed in the Vessel Map. To better see the positions placement, 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). To show the Vessel Map in original size, double-click it or use the Reset zoom button.



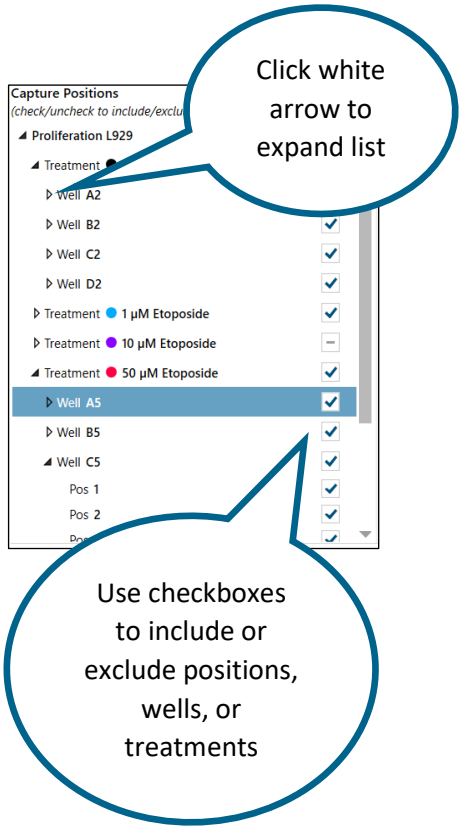
When a position is selected, the corresponding images are displayed in the Selected Image window.



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

The capture positions list

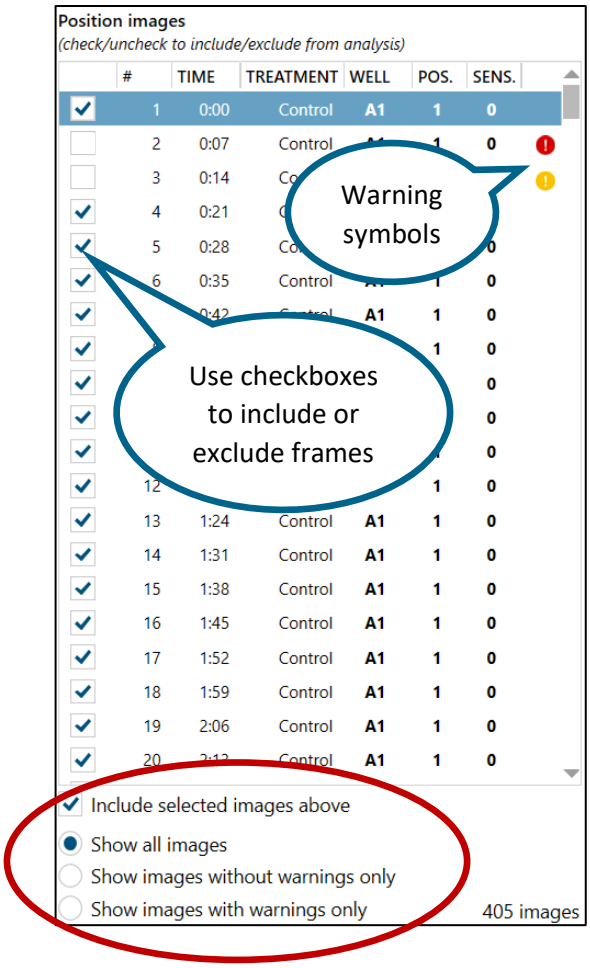
The Capture Positions list is organized as a tree view, showing the experimental hierarchy. Clicking an empty arrow expands the underlying hidden list.



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.

Check or uncheck the checkboxes to include or exclude individual images, or all images from a position or a well in the results.

When images are excluded or included, the corresponding changes to the graphs will only be implemented after clicking the Apply changes button.



The position images list

Images for the positions 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, time of capture, image treatment, well number and position number are given.

The image of the currently selected position will be shown in the Selected Image Window.

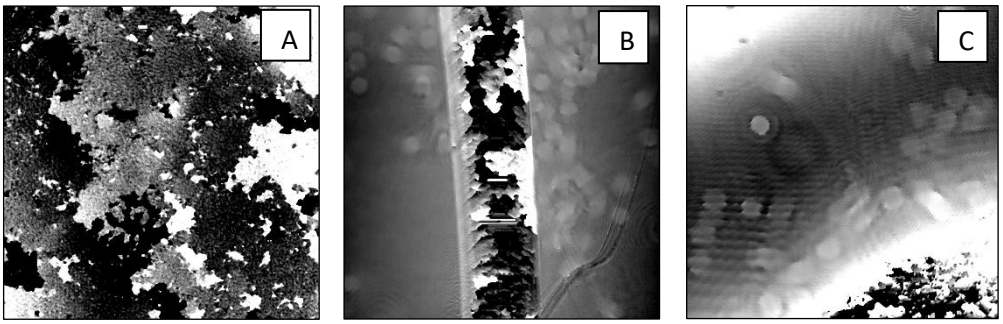
Image display functions are found below the Image List.

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

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 them 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.

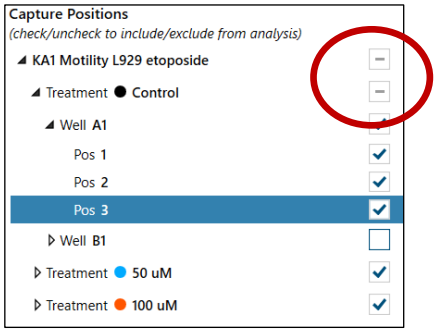


Sample examples of images with A. condensation, B. a scratch, and C. a corner of an air bubble, all of which cause insufficient image quality.

Activate Show Images with Warnings Only 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. The data will then be added to the results.

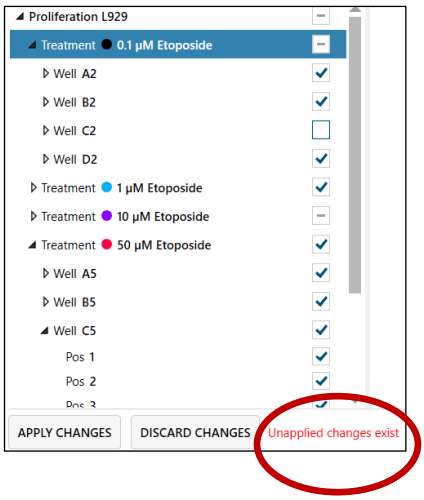
If the software has missed removing a bad quality image, it can be removed manually from the result. Activate Show Images Without Warnings Only to display all images without warnings that are selected in the Capture Positions list. By unchecking the boxes in the Position Images list, data is removed 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

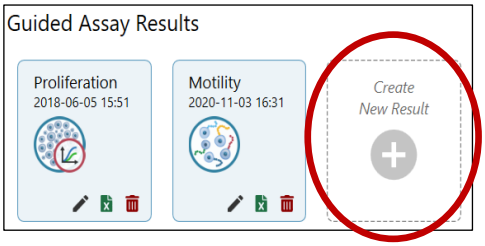
Checking and unchecking images or positions affect the data displayed in graphs and tables as well as exported data, but only if they are applied. To apply the changes and display them in the results graphs, click Apply Changes. To discard the unapplied changes, click Discard Changes. If there are any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



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.

Changes will be applied to the current results; they will not be shown as a new result.

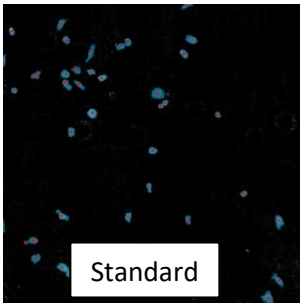
In order to keep the old results in parallel with new results, use the Create New Results button found in the Experiment Overview to create a parallel analysis (see Reanalyze results section for more information).

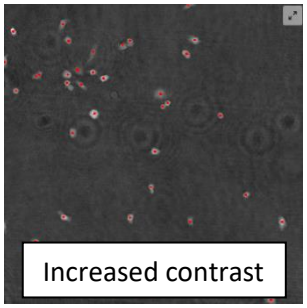


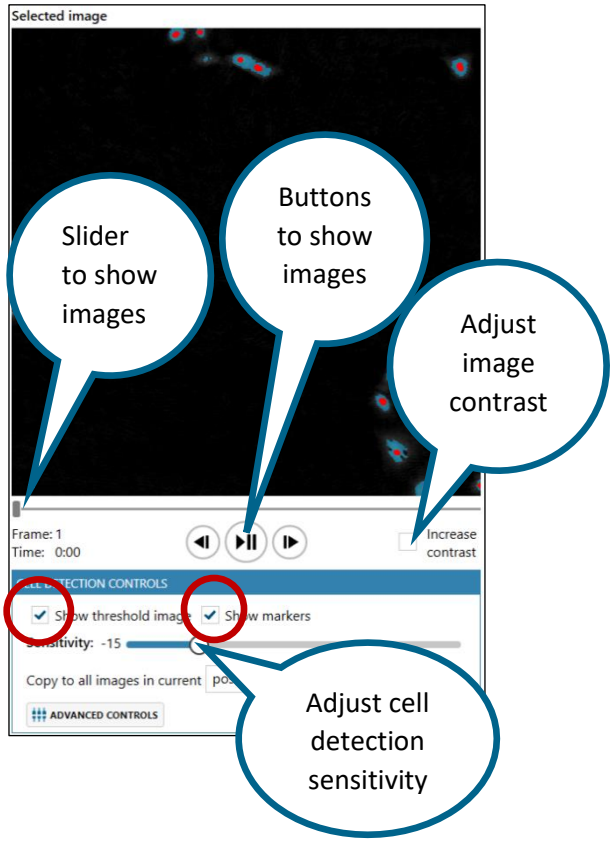
Viewing the analyzed images

To view an image in the Selected Image window, 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 play/pause 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.





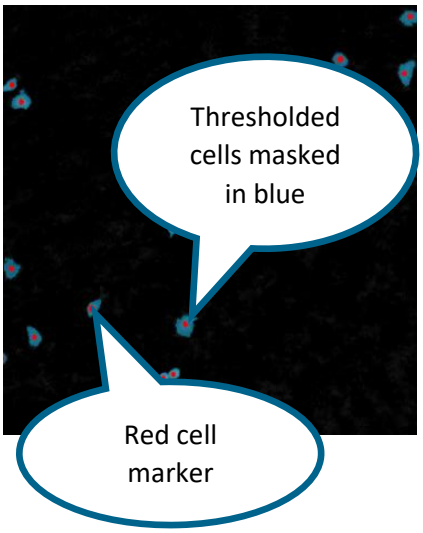


Automatic cell identification

The software automatically identifies the cells in all images.

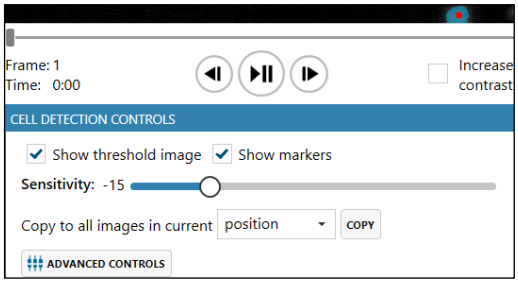
- The algorithm takes into consideration cell thickness, size and morphology to first separate the cells from the background, second split adjacent cells and third fine-tune the result.

- The algorithm identifies cells by setting a threshold that determines what is cell and what is background. This is shown as a blue area. Each identified cell is then marked with a red dot. Unchecking Show Threshold Image and Show Markers in the Cell Detection Controls window will hide these parameters.

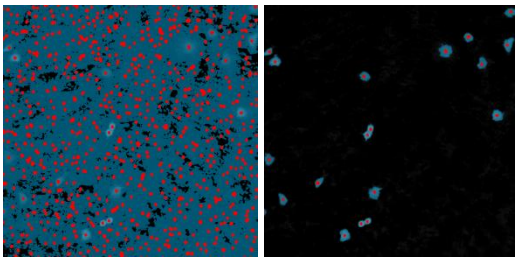


Adjusting cell identification sensitivity

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 in current:



- Selection or
- Position or
- Well or
- Treatment or
- Experiment

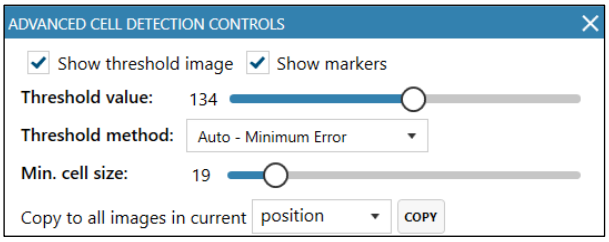


The examples show an image before (left) and after (right) threshold adjustment.

Advanced cell identification

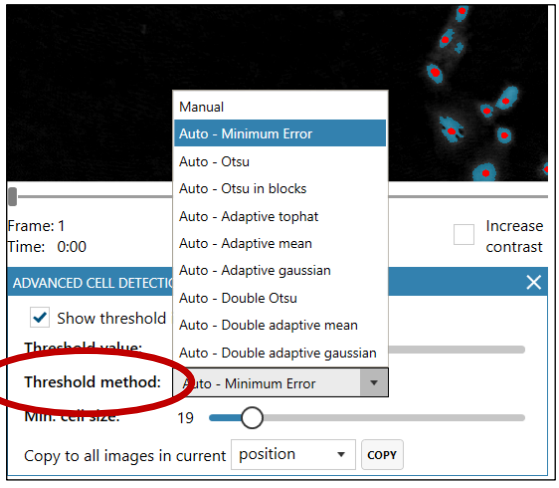
For more advanced cell identification adjustments, click Advanced Controls. The advanced cell identification adjustments are performed in three steps:

1. Determine which threshold calculation method that results in the best cell identification for the current experiment.
2. If needed, use the Adjustment slider to fine tune the threshold setting.
3. If needed, use the Min cell 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 threshold. 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.

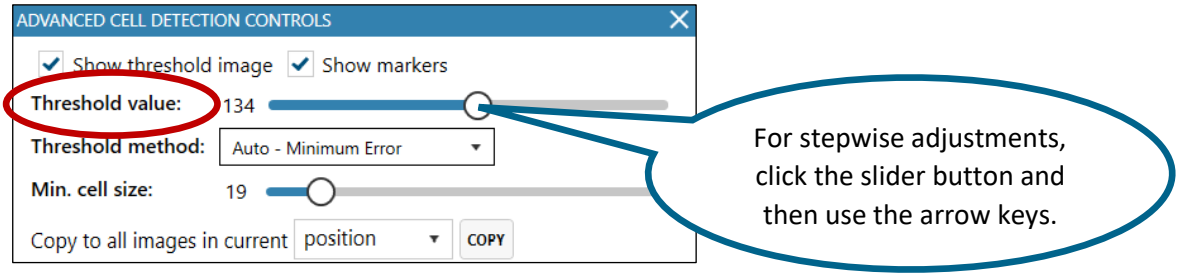
Double Otsu is a threshold method where both a wide and a narrow threshold mask are used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. Double Otsu uses double thresholding with Otsu global threshold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

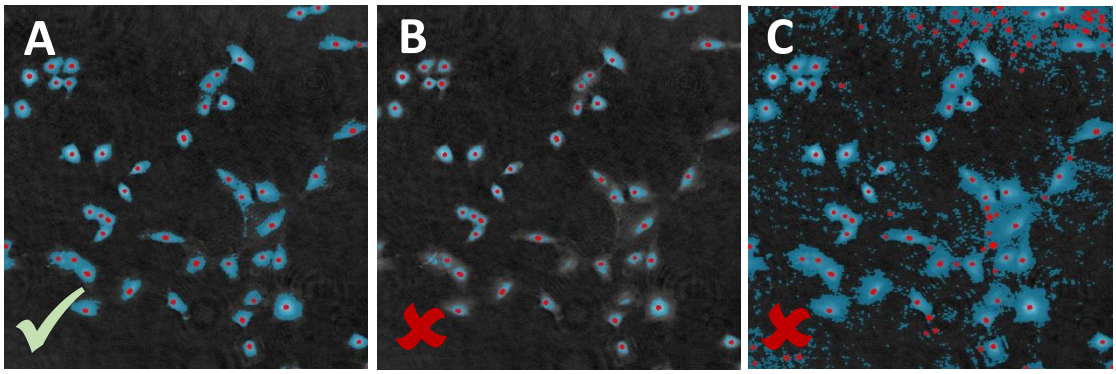
Double adaptive mean is the same as Double Otsu, but with two adaptive mean threshold masks.

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 Threshold Value slider.

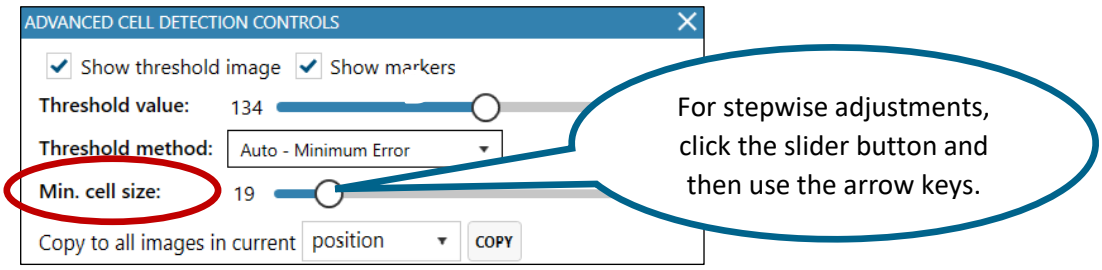


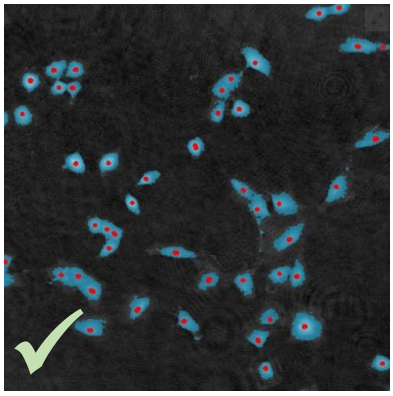


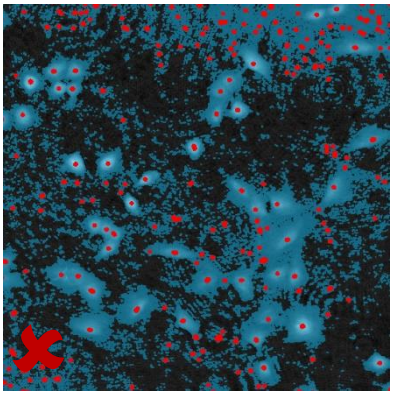
Adjusting the threshold between cell and background with the slider will adjust the minimum optical thickness of objects considered to be cells. Image A shows correctly thresholded cells, image B cells with a too high threshold, and Image C cells with a too low threshold.

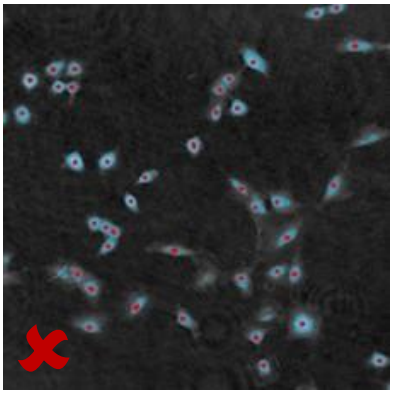
3. Adjusting object definition, i.e., cell size

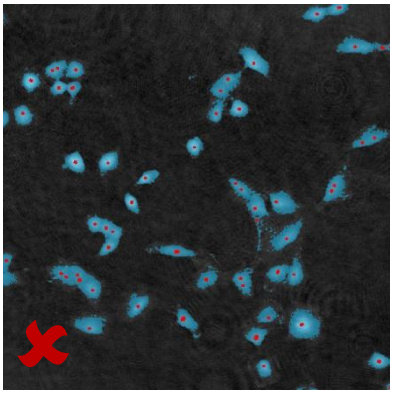
The minimum cell size is adjusted by using the Min cell size slider (see image above). The goal is to have one blue marker in every object considered to be a cell (see image A below). The slider values do not have a unit, but they are related to the diameter of the largest circle that can fit into the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

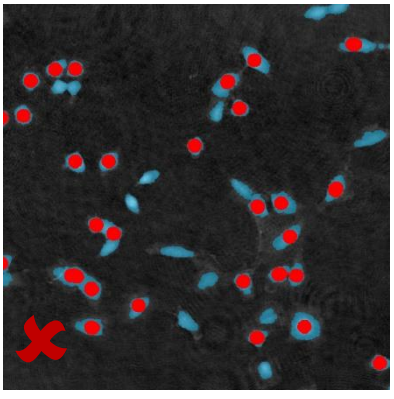












Common cell identification problems

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

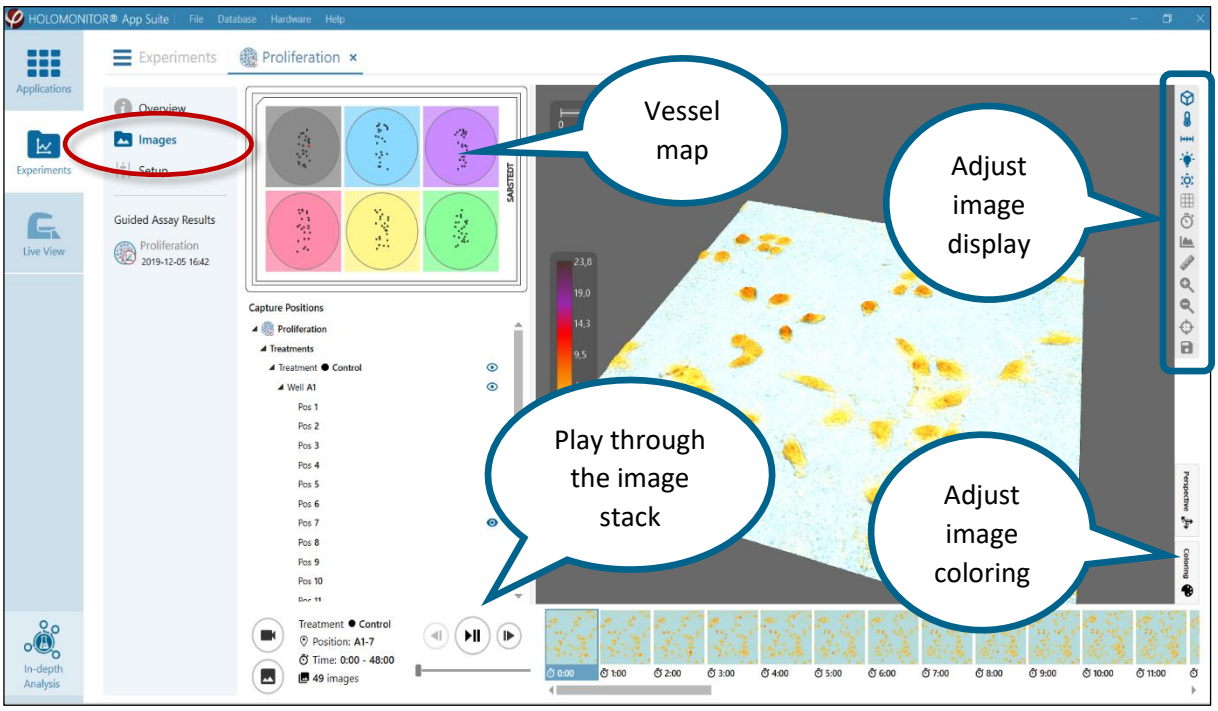
Pre-smoothing

Activate Use pre-smoothing to smooth the cell edges.

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 artifical coloring. Colored still pictures and videos can be created and exported in standard formats.

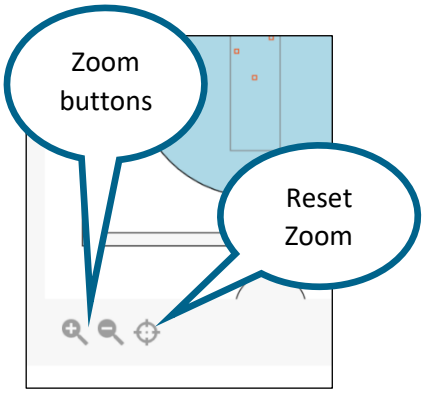


Move or zoom the captured image

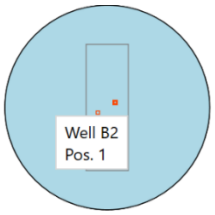
- To zoom the cell image, move the mouse cursor to the view area, click and scroll using the scroll button on the mouse.
- To move the cell image to a desired location, click, hold and drag the image using the left mouse button.
- To change viewing angle/perspective, click, hold and drag the image using the right 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.



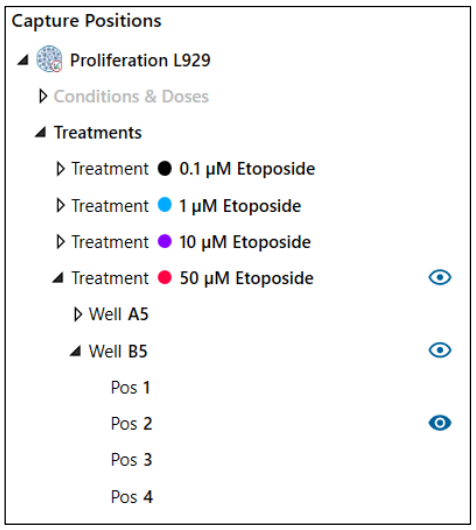
Select a position to view the corresponding images in the Image window.



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

The capture positions list

The Capture Positions list makes it possible to display 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 displays 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.
- if a treatment is selected all images included in that treatment will be displayed.

The filled eye icon indicates which position is currently shown in the viewing window. To view a time lapse showing one position, ensure that only one position is selected.

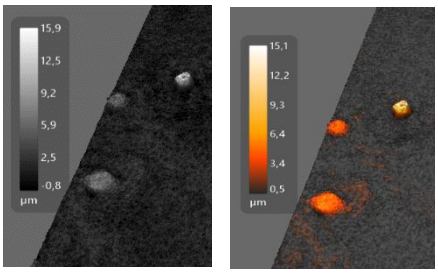
Images can be displayed by moving the slider below the selected images or by using the play/pause button or the forwards or backwards buttons below the selected image (see image to the right).

Adjusting image presentation

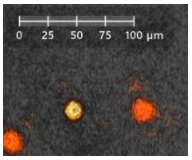
To the right of the 3D image view window there are several functions that adjust the presentation of the captured image (see image to the right). Blue functions are active while gray functions are inactive. All adjustments will be present in exported movies and images, when As seen on screen option is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

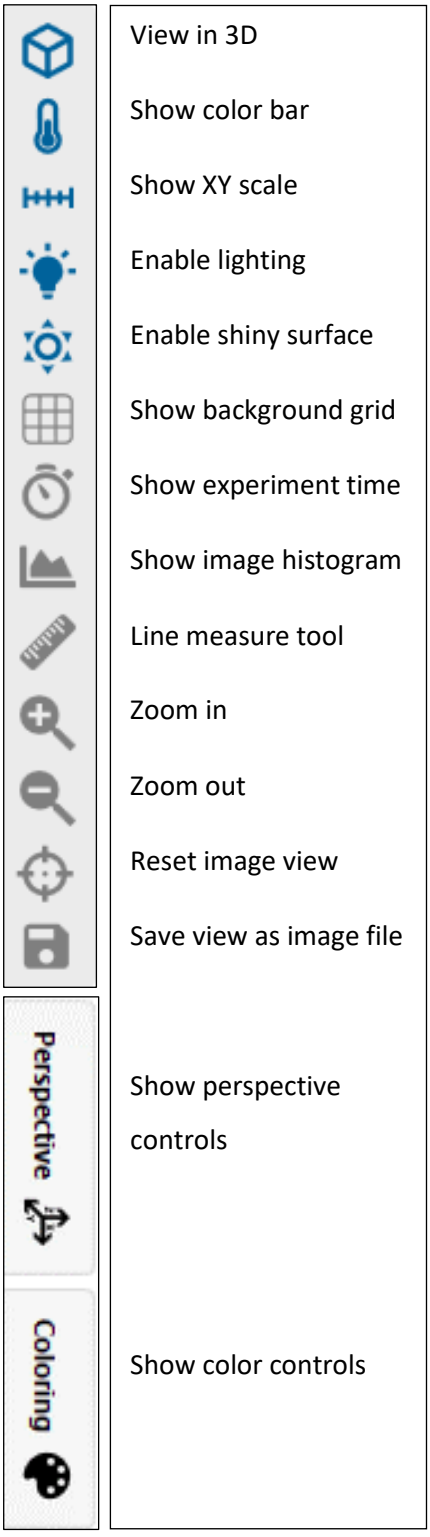


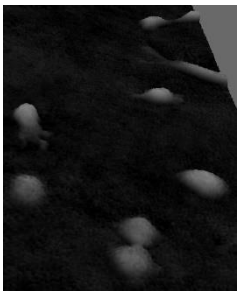
Show XY scale displays a horizontal scale bar representative of the distance in X and Y (See image to the right).



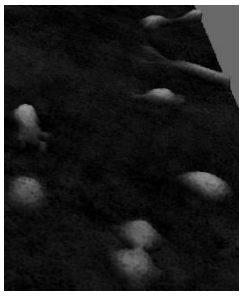
Enable Lighting applies an artificial light source to the image which may sometimes render an improved image (See images below).

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

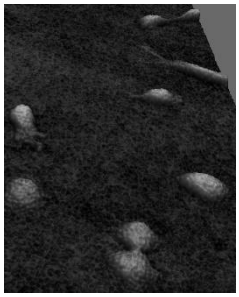




Standard image



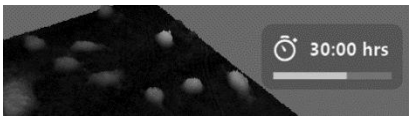
Enabled Lighting



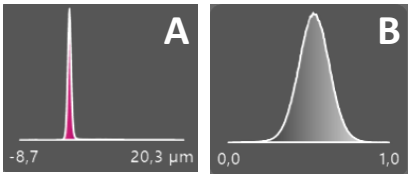
Enabled Lighting and Shiny surface

Show background grid adds a grid to the background.

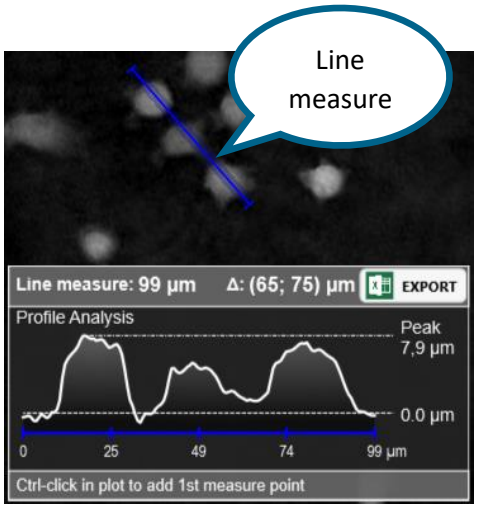
Show experiment time adds a clock to the image, showing the experiment duration at image capture. (See image to the right).



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.



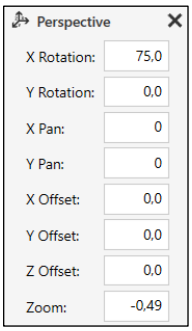
- It is possible to move and flip the image while the measuring function is activated.
- The line measure data can be saved as an image with the Save View button .
- The resulting data can be exported to Excel.
- When the line measure tool is clicked again, the measuring function is deactivated.

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, jpeg or png files.

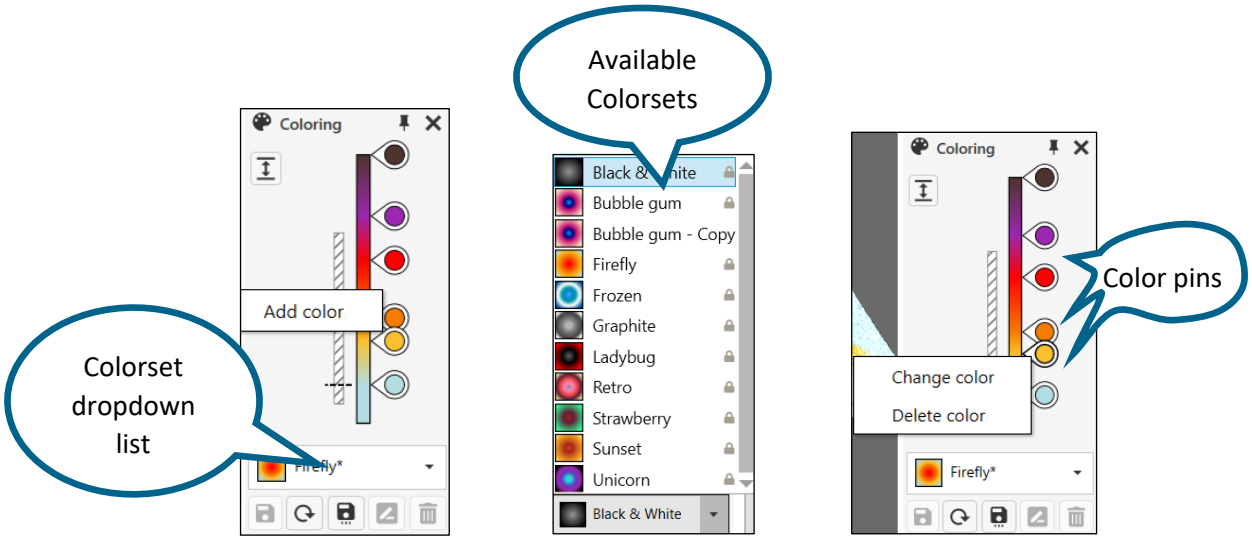
Perspective opens the Perspective window, where image perspective can be adjusted by adding the exact parameters. The image perspective can also be changed using the mouse by left clicking and drag the image for pan, left click and scroll wheel for zoom and right click and drag for tilting the image.



Coloring opens the Coloring window, which is used to adjust the color balance of the window and to color the image relatively to the cell optical thickness.

A set of colors that are saved together is called a Colorset. 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 automatically be applied to all captured images in that experiment.

Click the Rescale button to adjust the image color balance. This will rescale coloring from zero to the highest image value.



- 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 at the bottom of the Coloring window to:

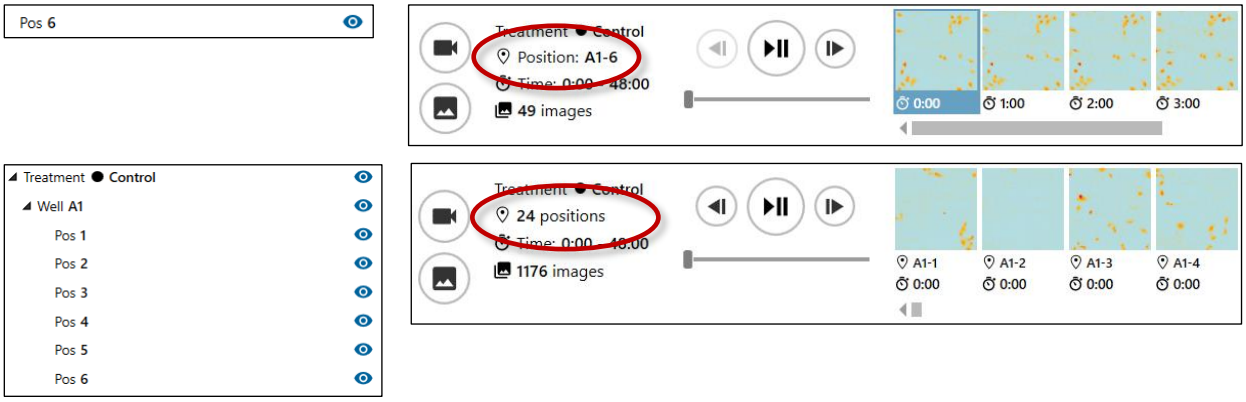
- Save a new colorset
- Go back to the last saved settings
- Save the colorset to a new name
- Rename the colorset
- Delete the colorset

Export images or a movie

Select images

Start with selecting the position from which a time lapse movie will be created, or images will be exported. Use either the Capture Positions list or click the desired position in the vessel pattern.

Selected frames are marked with a blue eye in the list. In the first example, only one position is selected, in the second example several positions are selected.



Include or exclude frames

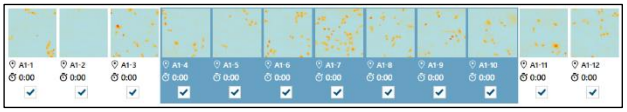
Click the Create a Movie button or the Export Images button to access selection functions.

Check frames that should be included in the export. By default, all selected image frames are included. Unchecked frames will be excluded.

Add all frames will check all frames, function is active only if there are excluded frames

Remove all frames will uncheck all frames

Add selected frames will check selected frames, function is active only if there are excluded frames



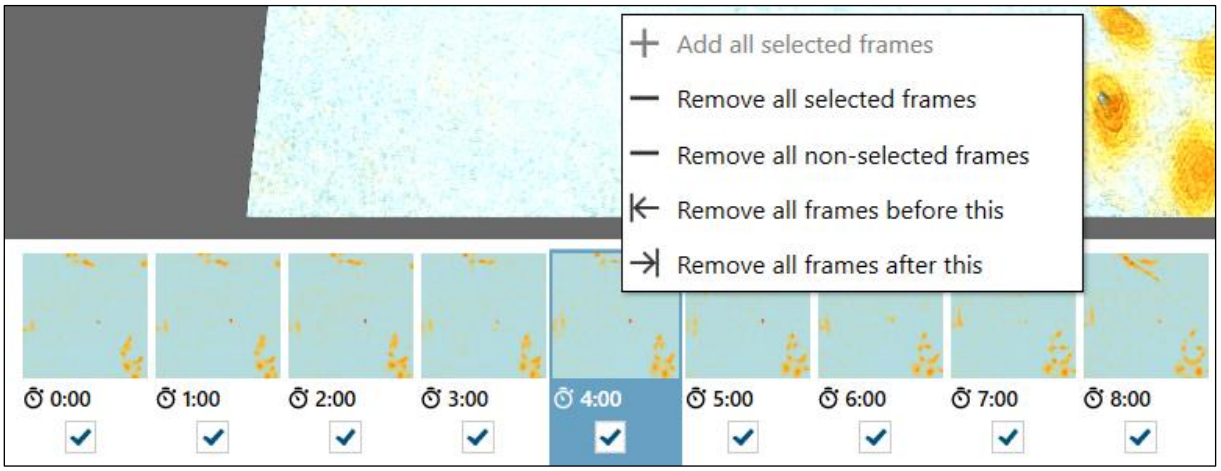
Remove selected frames will uncheck selected frames

Remove all frames before will uncheck all frames before the currently selected frame

Remove all frames after will uncheck all frames after the currently selected frame

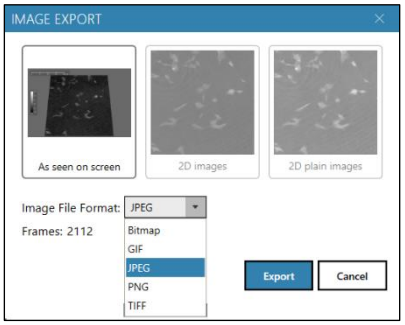
Only include every n^{th} frame will make it possible to include e.g. only every 2^{nd} or every 5^{th} frame in the movie. Click Apply to execute the selection. In the example below, every 3^{rd} frame is included.

All the above functions can be accessed through right clicking any frame.



Export images

Click Export Images to open the Image Export window (see image to the right). Select how to present the image and file format for the exported images, and then click Export. Jpg, png, bmp, tiff and gif files are available.

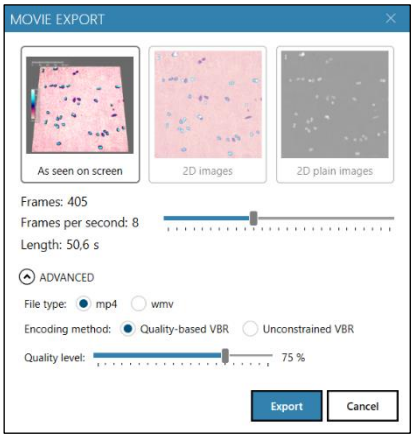


Selecting 2D Images uses the current image and color but shown in 2D with no overlays or effects.

Selecting 2D Plain Images uses a 2D image with greyscale coloring adapted to each image.

Export movie

Click Export movie to access the Movie Export window. Select how to present the images and movie format, and then click Export. The default movie format is mp4.



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.

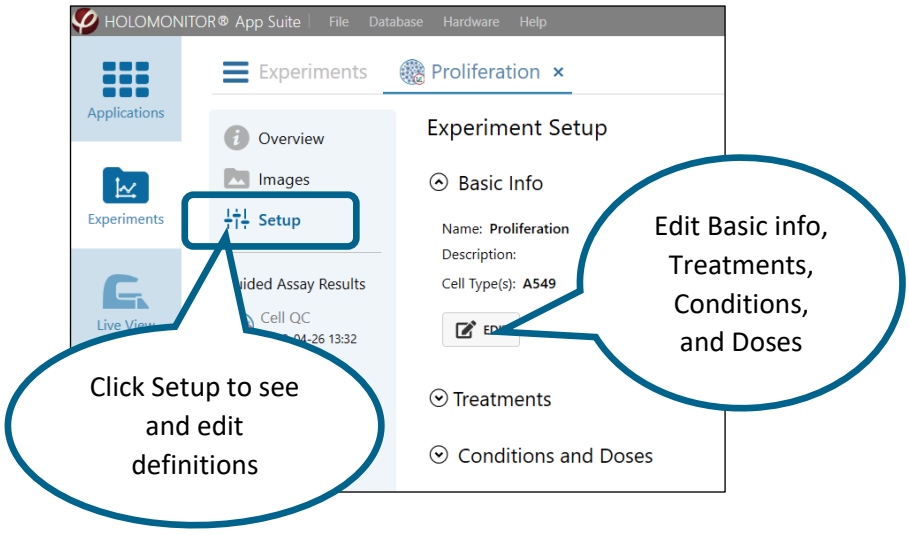
Click Advanced functions to adjust movie file type, encoding method and Quality level.

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.

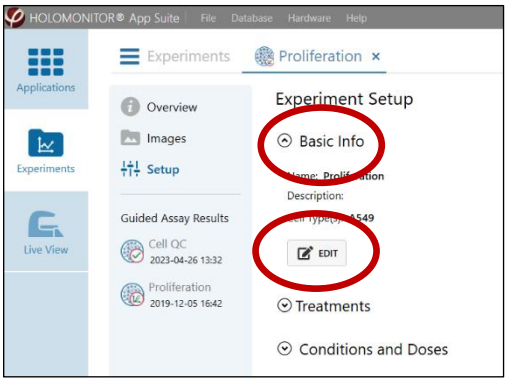
Setup edits

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.

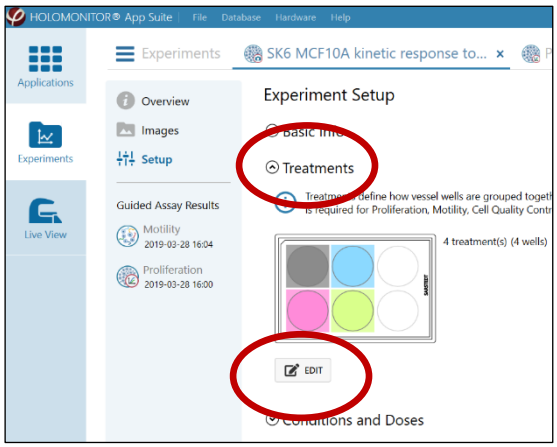


Treatments

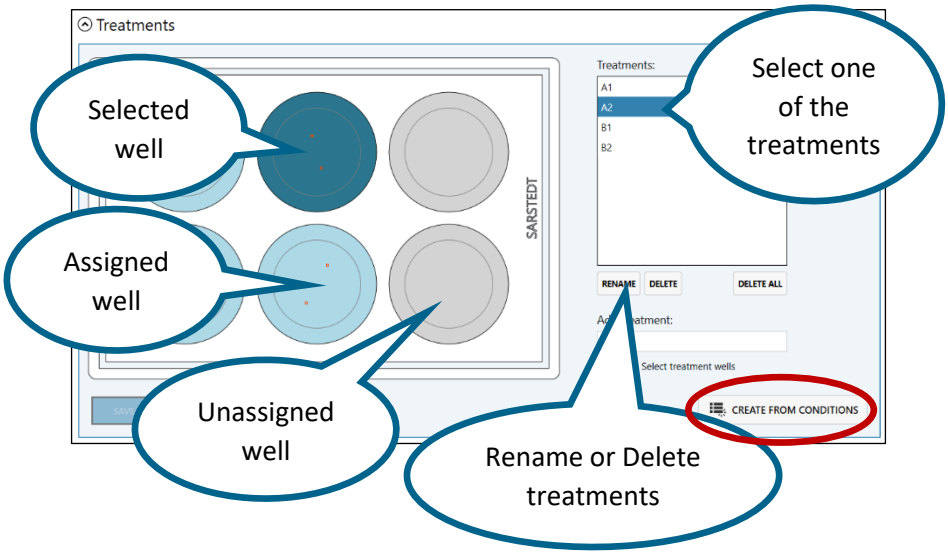
To add or edit Treatments, click Treatments and then Edit to open the Edit window.

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 an 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.

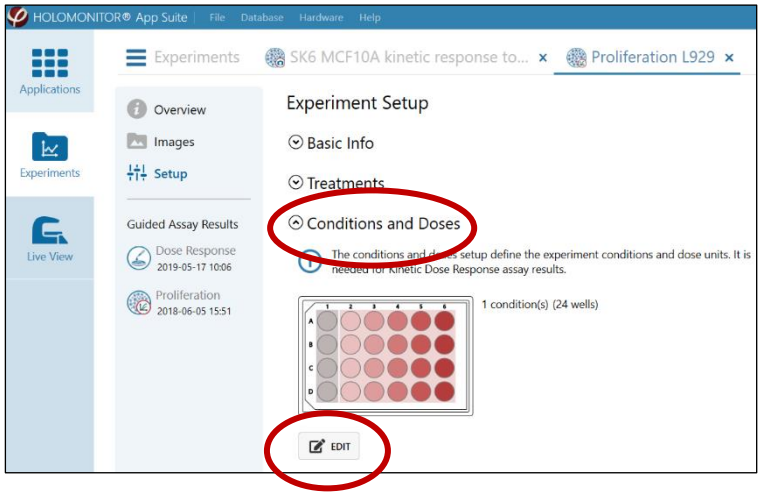
Click Save to add the changes and close the Edit function.

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.

If an experiment was set up with treatments, e.g., a kinetic proliferation experiment, Conditions and doses can be added to create a dose response curve.



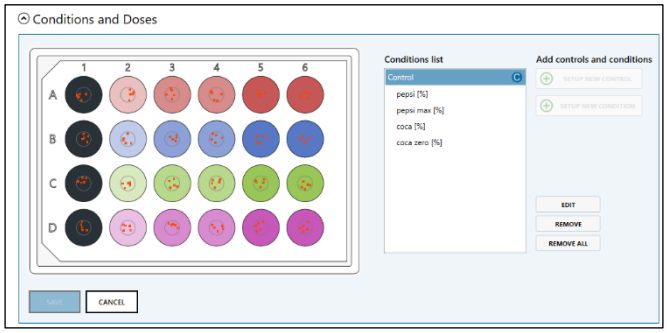
Edit conditions and doses

1. Click Edit to open Conditions and Doses.

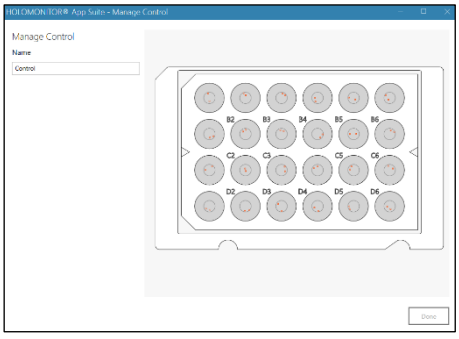
2. Select an existing control or condition from the list.

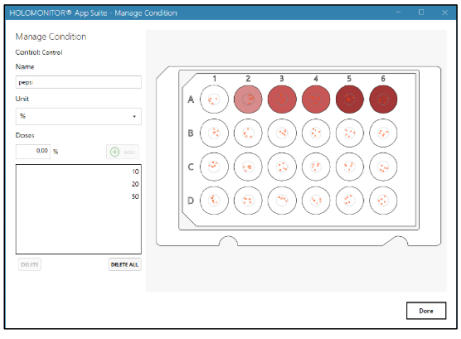
3. Click Edit to open Manage Control or Manage Conditions (see images below).

4. Edit the control or selected condition in the Manager, e.g., correct the name or Unit or add, adjust or delete Doses.



5. Save the changes.





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.

One control can be used for several different conditions.

Select one of the controls and then click Setup New Conditions in order to add the associated conditions. Click Done to add the changes and close the Edit function.

At least one control must be set before any conditions can be added.

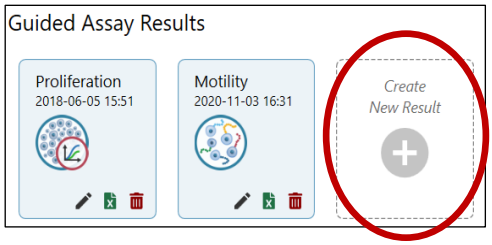
Controls must have unique names, otherwise it will not be possible to save them.

Editing conditions will cause previously saved settings and results to be deleted. To create parallel treatment setups, first create a copy of the experiment.

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

Create new result

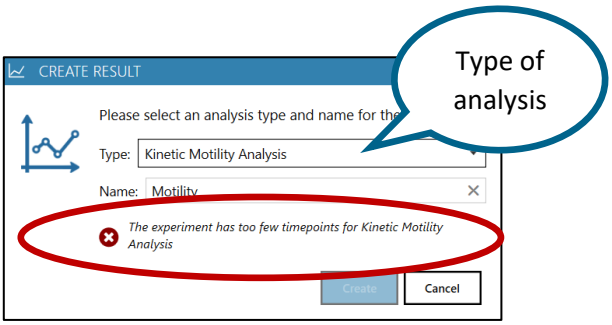
Experiments can be utilized for more than one analysis of the same type of results, or for more than one type of results. E.g. a proliferation assay can be reanalyzed for proliferation with results from certain wells excluded, or the proliferation data can be used to create a dose response curve if there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

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 used for a kinetic analysis.



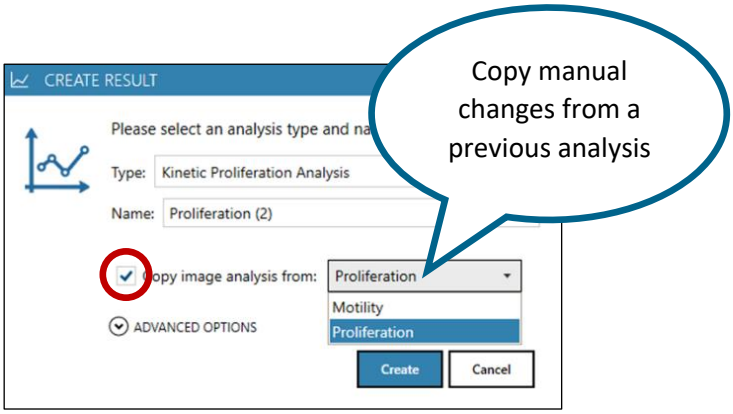
Before using a proliferation experiment to extract motility data, ensure that the timepoints are frequent enough to provide accurate data.

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

Most experiment results have manual changes to the settings. It is common to exclude or include images, or to change Cell Detection Control settings.

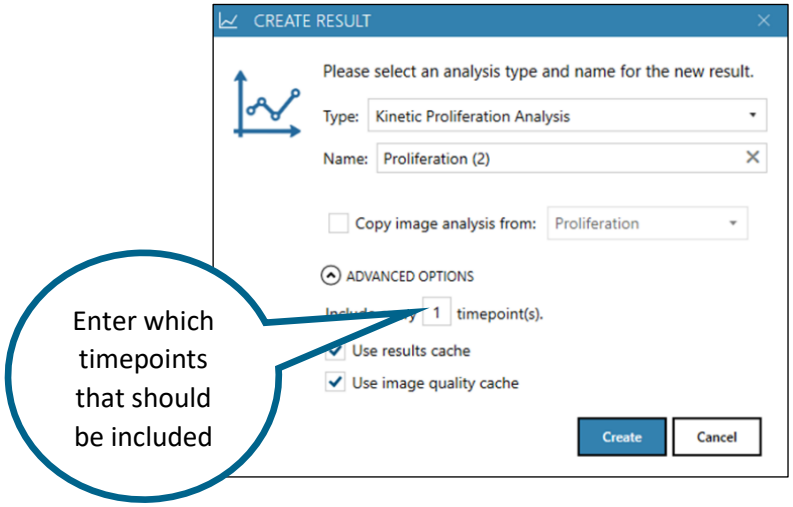
To re-use those manual changes when making a new analysis, check Copy image analysis from and select from which analysis the settings should be copied.



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.

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.

Not using the Results Cache and previous Image Quality calculations requires a lot of computing power and time. Depending on the experiment size and computer it can take from several minutes to several hours.

In-depth analysis

Software layout

This part of the software comprises six tabs. 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.

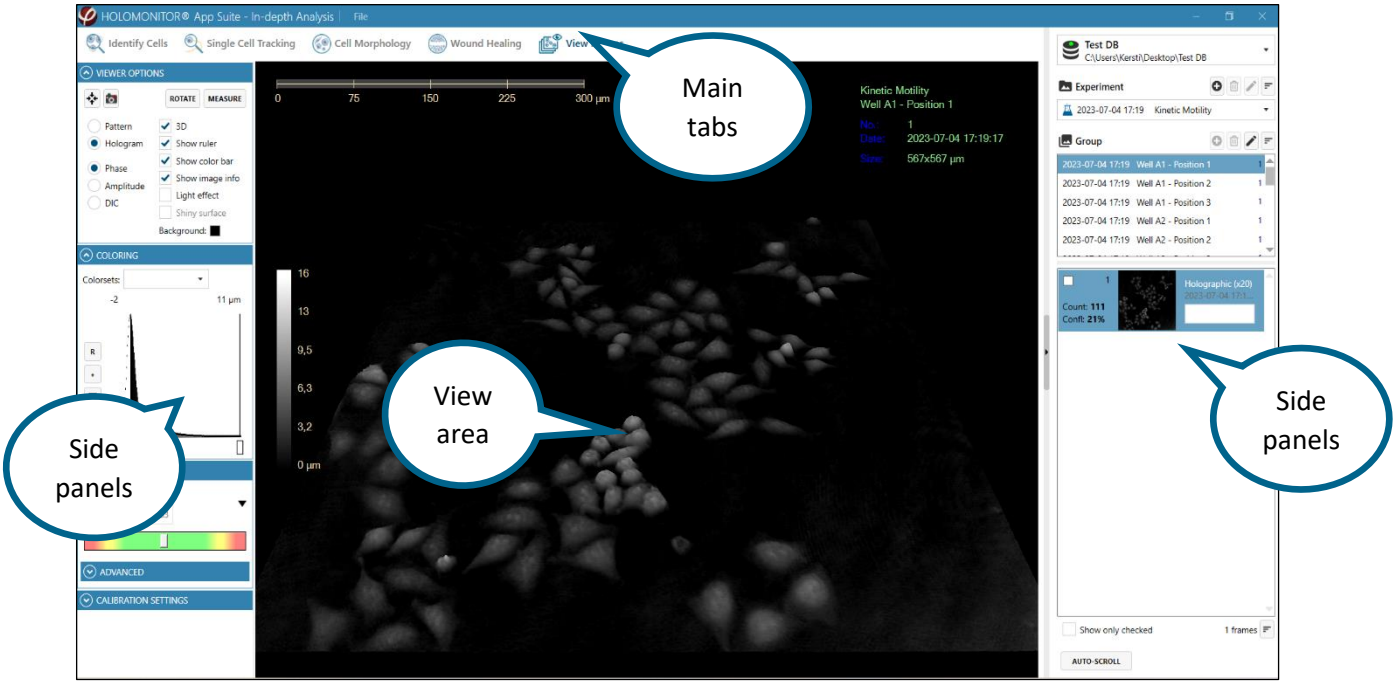
Cell Morphology analyses cell morphology in images and time-lapses, as well as displays and exports the results.

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

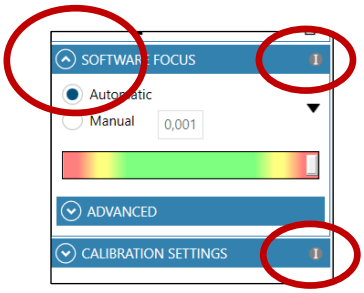
View images allows the user to view and color captured images and time-lapses and to adjust autofocus settings.

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.

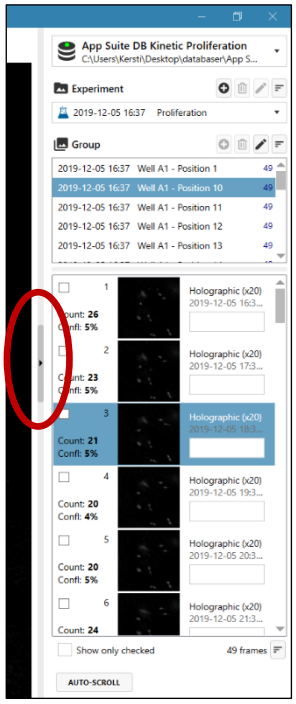


The left side panels can be collapsed or expanded by clicking the arrow tip found in every collapsible side panel header.



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

The right-side panel can be collapsed or expanded by clicking the little grey area with an arrowhead.

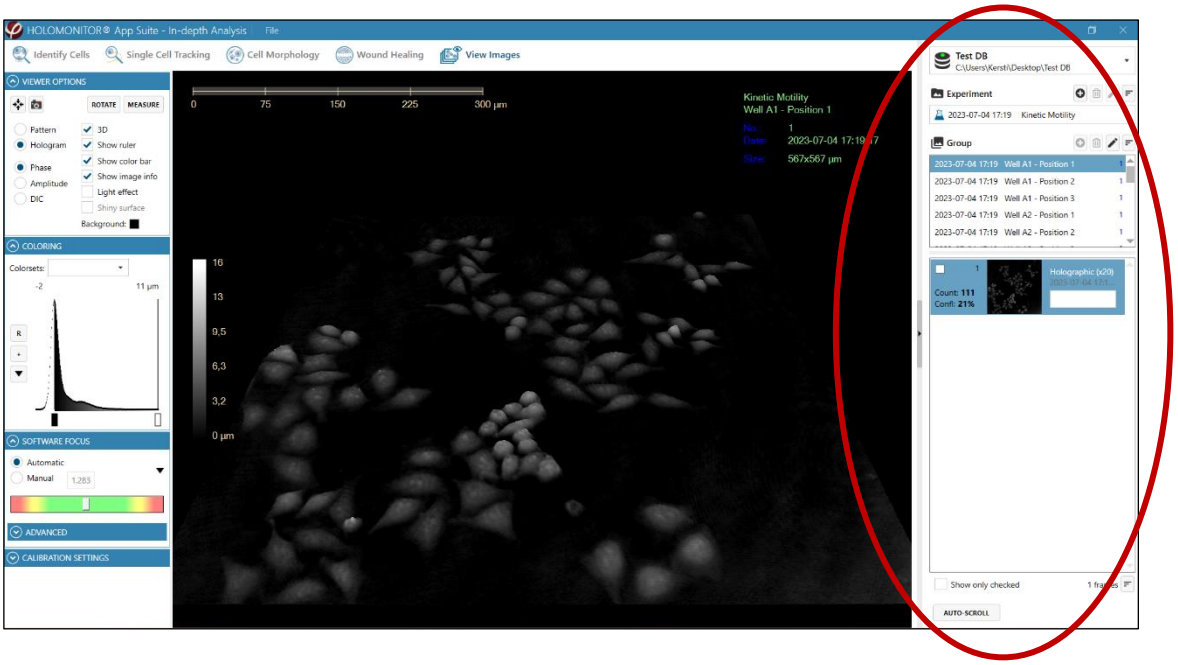


The image frame list

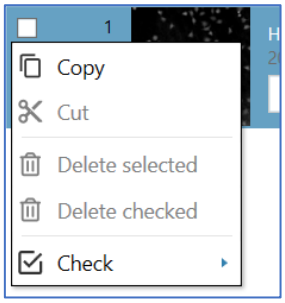
Images are grouped in Image Groups, which are organized into Experiments, which in turn are stored in a Database.

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

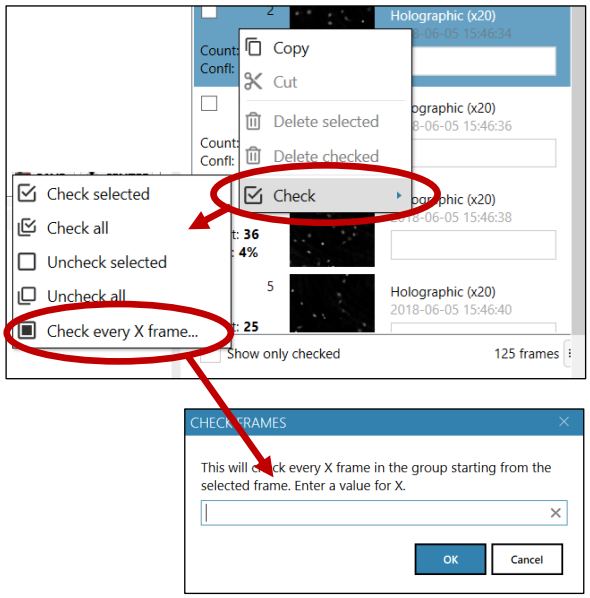


Copy, Cut or Delete Image Frames, work only with Hstudio data.

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.

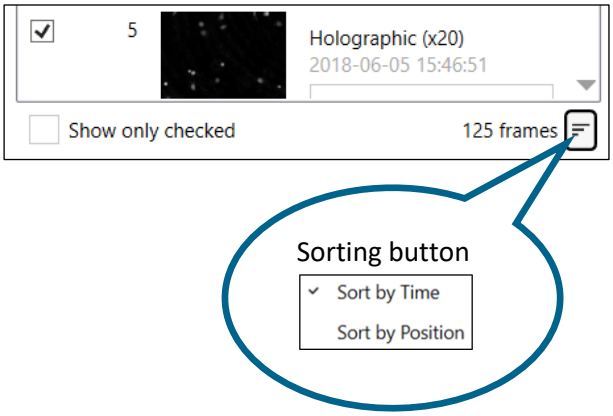
The Check every X frame is especially useful tool in combination with the Show only Checked function which is found below the Image Frame list.



The sorting button

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 boxes:

Show threshold displays pixels defined as belonging to a cell in red.

Show cell markers displays blue markers that identify individual cells.

Show outline outlines the cells.

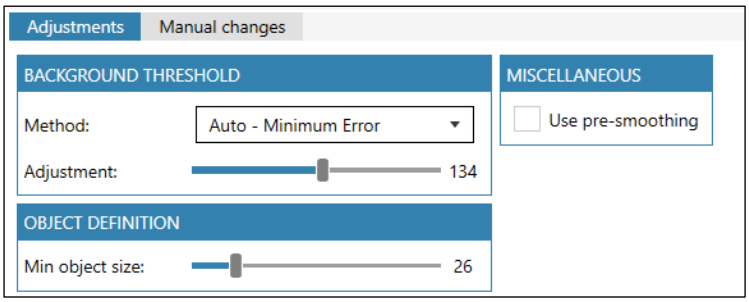
Show edge cells outlines cells touching the image edges.

View raw image displays the image without any adjustments to the coloring.

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

Cell identification adjustments

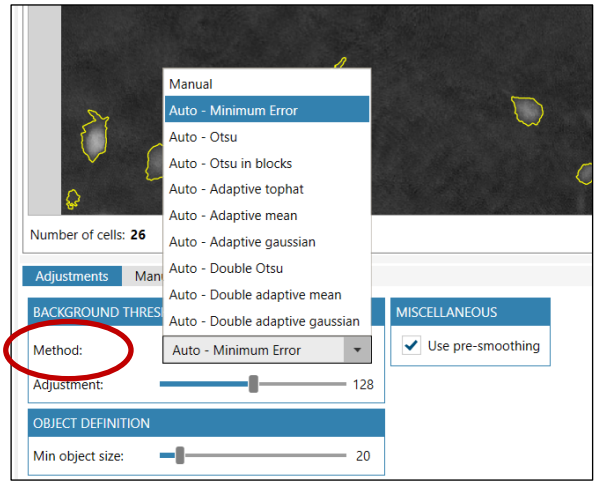
These adjustments encompass the entire frame and can be applied to other frames. The cell identification adjustments are performed in three steps:



1. Determine which threshold calculation method that results in the best cell identification for the current experiment.
2. If needed, use the Adjustment slider to fine tune 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.

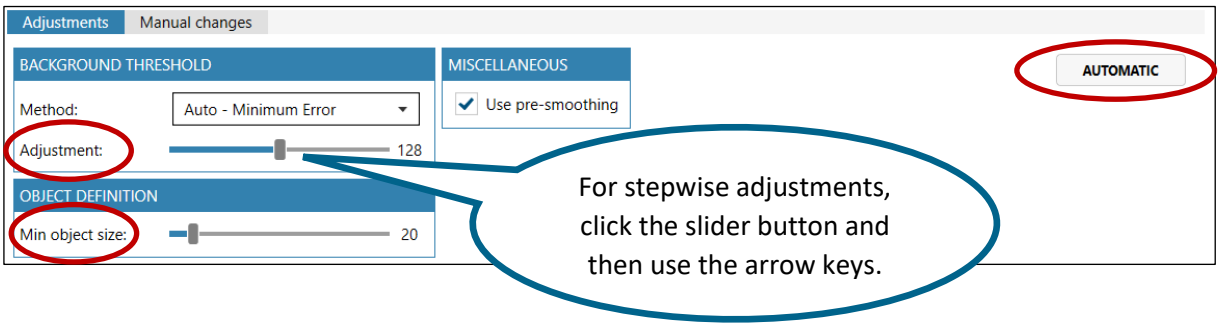
Double Otsu is a threshold method where both a wide and a narrow threshold mask are used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. Double Otsu uses double thresholding with Otsu global threshold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

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

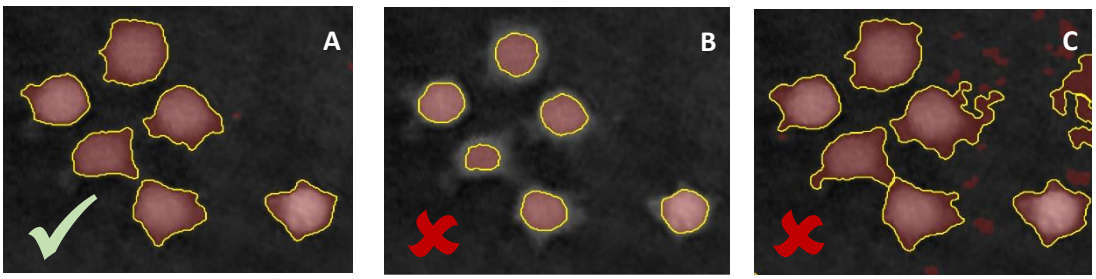
Double adaptive Gaussian is the same as Double Otsu, but with two adaptive Gaussian threshold masks.

2. Adjusting background threshold

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



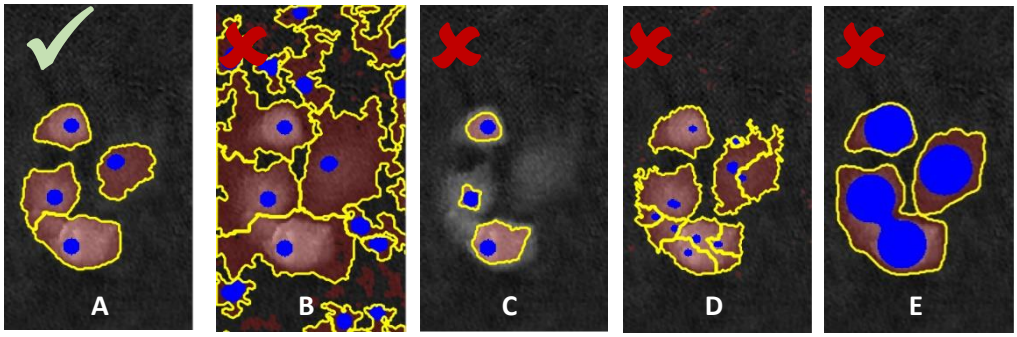
Adjusting the threshold between cell and background with the slider will adjust the minimum optical thickness of objects considered to be cells. Image A shows correctly thresholded cells, image B cells with a too high threshold, and Image C cells with a too low threshold.



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

The minimum cell size is adjusted by using the Min object size slider. The goal is to have one blue marker in every object considered to be a cell. 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



F. Threshold is OK, Object Definition is OK.
G. Threshold is set too low, too much of the background is counted as cell area.
H. Threshold is set too high, too little of the cell is included in the cell area.
I. Threshold is OK, Object Definition set too low, the cells are segmented into smaller units.
J. Threshold is OK, Object Definition set too high, the cells are merged into larger units.

Pre-smoothing

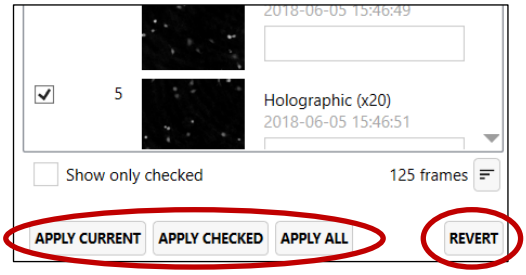
Activate Use pre-smoothing to smooth the cell edges.

Default values

Click Automatic to 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.



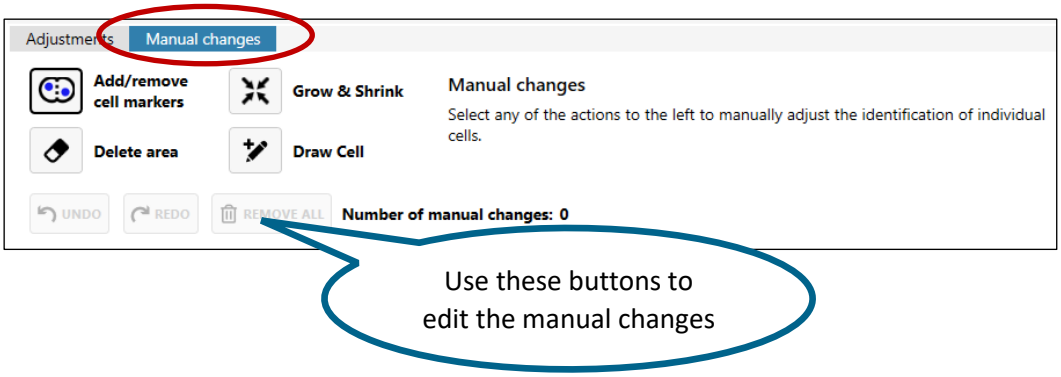
Revert cell identification adjustments

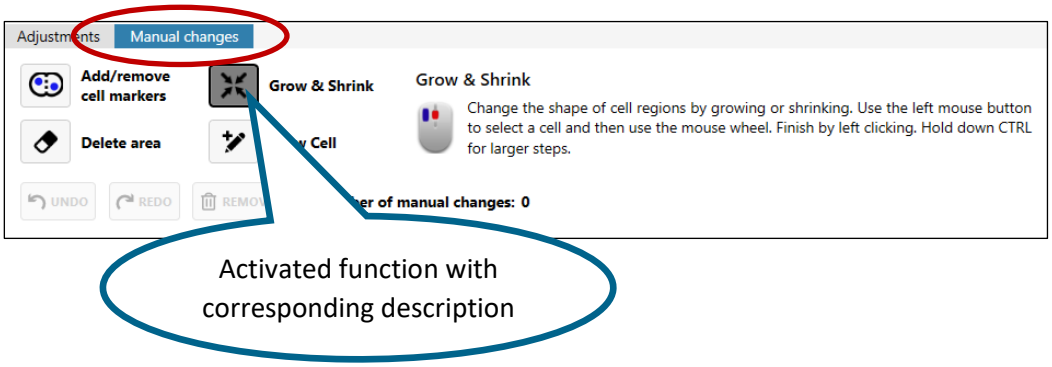
Use the Revert button to revert any changes back to saved settings for the current frame.

Manual identification adjustments for individual cells

Use the Manual changes tab, below the View area, to manually adjust he identification for individual cells. 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. Each function is described when the function is activated.

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





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 key for larger steps.

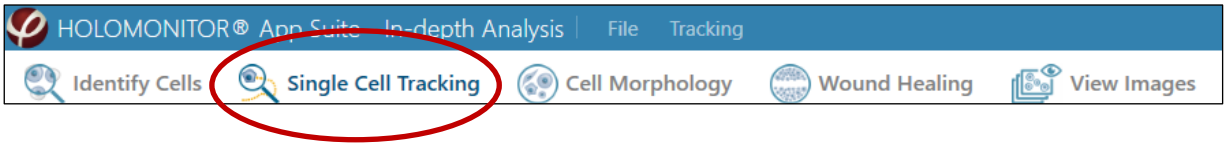
Delete Area removes threshold mask and marker from cell area. It can be used to remove small objects or disturbances created by floating cells that cannot be removed by other adjustments. Left click to select the area that should be deleted.

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 the selection.

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.



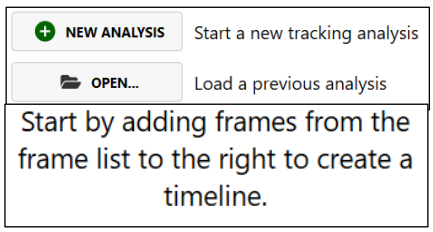
Cells must be identified in the Identify cells tab before using the Single Cell Tracking functions.

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. 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.



Tracking begins automatically and depending on the number of cells and the time-lapse length it can take several minutes for the software to track all the cells.

A short summary of how many cells that have been tracked, and how many cell families that have been detected is given once the tracking is finished.

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 reducing the amount of frames analyzed will decrea…es, making the process more efficient. Frames can be chosen by Selection, Sorti… …ded image frames are listed in the Frame List.

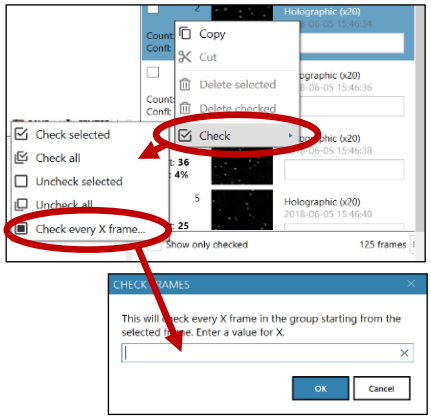
Cell tracking summary

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

Access the Check options by right clicking in the Image Frame list (see image to the right).

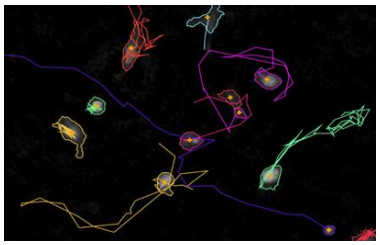
Use the Check options to analyze subgroups of images. Check every X frame is an especially useful tool in combination with the Show Only Checked function, which is found below the Image Frame list.

Tracking

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

Cell tracks

When the Timeline slider below the View area is moved by clicking the arrow buttons or dragging the black box, trails showing the cell movements will be displayed.

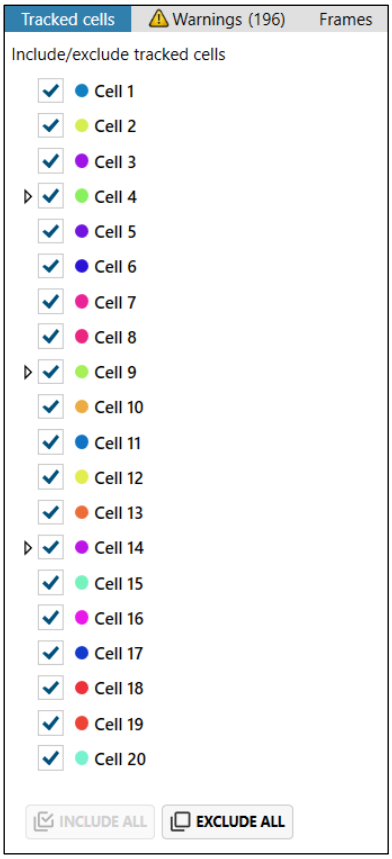


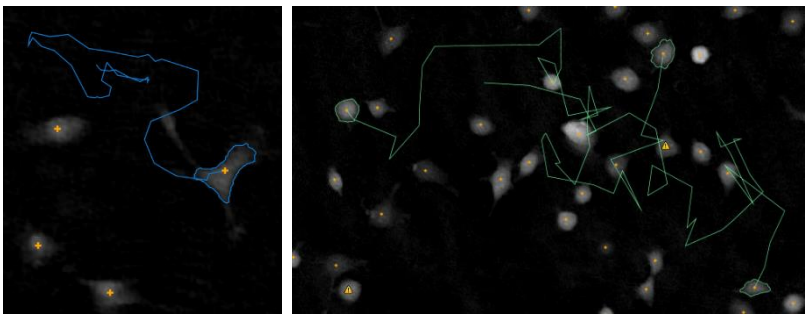
Use the checkboxes to hide or shorten the cell trails and to hide the cell outlines.

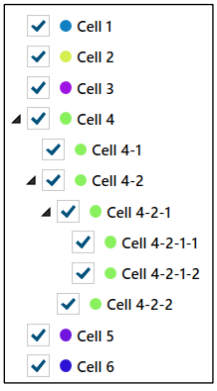
List of tracked cells

All tracked cells are shown in a list. The cells are numbered and color-coded.

To show only specific cell trails, use the checkboxes, and the Include/Exclude buttons. That way the trails of an individual cell or cell family can be shown.





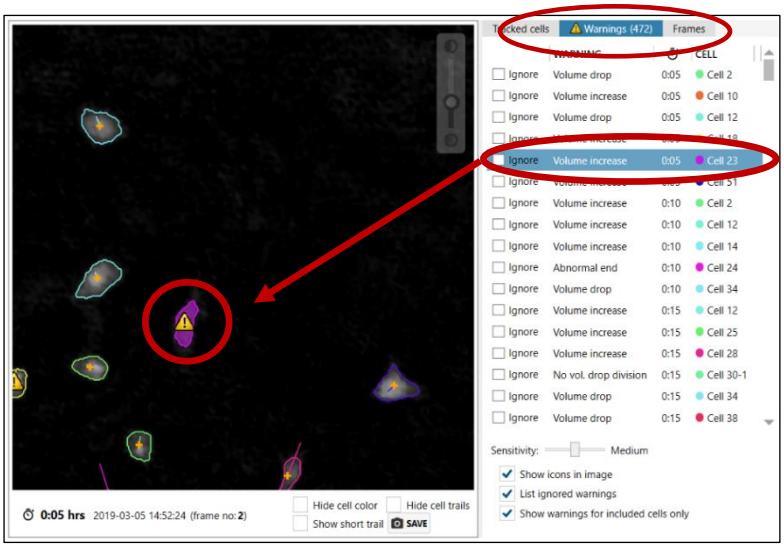


Cell families

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.

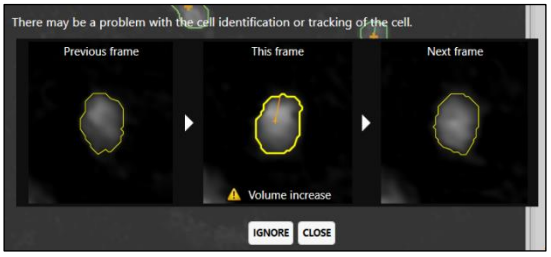
Cell tracking warnings

When the cell volume deviates greatly 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.

When the warning triangle is double-clicked, the cell identifications for the cell in the previous, current and next frames are shown. The user can then determine if the warning is appropriate or if it can be ignored.

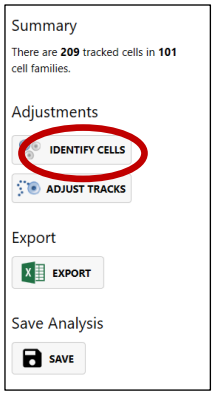


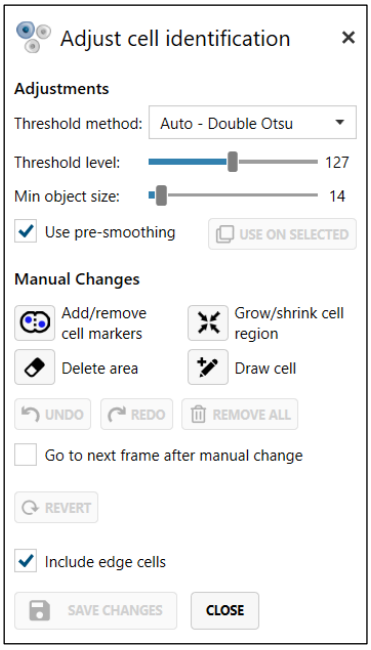
If the warnings are all deemed to be inappropriate, it is possible to do nothing with them. They will not disrupt the export of data.

- 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. The software automatically re-tracks all the cell tracks.





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.

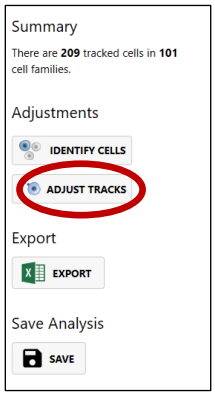
Include edge cells will include cells touching the edge of the image frame in the analysis for all frames.

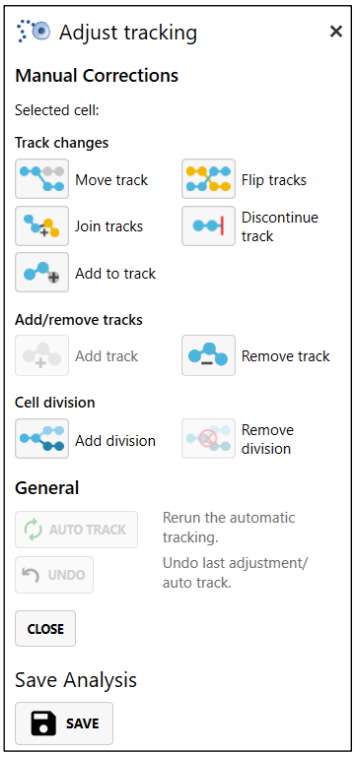
All other functions in this tab are equal to the functions in the Identify cells tab. The functions are described in detail in the Identify cells section of the manual.

All changes made in the Adjust Cell Identification tab will be applied globally to all results, thus changing other analyses (e.g. Cell Morphology) 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, press Auto track and close the tab.





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

Flip tracks is used to flip the 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.

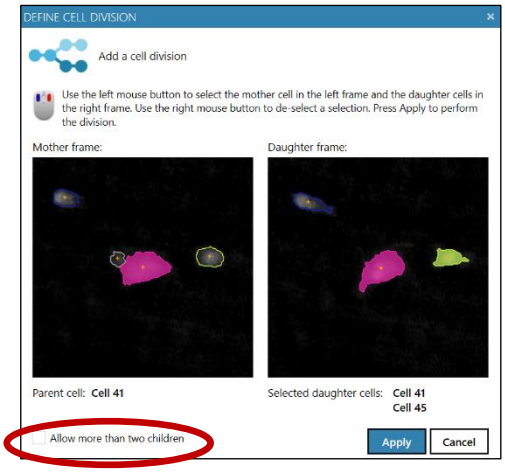
Discontinue track is used when a cell track needs to be terminated. After selecting the cell to be changed, click Discontinue Track. The tracking will be discontinued from the present frame and onward.

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 Add to track 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 Add track button, then follow the instructions.

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

Add division is used to add a cell division that was not recognized by the software. Start by selecting the mother cell,click the button, then follow the instructions as prompted. 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.

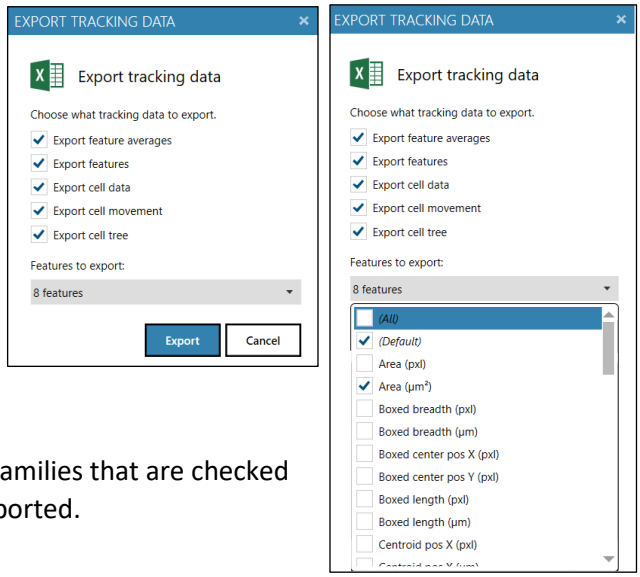
Auto track is used to re-run the automatic tracking while incorporating all manual changes to the data set, e.g., divisions and removals. It is recommended to use this function after any manual changes were made to cell tracks.

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.



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

Save analysis

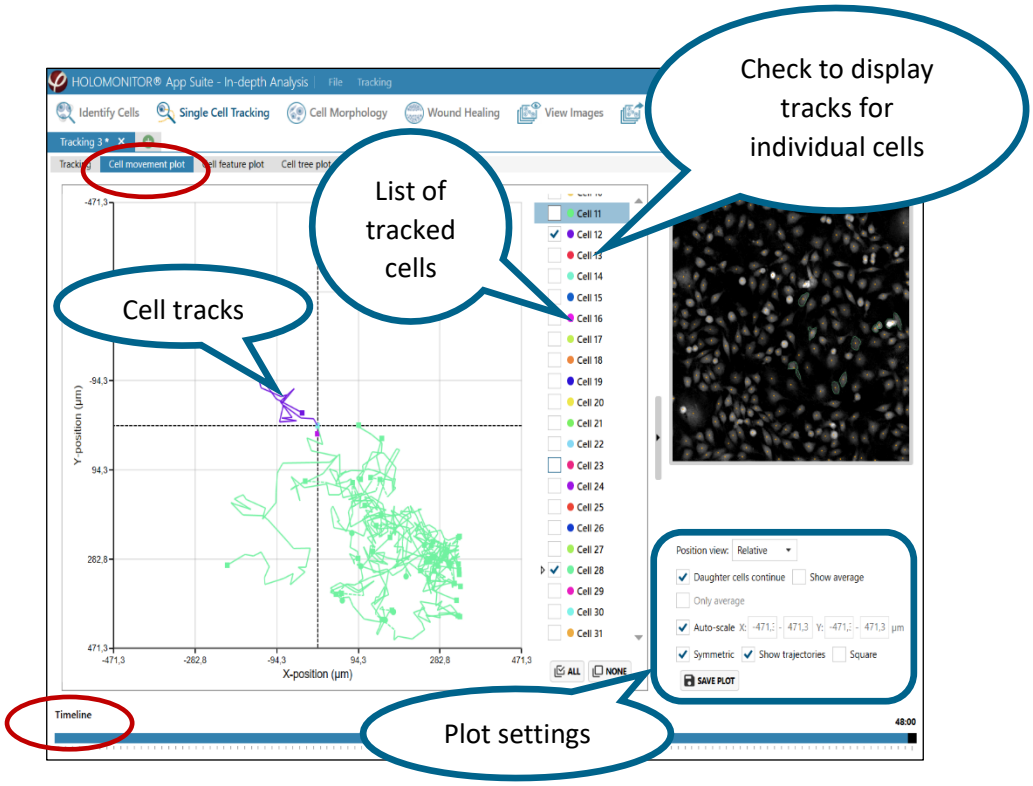
Click the Save button to save the analysis. If the same database is accessible, the analysis can be re-opened for continued use at a later timepoint.

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

Cell numbers might change in the re-opened tracking file.

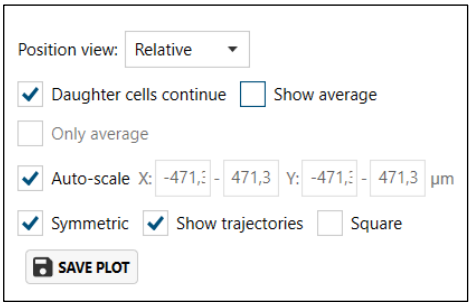
Plotting cell movements

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. Use the timeline slider to view the tracks at different timepoints.



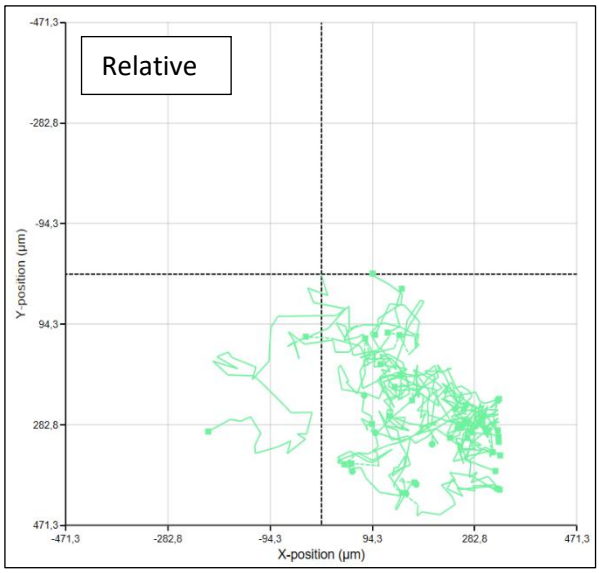
Plot settings

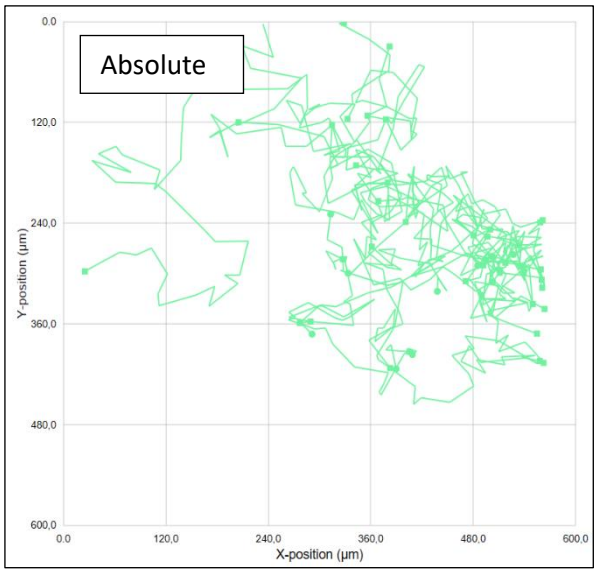
Plot settings are found below the Image view.



Position view

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





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

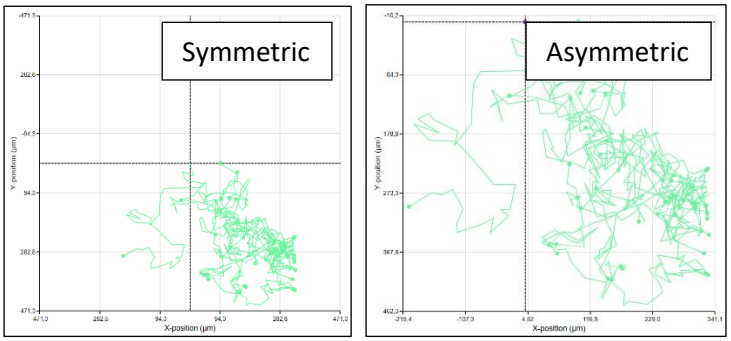
With only average checked, all other tracks will be hidden.

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.

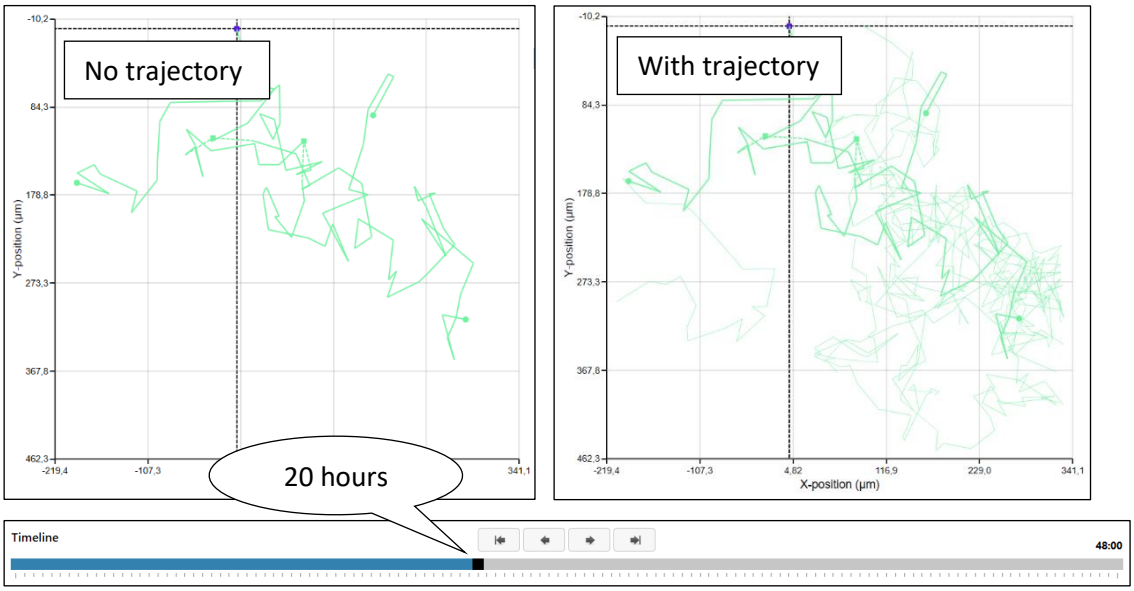
Symmetric

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 tracks.



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

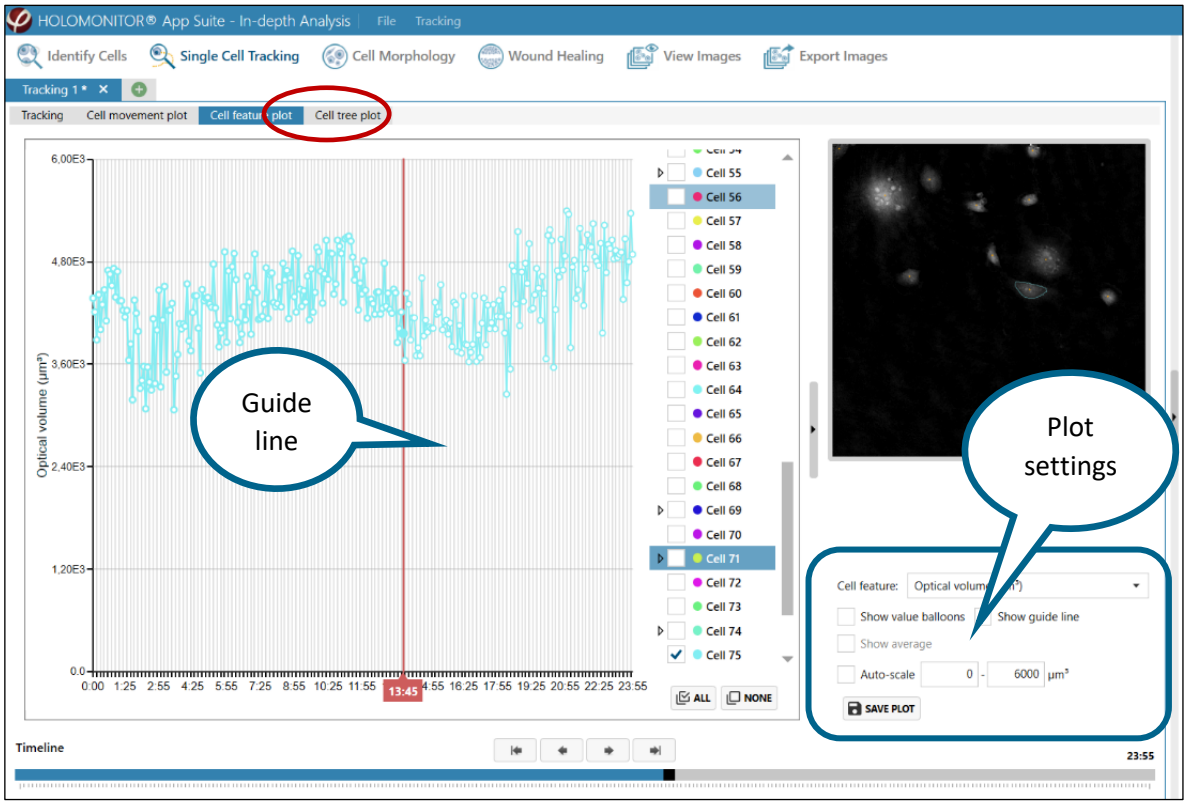
Checking Square shows a plot that is square, meaning X and Y axis min-max values are the same.

Save plot

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

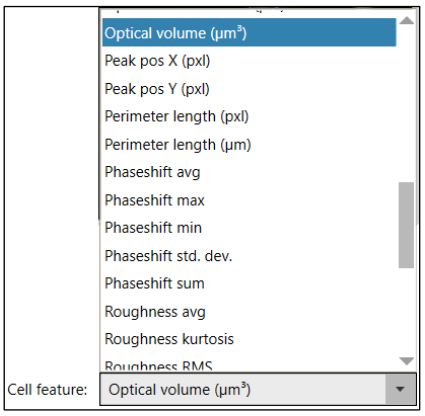
Tracking cell morphology

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



Plot settings

Plot settings are used to adjust the plot display.



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 Analysis Feature section 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, .bmp, or .gif format.

Cell tree analysis

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

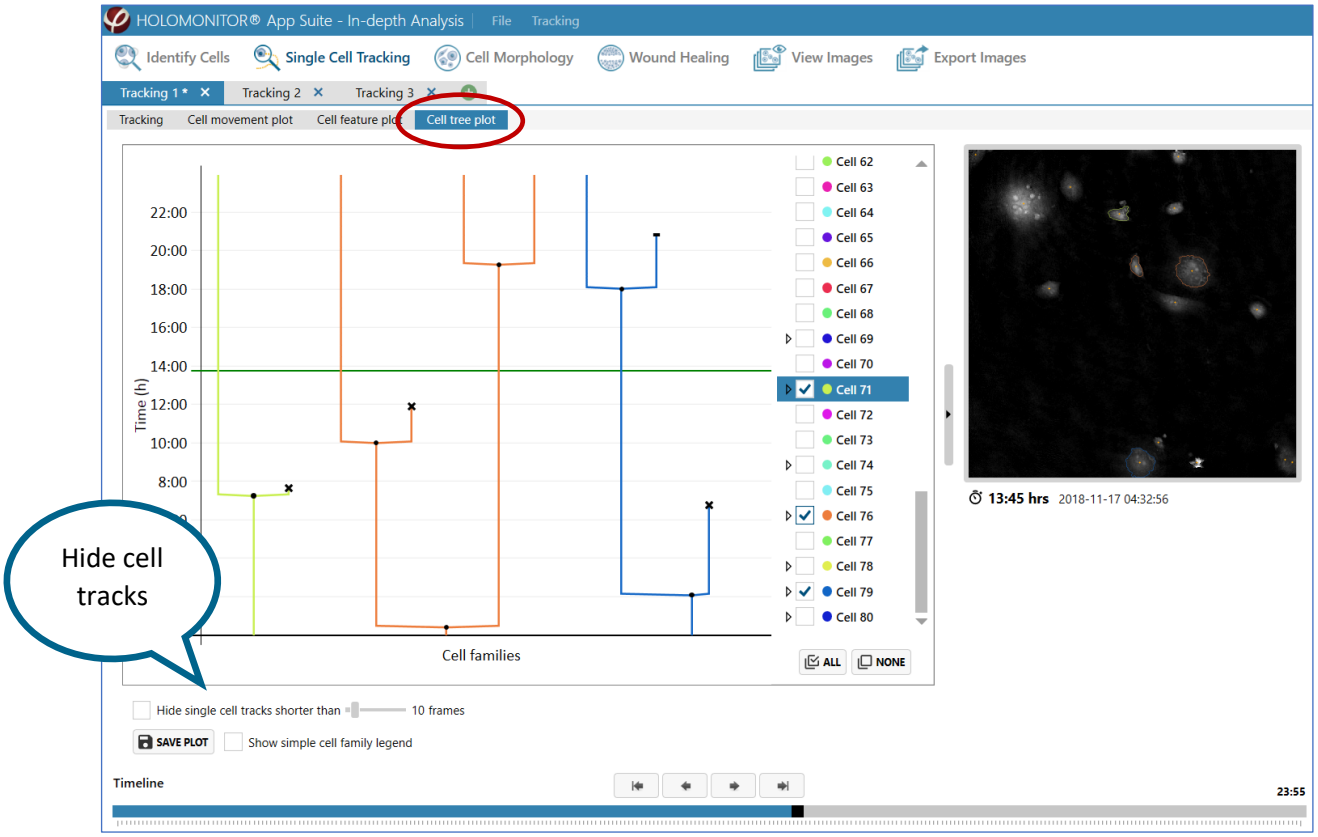
x symbolizes that the tracking of the cell ends within the frame, which can be caused by, e.g., cell death or cell engulfment.

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

• symbolizes cell division

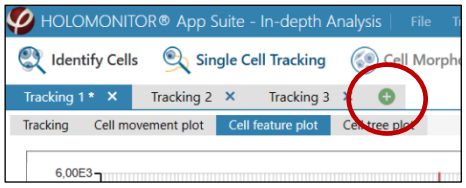
In the tracked cell list, cell families are numbered and color-coded for easy identification. When Cell-4 divided, the daughter cells were named Cell 4-1 and Cell 4-2. When Cell 4-1 divided, the daughter cells were named Cell 4-1-1 and Cell 4-1-2 and so on. All the cells in a family have the same color.

There is an option to hide cell tracks. Using the slider towards the bottom of the screen, tracks which are present during less 10 frames can be selected to be hidden, based on the number of frames in which they are present.



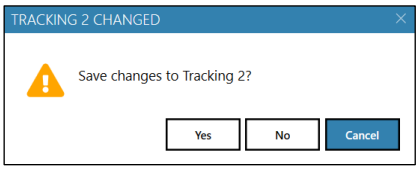
Multiple analyses

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

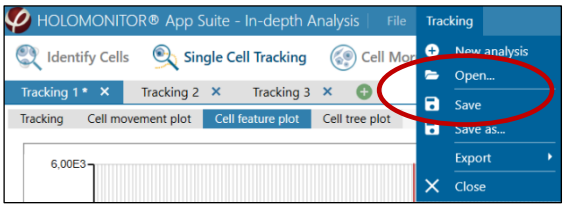


Save or open tracking analyses

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 is available.



There is a Save option in the top Tracking menu as well. Previously saved trackings can be opened from this menu as well.

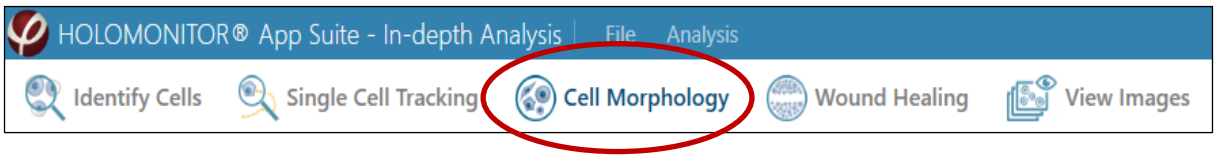


The analyses are saved as separate files to the computer. They are not integral parts of the software and need to be backed up separately.

In the saved all analyses for all tracks/cell families will be saved irrespective of whether they are selected or not.

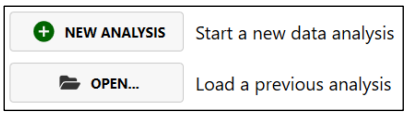
Cell morphology

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 one. When New Analysis is selected, a request to add image frames to the analysis appears.



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

Add image frames to the analysis

Select an Experiment 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 table.

Sometimes not all images are needed to get good results. Selecting only a portion or subsection of the total frames may improve experiment throughput and time efficiency.. The frames can be chosen by selecting, sorting or checking options.

Image selection

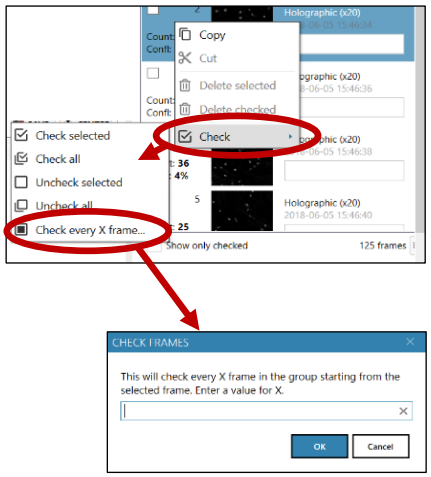
Several images can be added simultaneously if they are all selected. The SHIFT key can be used to select several consecutive images and the CTRL key to select non-consecutive image frames.

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. The Check every X frame is especially 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 frame that is selected in the Source frame list.

Add data from different experiments or treatments

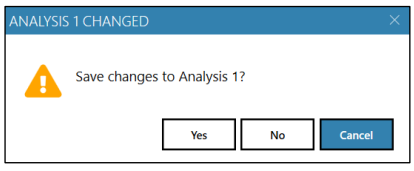
Data from different experiments and treatments can be added to the same analysis if they are stored in the same database.

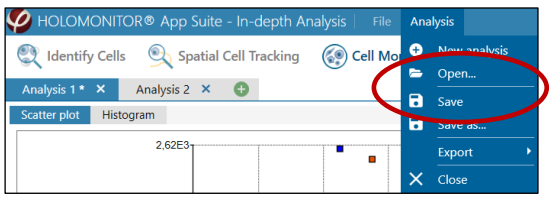
Multiple analyses in parallel

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 closes, a choice to save the changes to the analysis appears. The saved analysis can be opened in the top Analysis menu as long as the database with the data is available. There is also an option to open saved analyses.



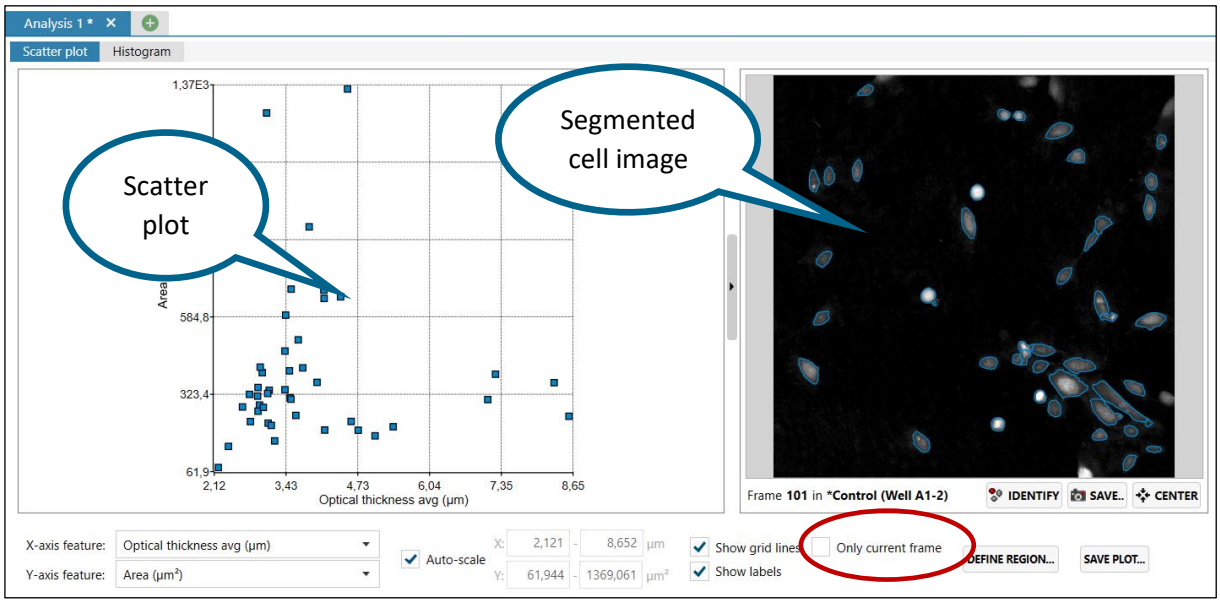


The analyses are saved as files to the computer, and are not integral parts of the software, so theyneed to be backed up separately.

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.

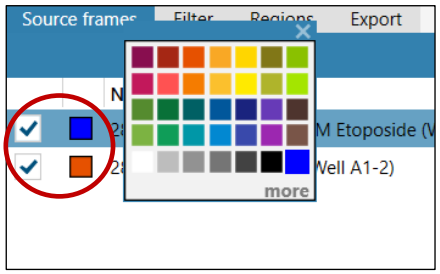
If several frames are represented in the plot, the data points from all images will be displayed in the plot simultaneously, while the frame that is selected in the Source frames table is displayed in the viewer.

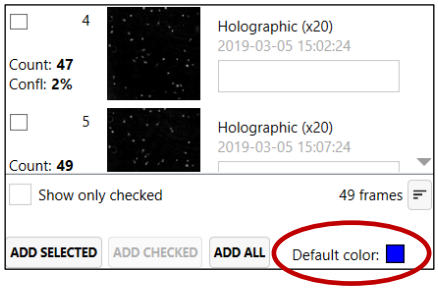


Data point coloring

The data points will be displayed in the plot with the color shown in the color box in the Source frames table, 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,time points or other conditions, 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.

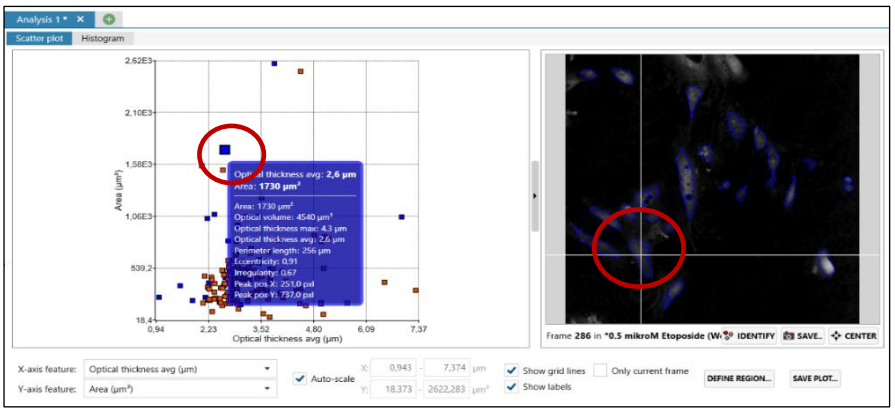
By changing the default color before adding the frames, they will have the designated color when added.



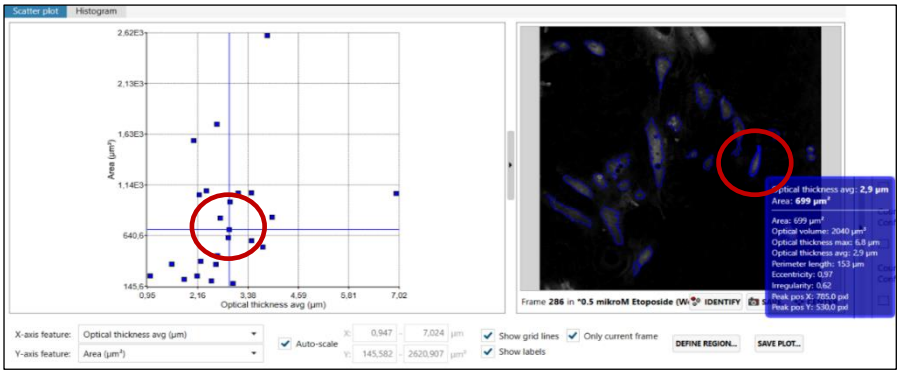


Identify which data point corresponds to which cell

Hovering the mouse cursor over a data point identifies the cell that is connected to that data point. The corresponding cell will then be identified in one of the cell images by a crosshair. Cell feature information for that cell will be displayed simultaneously in a box.

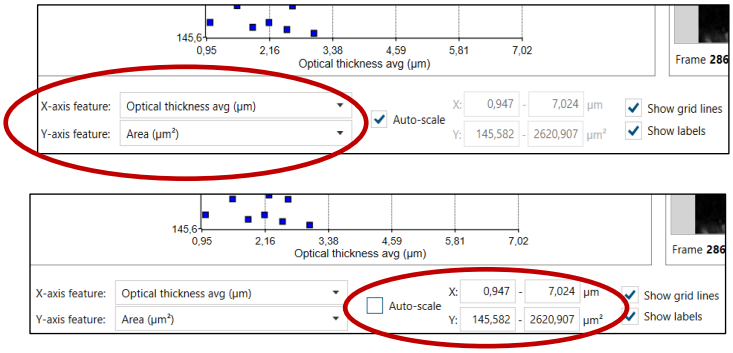


It works the other way around as well. Hover over a certain cell in an added image frame to identify the corresponding data point by a crosshair. Cell feature information for that cell will be displayed in a box.



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 Analysis Features at the back of the manual.



Set the scatter plot axis range manually

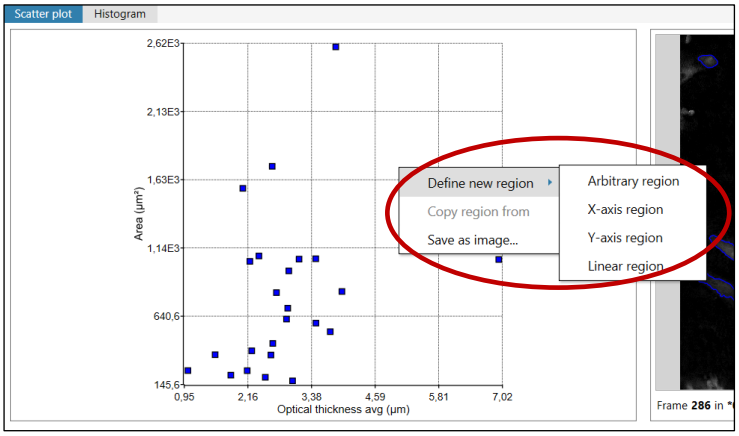
The scatter plot settings are found below the scatter plot. The area of the scatter plot (i.e., the values of the x- and y-axis) is set automatically to fit all values from all added frames. To set the plot area manually, deactivate Auto-scale and enter desired x- and y-minimum and maximum values.

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 with 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.

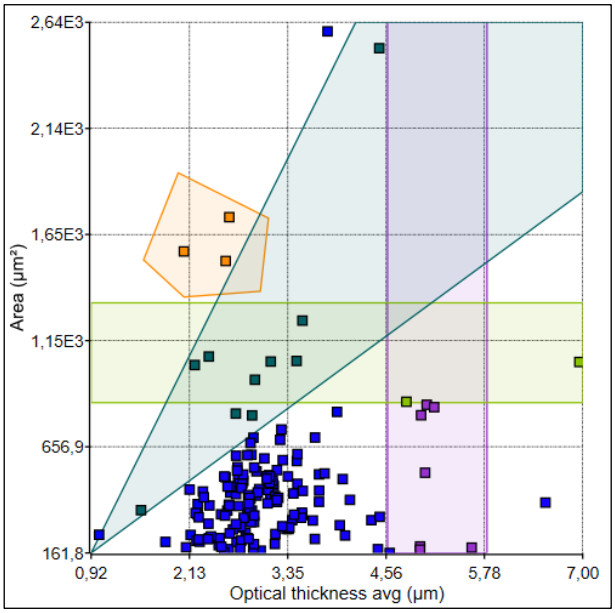
Select a region type by clicking it. There are four region types:

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.

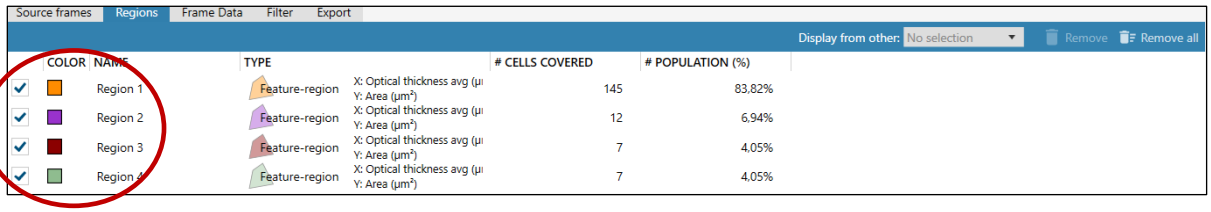
Linear region (dark green) where clicking in the diagram results in straight lines that cut off the region from origin (X=0, Y=0). First mouse click in the plot determines the starting point and second click the end of the region.

Arbitrary region (yellow) where repeated clicking in the diagram results in an irregular shape 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.

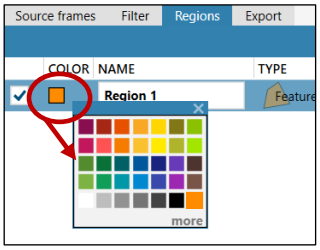


The regions tab

The regions are summarized in the Regions tab, which is found below the Scatter plot tab.

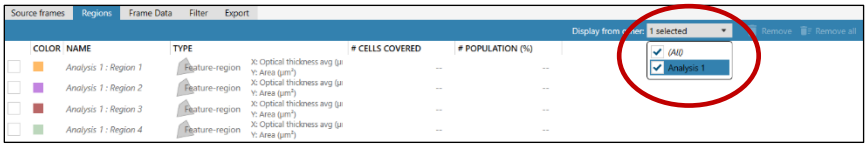


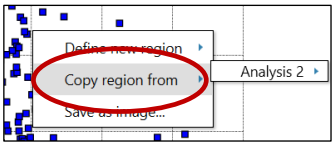
Regions can be hidden by unchecking.



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

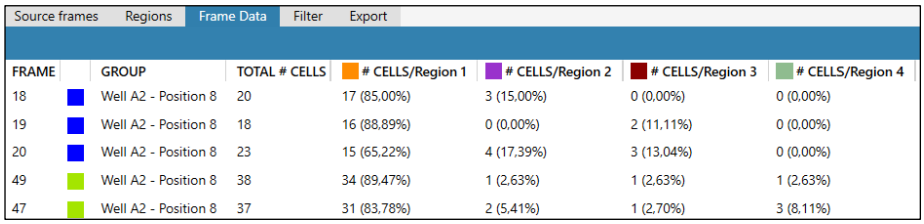
Regions can be copied from one open analysis to the next by using Display from other in the Regions tab or by right clicking the mouse on the scatter plot and selecting Copy region from and selecting a region to copy.





The frame data tab

In the frame data tab, total cell number for each frame and how many of those cells that are found in respective region are given.

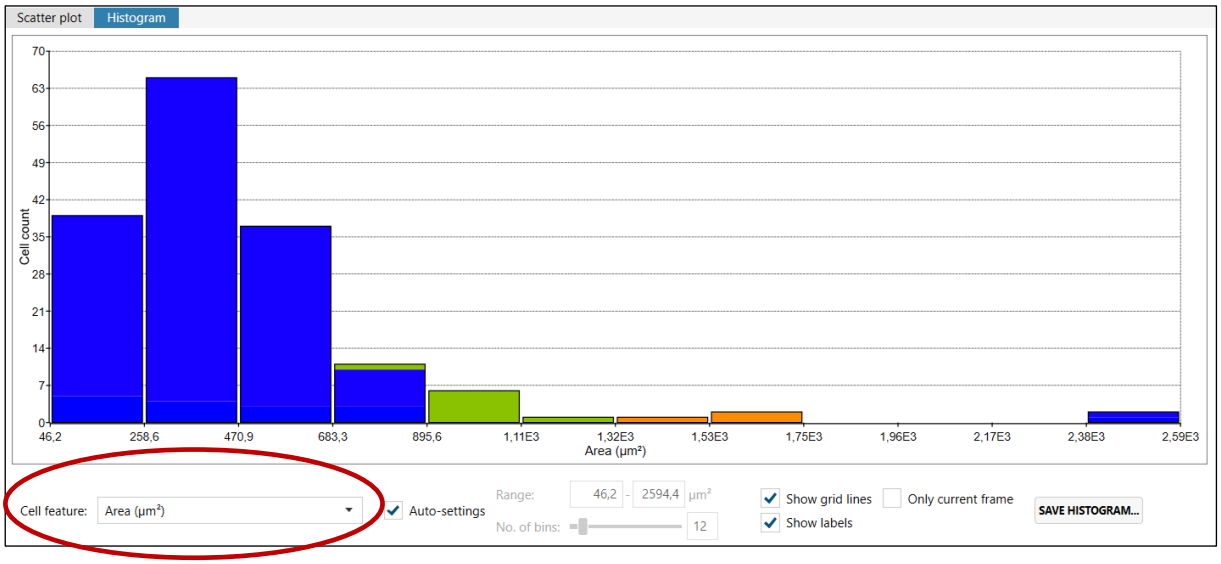


Display results as histograms

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.

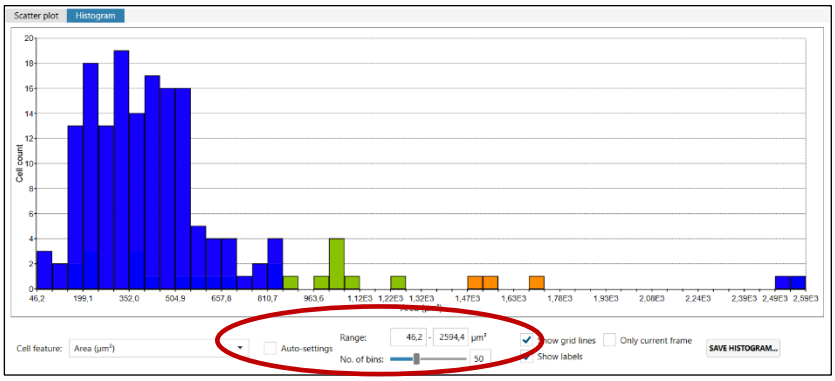
Change the histogram x-axis units

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 Analysis Features section at the back of the manual.



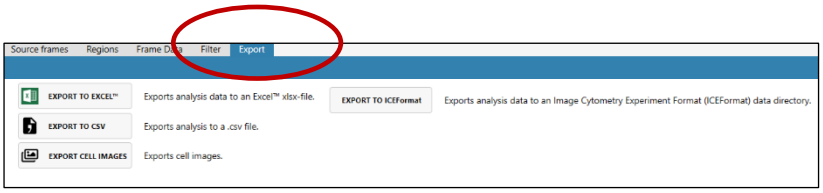
Change the histogram x-axis intervals and bins manually

The histogram x-axis interval is set automatically to fit in all values from all added frames. To make these changes, Auto-settings must be unchecked and enter the minimum and maximum values. The number of bins can be changed by using the slider of typing a number in a text box.



Export cell data

In the Export tab, cell data from the current plot can be exported as Excel, CSV or ICE files. Images of individual cells can also be exported.



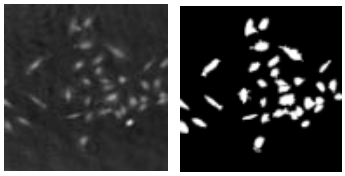
Click one of the Export buttons. A file browser window will open. The file will automatically be named and dated, but it is possible to rename the file. Select a save location. All data relating to the image frames added to the current plot are exported.

Excel exports

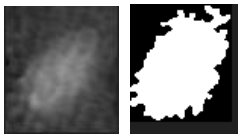
Excel, ICE, and CSV exports will contain morphological information regarding every individual cell in all frames added to the analysis as well as the cells belonging to every region. Average morphological data for every image frame will also be available.

Export Cell Images

Cell image export applies to all the cells in all frames added to the analysis. Several different types of images are exported.



Holography image frames together with the respective masked image for cell identification

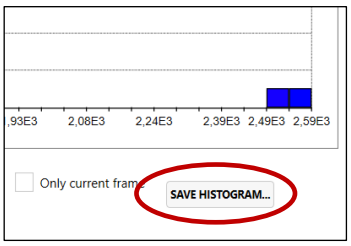


Separate images of every individual cell and the cell identification masks belonging to every cell.

An index image containing a cell map based on pixels that are colored by cell index number. The indexed masks image is a 16-bit png image of the whole frame where the mask of each cell has the greyscale color value of the cell's index. Opening the image with a photo editing software, e.g., Image J, GIMP or Photoshop, will display the greyscale colors.

Save images, plots and histograms

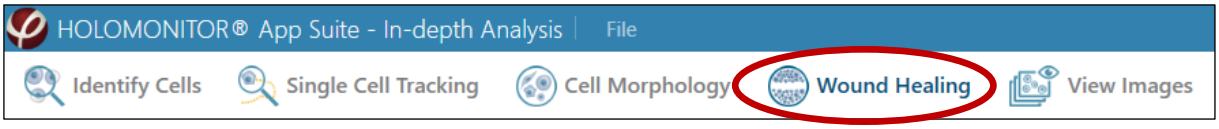
Images of the current cell image, plot and histogram can be saved as BMP, GIF, JPEG, PNG or TIFF by using the Save, Save Plot and Save Histogram buttons.



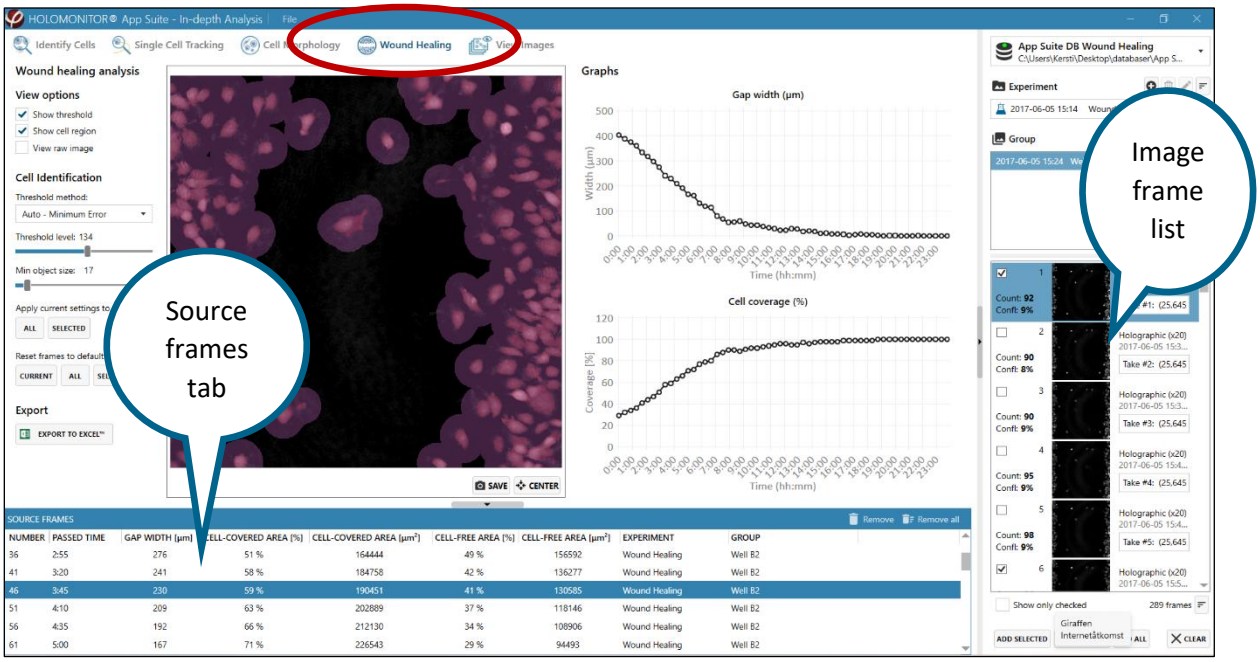
- Click the respective Save, Save plot or Save histogram button. A file browser window will then open.
- The file will automatically be named but it is possible to rename the file.
- Select an image format (bmp, gif, jpeg, png, tiff).
- Save the file to an appropriate location.

Wound healing

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



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.

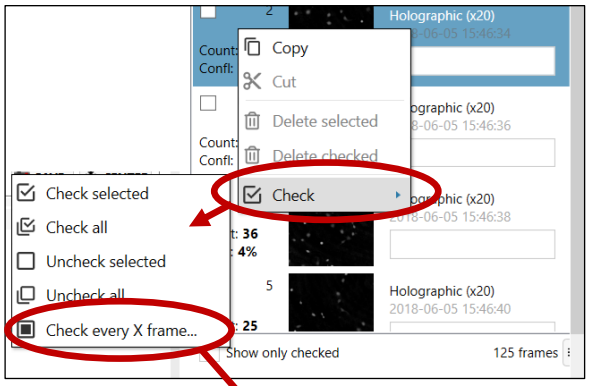
Begin by adding frames from the frame list to the right.

Add images

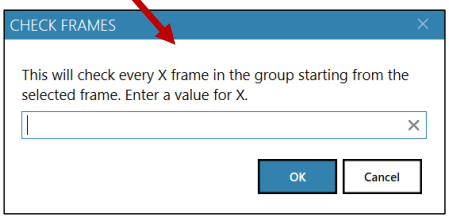
Select an Experiment and a Group in the Image Frame list, which is found to the right of the Viewing area. Then select image frames and transfer them to the analysis by using the Add buttons, found below the Image Frame list, or by drag and drop into the source frames list.

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.

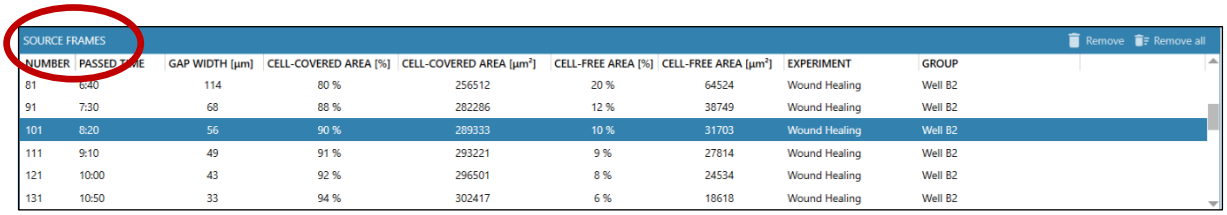
Often, for wound healing analyses, only images captured every half hour, or every hour are needed. As an example, if images have been captured every five minutes, but only every 30 minutes (i.e., every 6 images) are needed, use the check-functions to select every 6th image.



- 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.

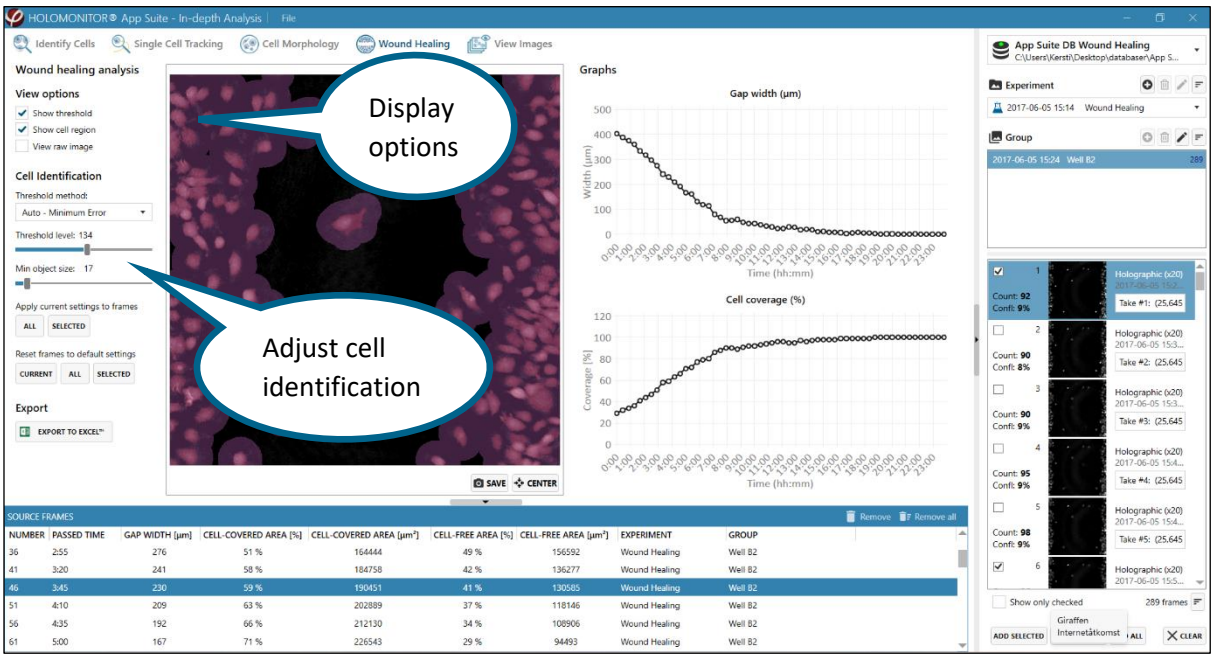


The images that are included in the analysis are listed in the Source frames tab, found below the Viewing window.



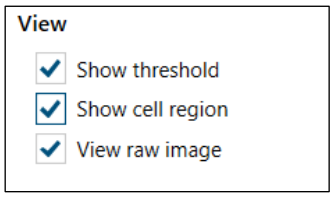
Masking the gap

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.



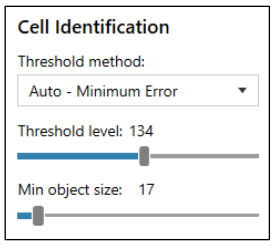
Show threshold displays the identification of cells.

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 Minimum error 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 experiment. The method that suits the experiment 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.

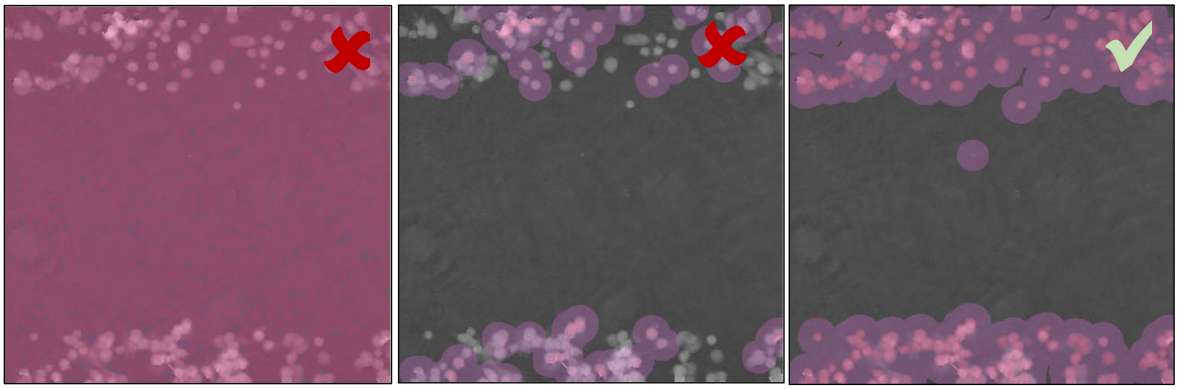
Double adaptive mean is the same as Double Otsu, but with two adaptive mean threshold masks.

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

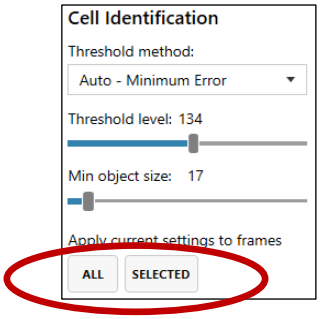
In addition to the different automatic calculation methods for cell identification, the user can adjust the settings using the sliders in the Identification panel. Use the threshold level slider to adjust the

cell areas. Adjust the minimum cell size with 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 settings to frames, All or Selected. The software will then apply the threshold and analyze the gap width. This might take several minutes, depending on the number of source frames.



In short, 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, both in a table and as graphs

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 increase steadily without big deviations between frames (see image below). 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.

SOURCE FRAMES

Remove | Remove all

NUMBER	PASSED TIME	GAP WIDTH [µm]	CELL-COVERED AREA [%]	CELL-COVERED AREA [µm²]	CELL-FREE AREA [%]	CELL-FREE AREA [µm²]	EXPERIMENT	GROUP
1	0:00	404	29 %	92317	71 %	228718	Wound Healing	Well B2
11	0:50	375	34 %	104426	66 %	212609	Wound Healing	Well B2
21	1:40	334	41 %	131180	59 %	189455	Wound Healing	Well B2
31	2:30	298	47 %	151074	53 %	168661	Wound Healing	Well B2
41	3:20	241	58 %	184758	42 %	136277	Wound Healing	Well B2
51	4:10	209	63 %	202889	37 %	118146	Wound Healing	Well B2

Export data to excel

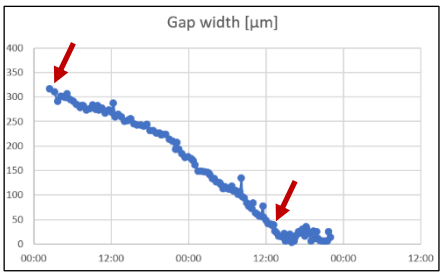
Use the Export button to export the data to Excel. In the resulting file, graphs and data for gap width in µm, as well as cell covered area, and cell free area, in % and µm^2, are given.

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. Then divide with the difference by the number of hours between the values.

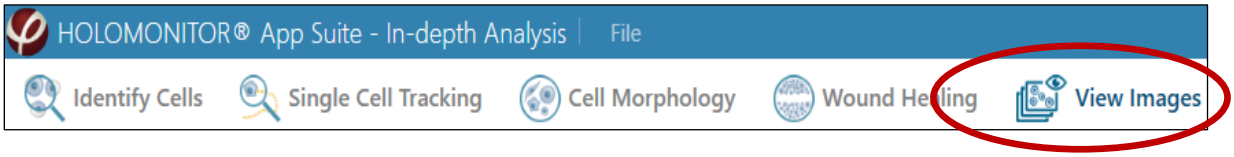
In this example: (321-25) µm/36 h = 8 µm/h

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.

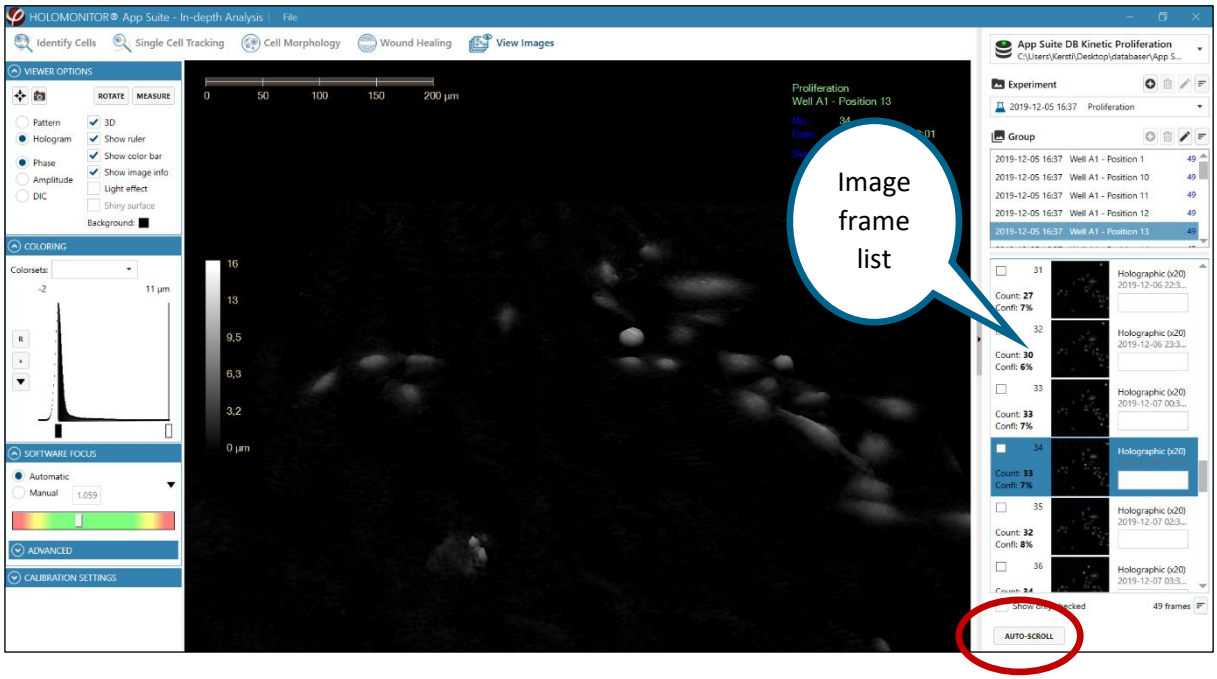


View an image

Select an Experiment 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 time-lapse.



Move, flip or zoom the cell image

To zoom the cell image, move the mouse cursor to the view area, then 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.

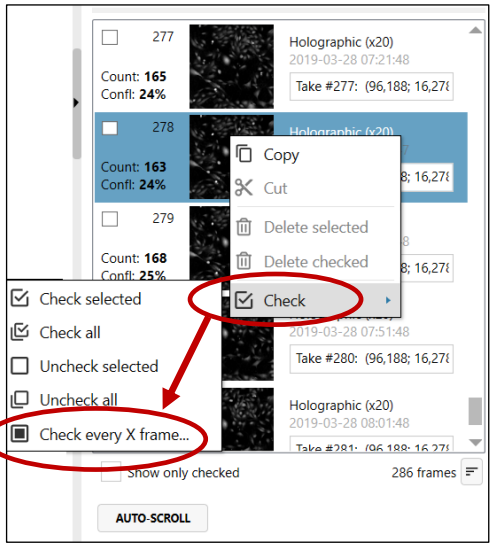
To flip and move a holographic 3D image, click, hold and drag the image using the right mouse button on the image.

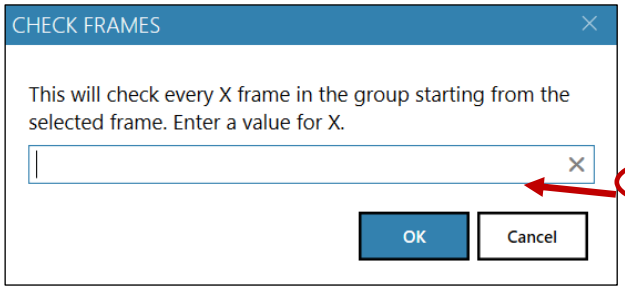
For Hstudio images, view one position of several in parallel time-lapses

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 then 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.



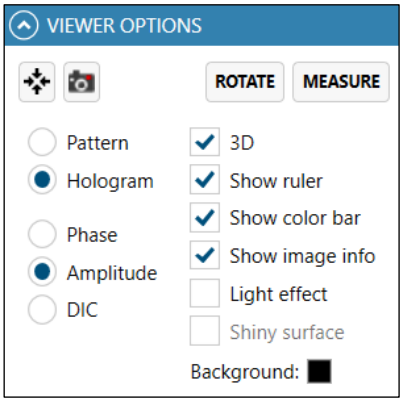


For Hstudio images, view all positions in a group as sequential time-lapses

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 changed 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.

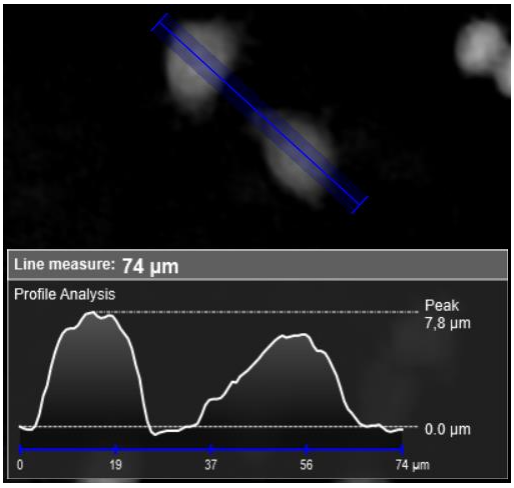
Light effect applies an artificial light source to the image which may sometimes render an improved image.

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 Line Measure, a simple tool for interactively measuring distances and cells.



Once the tool is activated, the measurement is started by left clicking anywhere in the image. The measurement is finished by left clicking at a new point in the image.

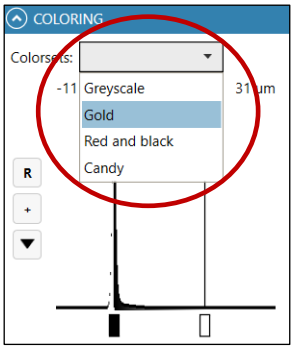
It is possible to move and flip the image while the measuring function is activated.

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

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

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 the 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. This will overwrite the settings previously saved.

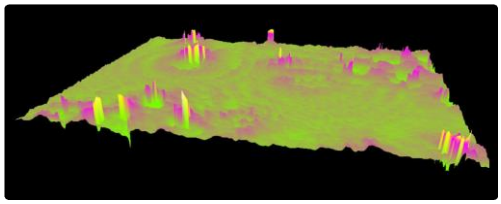
Delete a Colorset by selecting it in the drop-down Colorset list. Then left click the button and select Delete colorset.

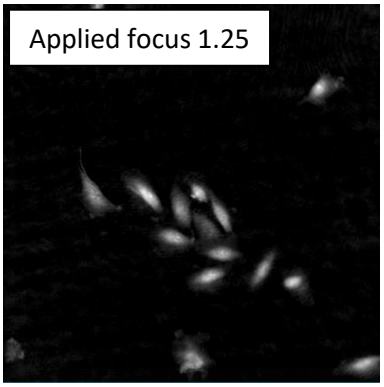
Colorsets are stored with the databases. Thus, if a colorset is created in one database, it is not found in a different database, even on the same computer or harddrive.

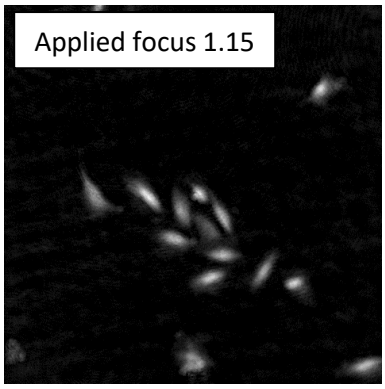
Colorsets do not carry over from the App Suite assays to In-Depth and vice versa.

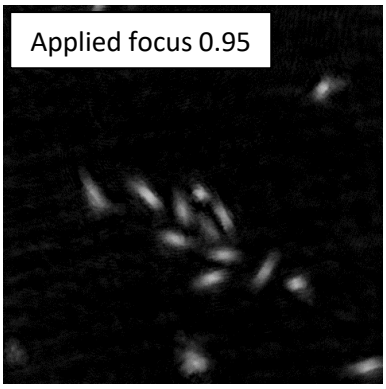
Re-focus a captured image

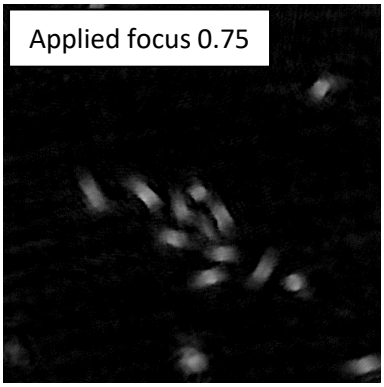
After image capture, most images are well focused. Some images may need to be re-focused manually after capture. If an image is not correctly focused, as in this example, recalculating the software focus may restore focus. The image below has been manually set to different software focus values.

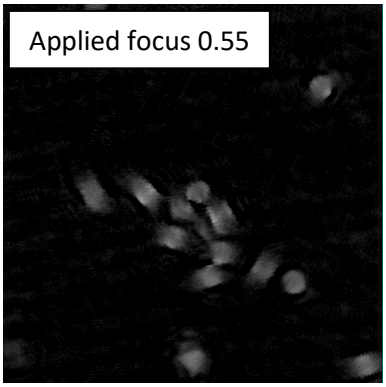


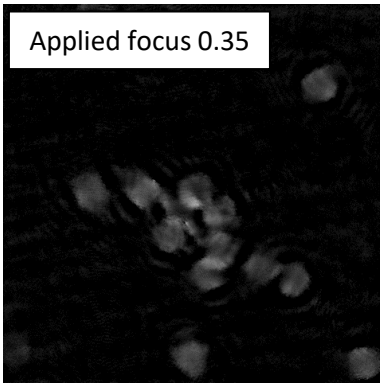


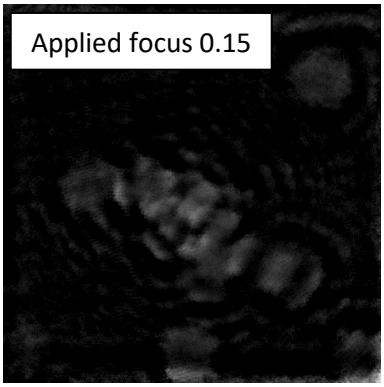






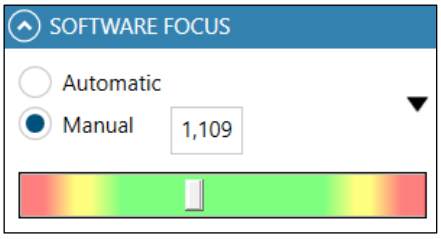






Recalculate focus manually

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.

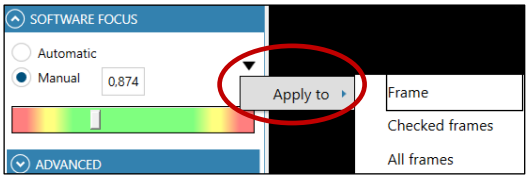
Use the ▼ button to apply the update to either the current frame, all frames or all checked frames.

Recalculate focus automatically

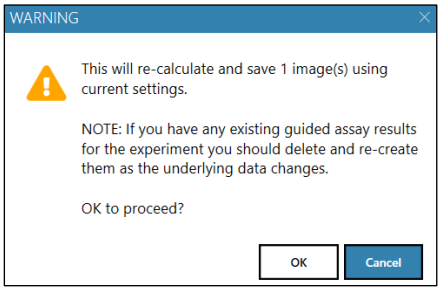
To replace the manual changes with the automatic focus, activate Automatic in the Software focus panel and then use the ▼ button to apply the update to either the current frame, all frames or all checked frames.

Activating Automatic focus will reset the software focus to the original values. These are the values that were applied when the image was captured. They may not be the optimal focus settings.

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

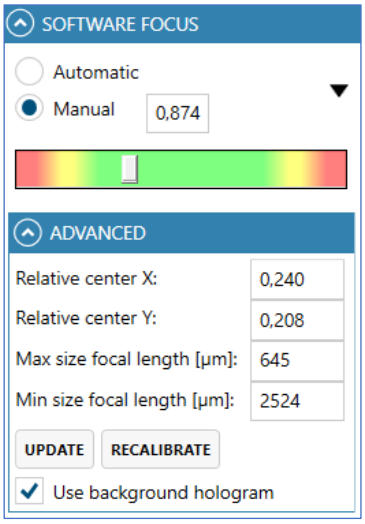


If the software focus is recalculated in a guided assay experiment, this will not automatically transfer to existing results. Instead, the results have to be re-created.



Advanced

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

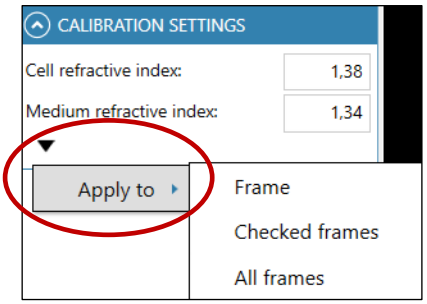


Update adjusts the current image display using the set values.

Recalibrate calculates new values based on the image.

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 optical thickness in µm.

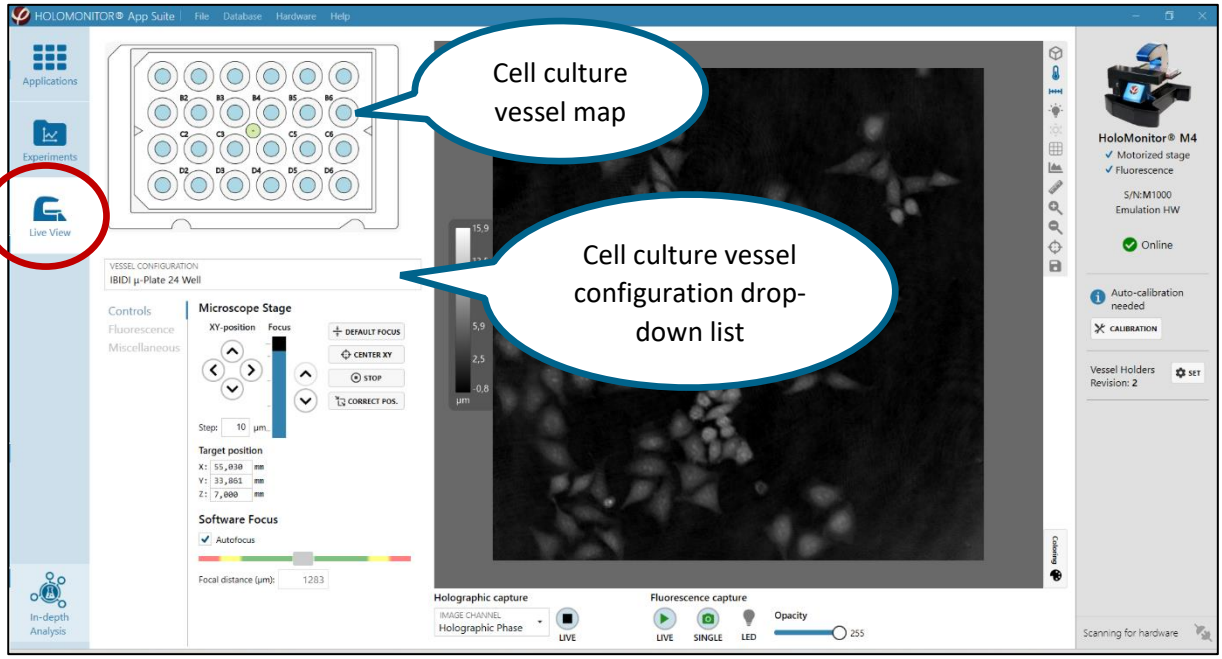


The default refractive index settings can be changed in the Calibration side panel.

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.

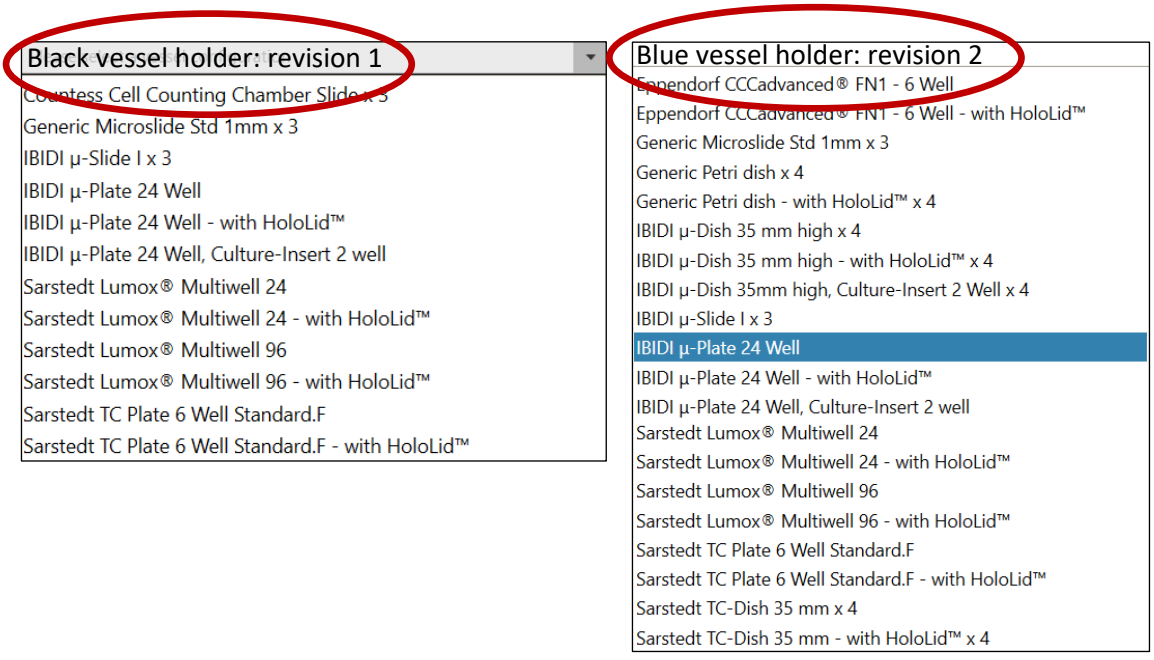
Live view

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



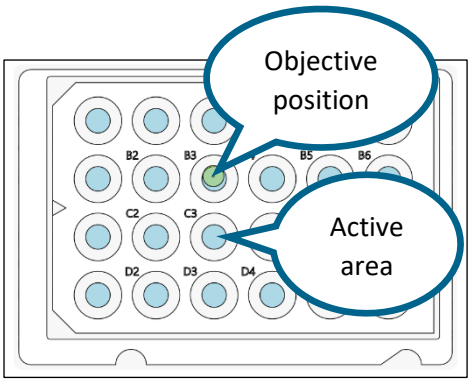
Cell culture vessels

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 list. The following vessels, depending on the vessel holder, are supported:



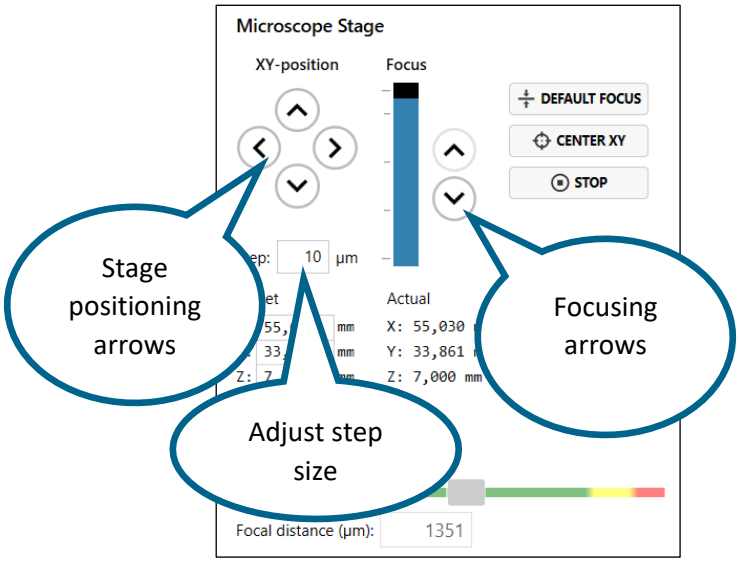
Moving the motorized stage

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.

The step length applies to all directions of stage movement: X, Y and Z. Large 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.

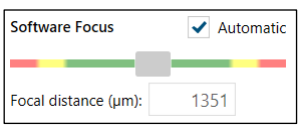
Clicking the Stop button will immediately stop all stage movement.

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

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.



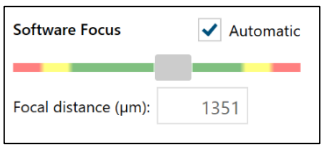
HoloMonitor has both a coarse mechanical and a fine software focus. They need to cooperate for the image to be in focus. Use the mechanical focus, *i.e.* move the motorized stage, to move the cells to the approximately correct distance from the objective. The software focus then finds and calculates 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.

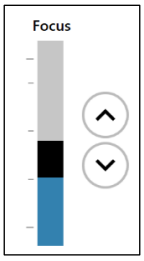
Focusing in practice - semi automatic focusing

By default, Automatic focus is activated.

For good focus, the grey marker in the Software Focus indicator bar 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 in order to shift the marker to the middle.



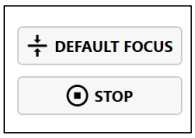
The black box in the Focus bar represents the motorized stage. Move the black box up or down to move the motorized stage up or down. Click the two arrows for smaller stage movements up or down.



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

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.



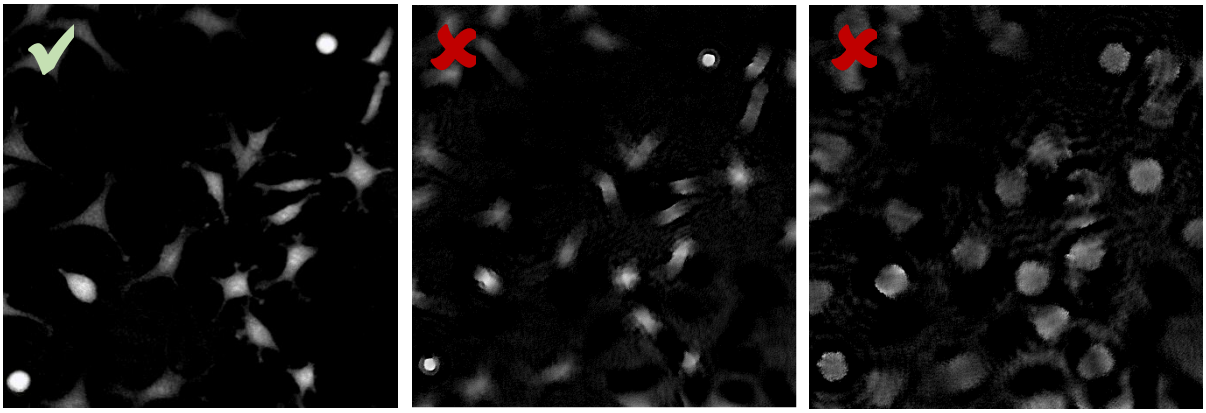
Manual focusing

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

- Uncheck Automatic in the Software focus panel.
- First adjust the software focus settings. Use the mouse cursor to move the grey marker in the Software Focus indicator until it is centered in the green area.
- Then adjust the mechanical focus by moving the stage 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 stage until the cells are in focus.

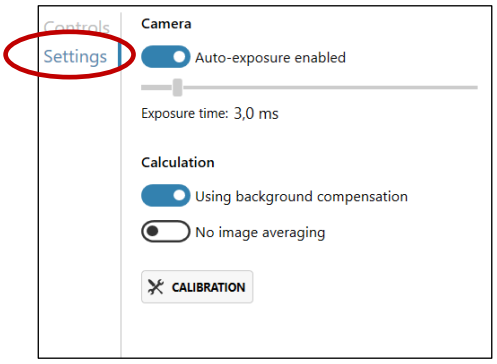
Images in and out of focus

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 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.



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.

Auto-calibration

Auto-calibration gives a background image that is used by App Suite to lower background noise and increase image quality. There is a more detailed description in the Image calibration section in the HoloMonitor Installation chapter.

Live image

Move 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.

Moving the image will not move the motorized stage to a new position.

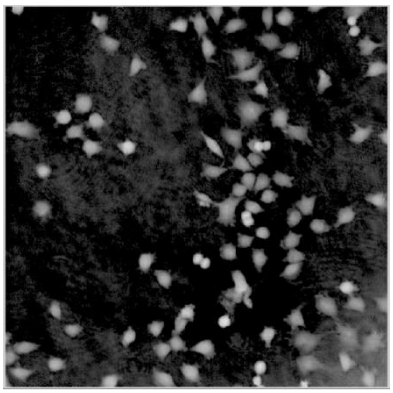
Image channels

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

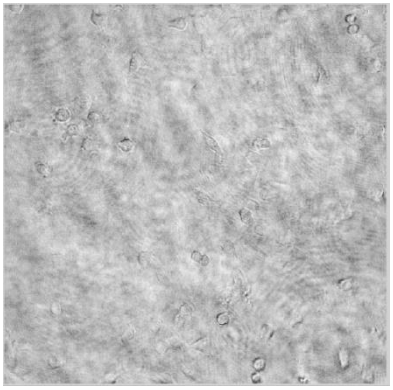
The Holographic Phase Image is a reconstructed image based on the light phase information in the hologram.

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

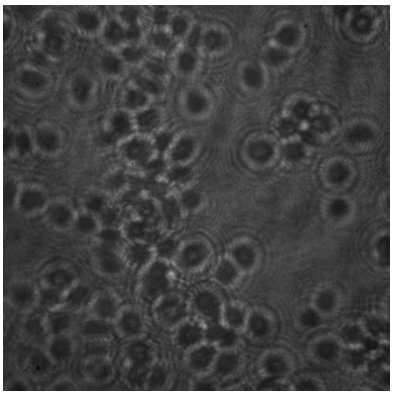
The Hologram image is captured by the camera and is the result of interfering light waves. The hologram is the basis for the reconstructed holographic cell images.



Holographic phase image



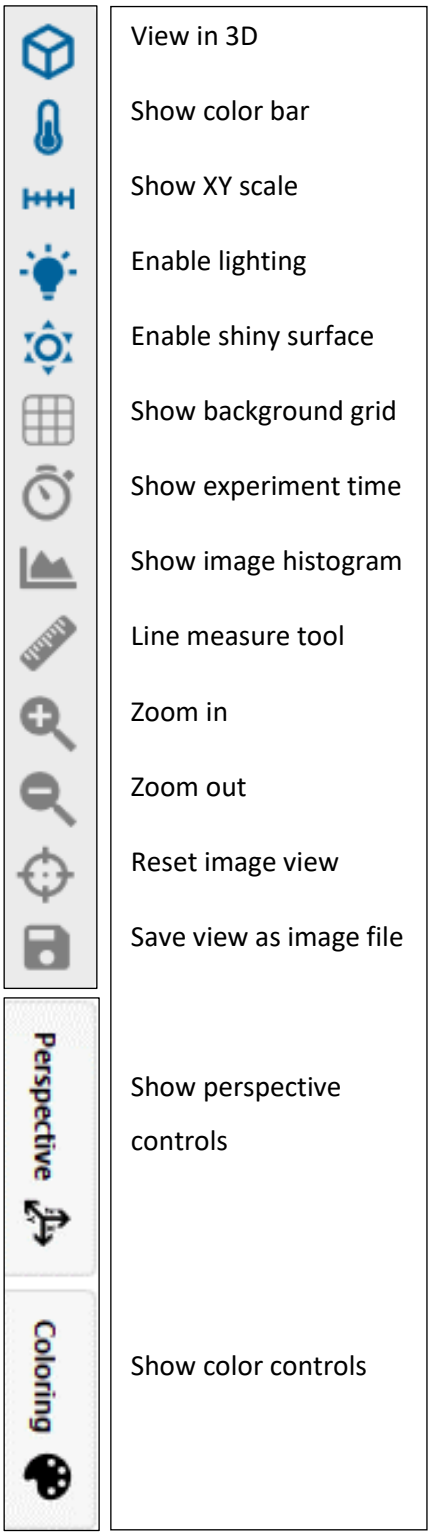
Holographic amplitude image



Hologram image

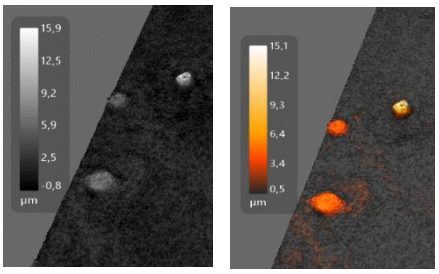
Adjusting image presentation

To the right of the Live image view window there are multiple functions that adjust the presentation of the live image (see image to the right). Blue functions are active while gray functions are inactive.

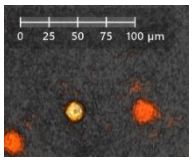


View in 3D displays the image in 3D. To flip and move a holographic 3D image, click, hold and drag the image using the right mouse button on the image.

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

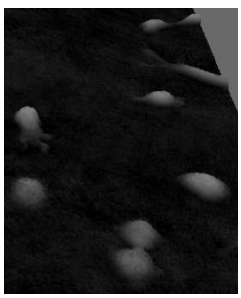


Show XY scale displays a horizontal scale bar representative of the distance in X and Y (See image to the right).

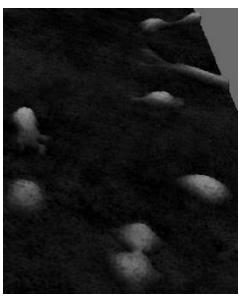


Enable Lighting applies an artificial light source to the image which may sometimes render an improved image (See image below).

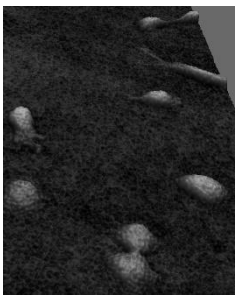
Enable Shiny surface shows the rendered surface with a reflective visual effect. Shiny surface is only active when Lighting is active.



Standard image



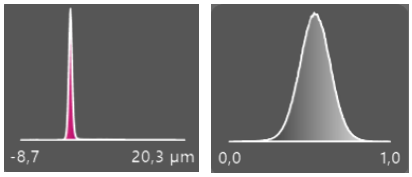
Enabled Lighting



Enabled Lighting and Shiny surface

Show background grid adds a grid in the background.

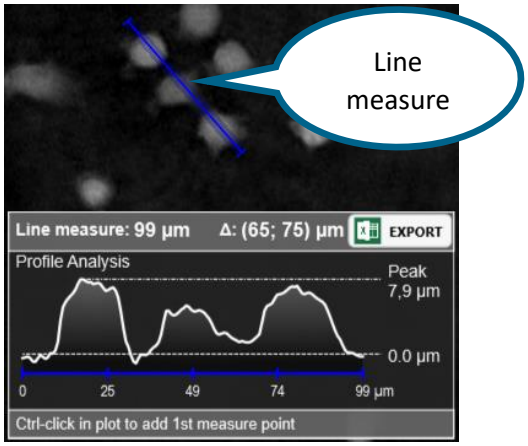
Show image histogram shows a histogram overlay of the pixel values in the image. For a phase image this equals a histogram of the optical thickness values (left image) and for other image channels such as camera it shows a histogram of the pixel intensity values (right image).



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.

The measure can be saved as an image with the Save View button . The resulting data can be exported to excel.

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



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

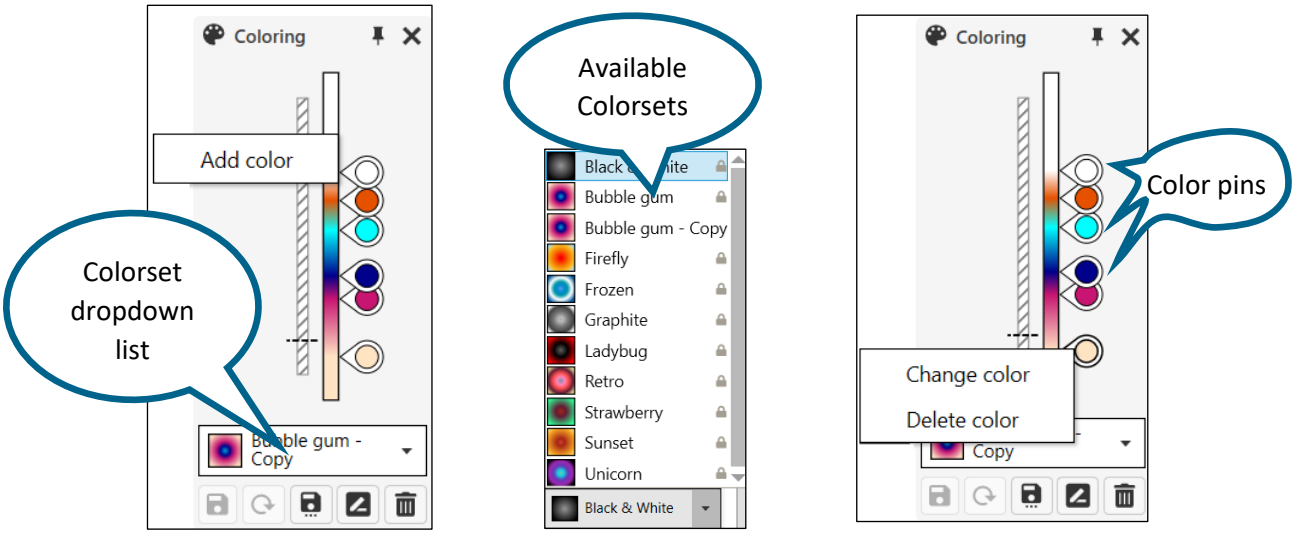
Reset image view will return the image view to default size and center position.

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 Coloring (see image below) which is used to adjust the color balance of the image and to color the image relative to the cell optical thickness. 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.

- Switch to a pre-existing colorset by using the Colorsets drop-down 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).

Colorsets adjusted in the Live view do not automatically apply to the experiment set up colorsets.



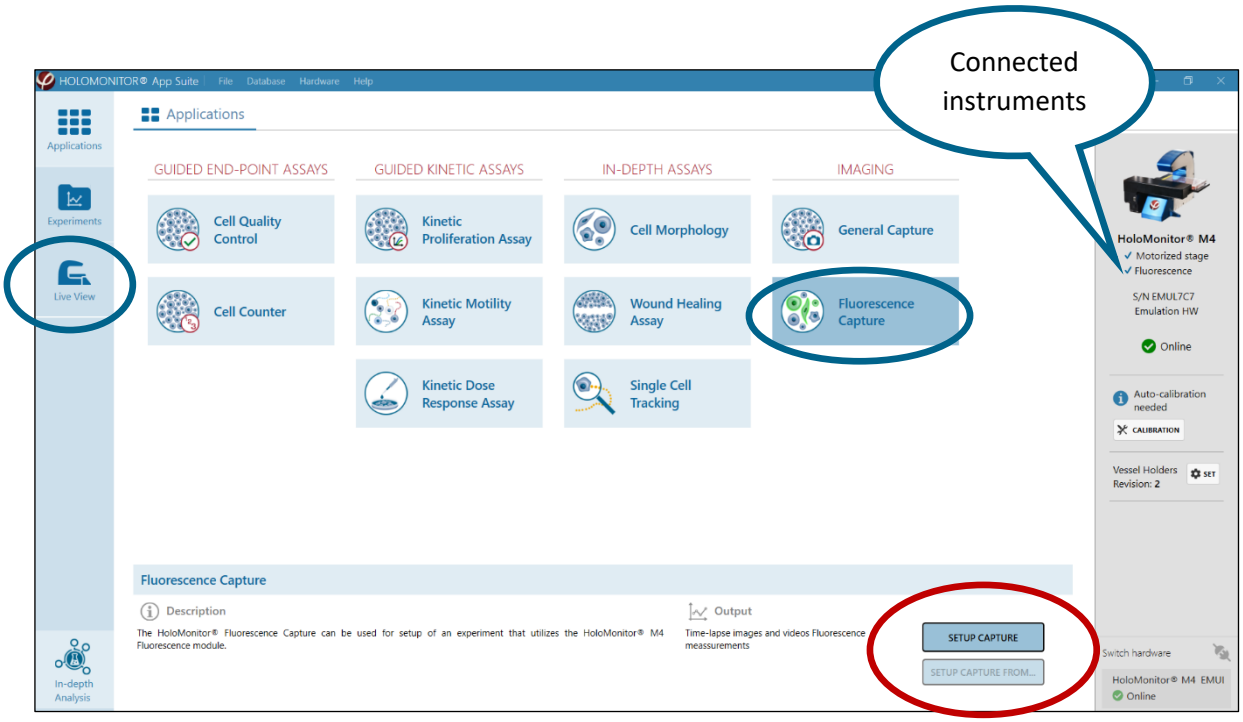
The buttons below Coloring will

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

HOLOMONITOR® APP SUITE FLUORESCENCE SOFTWARE

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

Fluorescence experiments can only be captured using the Fluorescence Capture Application. Before each experiment, the fluorescence focus must be freshly calibrated using Live View.



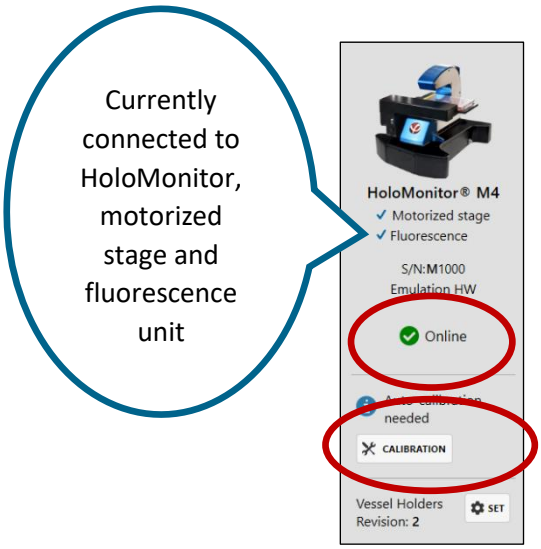
Before each experiment, the fluorescence focus must be freshly calibrated using Live View.

The holographic images in fluorescence experiments can be reanalyzed in other versions of the software, but without the fluorescence data.

Results created using the fluorescence version of the software cannot always be opened in other versions of App Suite.

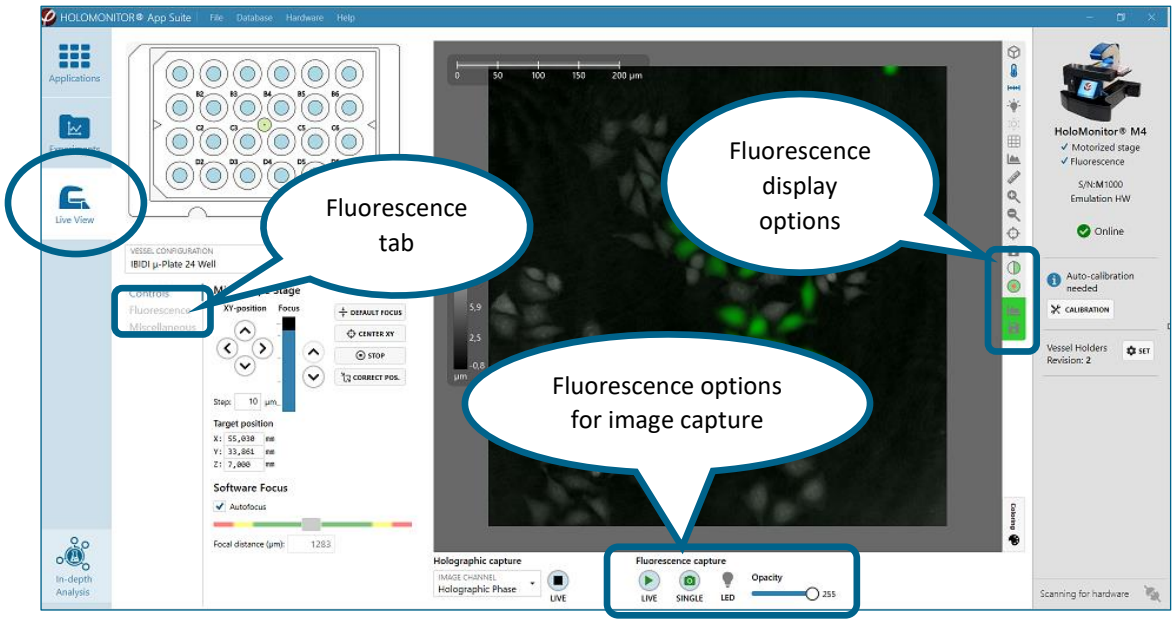
Connected instruments

Connected instruments will be presented in the right-side panel. The software first initializes the connection to HoloMonitor. Green check mark symbols will appear when the connections to the motorized stage and the fluorescence unit are established.



Live view

The Live View tab comprises controls for focusing and image adjustments for both holographic and fluorescence imaging. The holographic options are displayed when the tab opens. The fluorescence options below the image view are available at all times, while the fluorescence options to the right of the live view become available once fluorescence Live capture has been started.

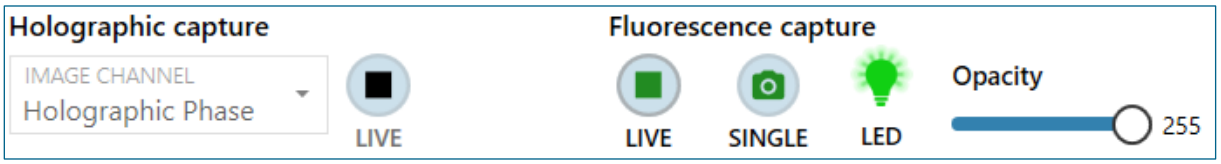


Holographic and fluorescence image capture

In order to capture fluorescent or holographic Images, use the functions below the Live View window.

Note that only one camera will work at a time, either holography or fluorescence.

Fluorescence capture



The LED light bulb will indicate when the laser light is on.

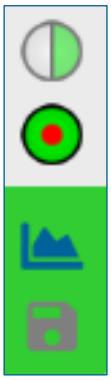
Auto-LED on automatically turns the LED on when images are captured.

Clicking Single results in a single fluorescence image, while Live results in continuously updated fluorescence images.

The Opacity slider determines the opacity of the fluorescence image and makes it possible to view either the holographic image (value=0), an overlay of fluorescence onto the holographic image (intermediate values) or only the fluorescent image (=maximal value).

Fluorescence display options

Toggle between holographic and fluorescence frames allows the user to display either only holographic, only fluorescent, or a combination of both techniques.



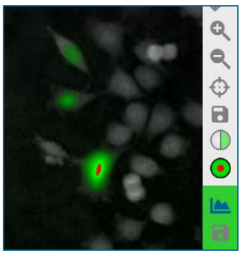
Toggle between holographic and fluorescence frames

Highlight saturated fluorescent areas

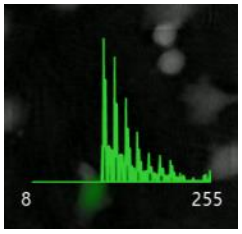
Show fluorescent image histogram

Save fluorescent image

Highlight saturated fluorescent areas indicates whether the image is saturated and needs less exposure time. Saturated areas are indicated with red.



Show fluorescent image histogram displays a histogram showing the variation in fluorescence intensity in the image. The intensity varies between 8 and 255. Set the exposure time to have no or only very few pixels at 255.

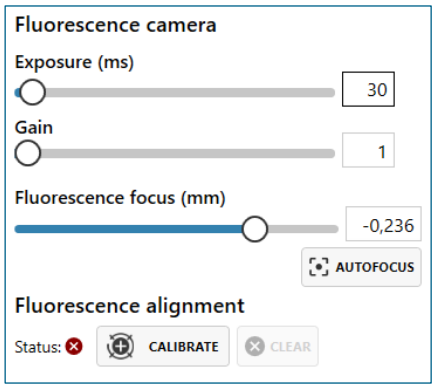


Save fluorescent image saves a screen shot of only the fluorescent part of the image.

Fluorescence tab

Opening the fluorescence tab gives access to exposure time settings, as well as functions necessary for the calibration of the fluorescence focus.

Fluorescence camera

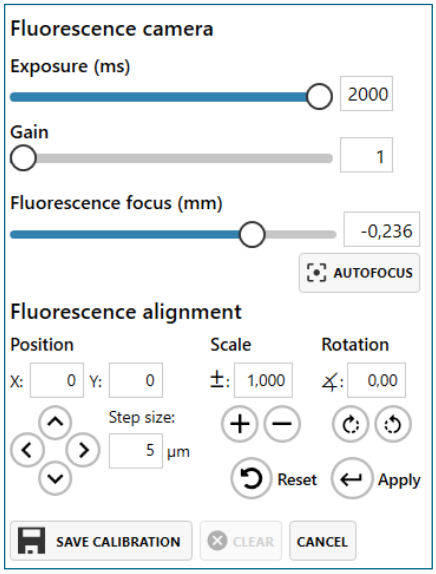


Exposure can be set from 0-2000 ms. Adjust until the image is as bright as possible without being saturated. Use the Highlight saturated areas or the Show fluorescence histogram (see paragraph above) functions to determine whether the image is saturated.

Fluorescence focus regulates how the software focus automatically adjusts depending on the holographic focus.

Autofocus performs automatic focusing.

Fluorescence alignment



Click the Calibration button for the Alignment and Focus calibrations that is necessary before each experiment.

Use the Fluorescence Focus slider to adjust the Fluorescence offset from the holographic focus. Click the slider to use arrow keys on the keyboard for smaller steps.

Use the Alignment functions to move position, scale, and rotate the fluorescence image until it aligns with the holographic image.

Decrease the Step size to make finer adjustments if needed.

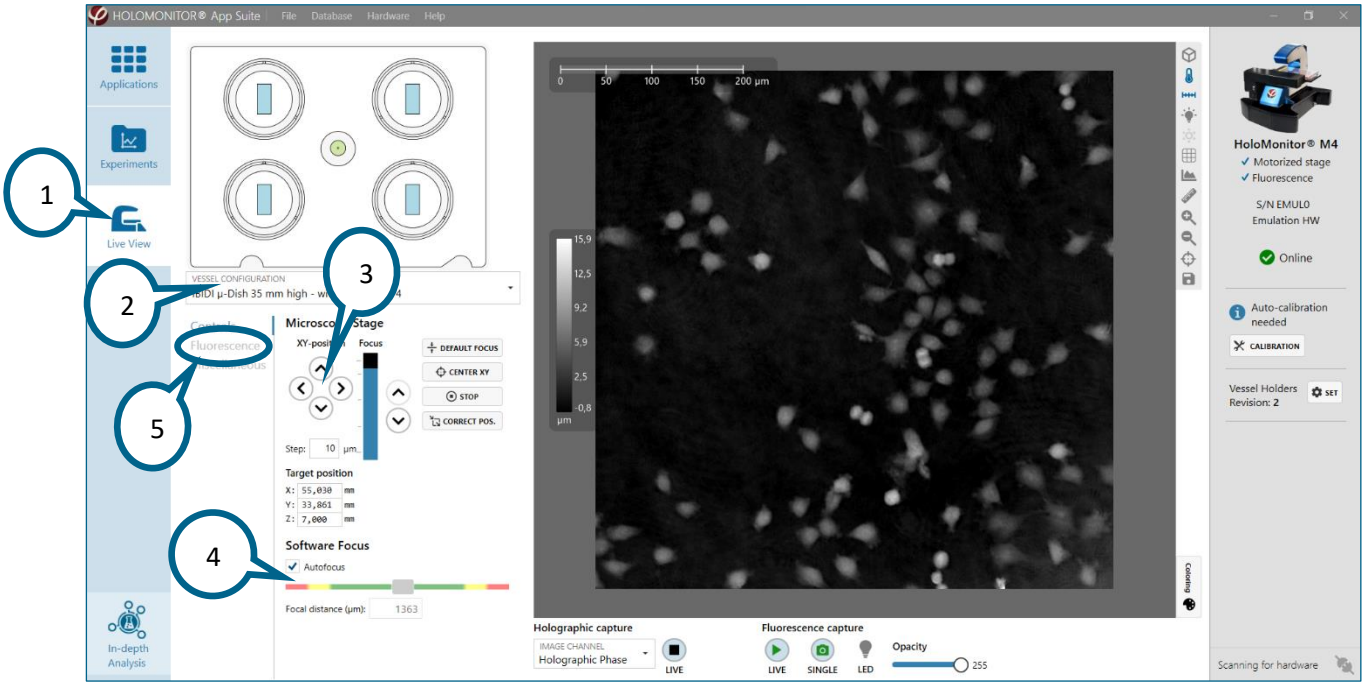
Reset will revert the alignment changes.

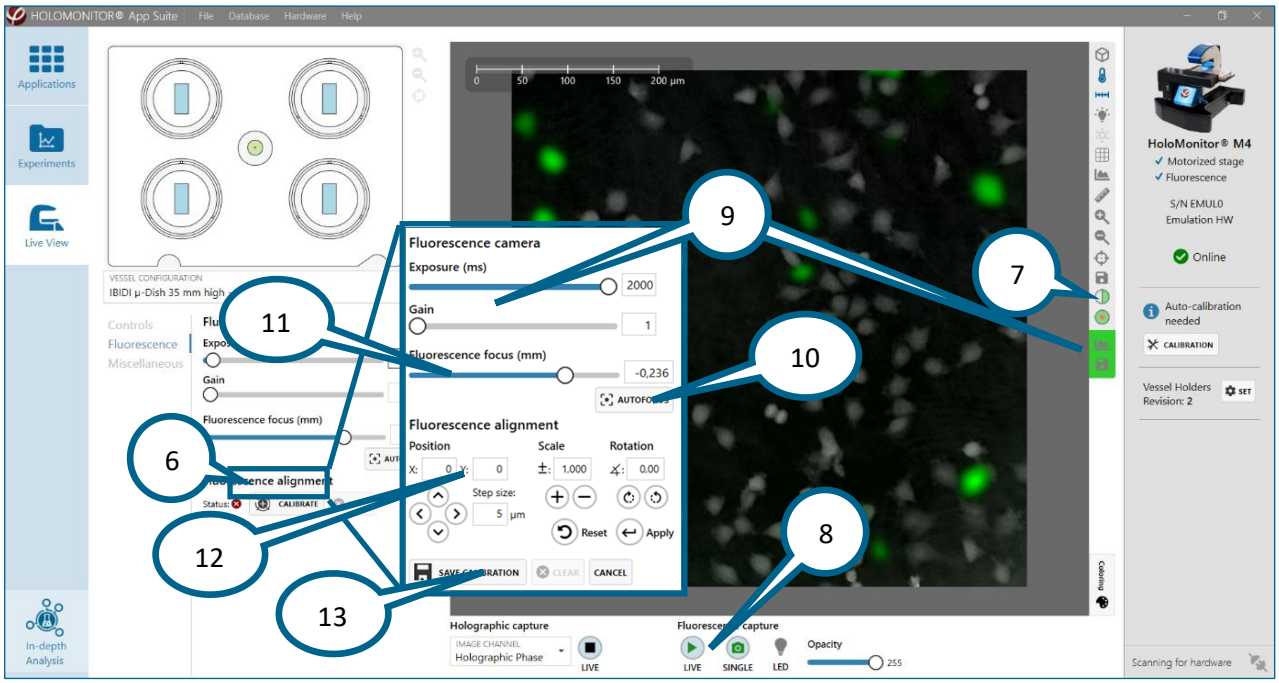
Clicking Save Calibration will store the settings for the coming experiment and then close the Alignment and Focus calibration. Use Apply to apply the settings without closing the Alignment and Focus calibration.

To reduce the fluorescent exposure during calibration, turn off fluorescence live capture. Instead, take a fluorescent snapshot every time you need to confirm a change during the calibration.

Calibrating fluorescence image focus

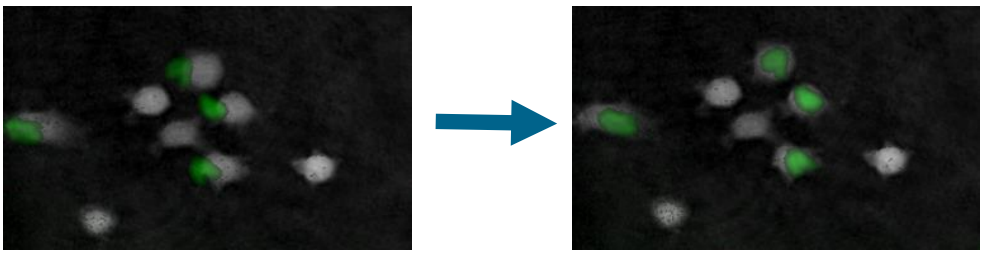
Calibrating the fluorescence is a procedure with several steps and it is performed in Live View.





1. Go to the Live View Tab on the left side panel. The holographic options will be displayed when the tab opens.
2. Select the correct vessel from the drop-down list.
3. Find a position with cells. Preferably, the position should be as centered in the cell culture vessel as possible.

4. In Controls, make sure that the holographic image is in focus by adjusting the Z-position of the stage using the up/down arrows. Ensure that Autofocus is ticked and that the chosen Z-position gets the Software focus bar to 1300±5.
5. Go to the Fluorescence tab in Live View.
6. Click Calibrate.
7. Check that the fluorescence channel view is set to mixed (to the right of the Live View image).
8. Turn on Fluorescence live capture by pressing the fluorescence Live button (or take a Single images repeatedly) while manually adjusting the Fluorescence focus slider until a fluorescence signal is seen. Use the arrow keys on the keyboard for small step adjustments.
9. Adjust Exposure time and Gain. Use the Highlight Saturated Areas or the Show fluorescence histogram function to the right of the Live View window to determine whether the image is saturated. Increasing gain value might be beneficial when the fluorescence signal is weak, and App Suite struggles to find focus automatically.
10. Click Autofocus to automatically set optimal focus offset.
11. If the software fails to find optimal focus, adjust it manually by adjusting Fluorescence focus slider. Move the slider until the fluorescent image is in focus when it is aligned with the holographic image. Use the arrow keys on the keyboard for small step adjustments. The Fluorescence focus slider represents how much the fluorescence focus is offset from the holographic focus, thus allowing the fluorescent images to be captured in focus. The offset is calibrated when the fluorescence unit is first mounted on the M4 but needs to be adjusted for each experiment.
12. Use Position, Scale and Rotation to adjust the fluorescence overlay to fit the holographic image. If needed, step size can be decreased to make finer adjustments. The opacity of the fluorescence overlay can also be adjusted to compare it better with the holography image. To reduce the fluorescence exposure during calibration, turn off fluorescence live capture, and take a single fluorescence image as needed to confirm a change during the calibration. Please note that this is an iterative step. Thus, position, scale, and rotation adjustments do not need to be performed sequentially.

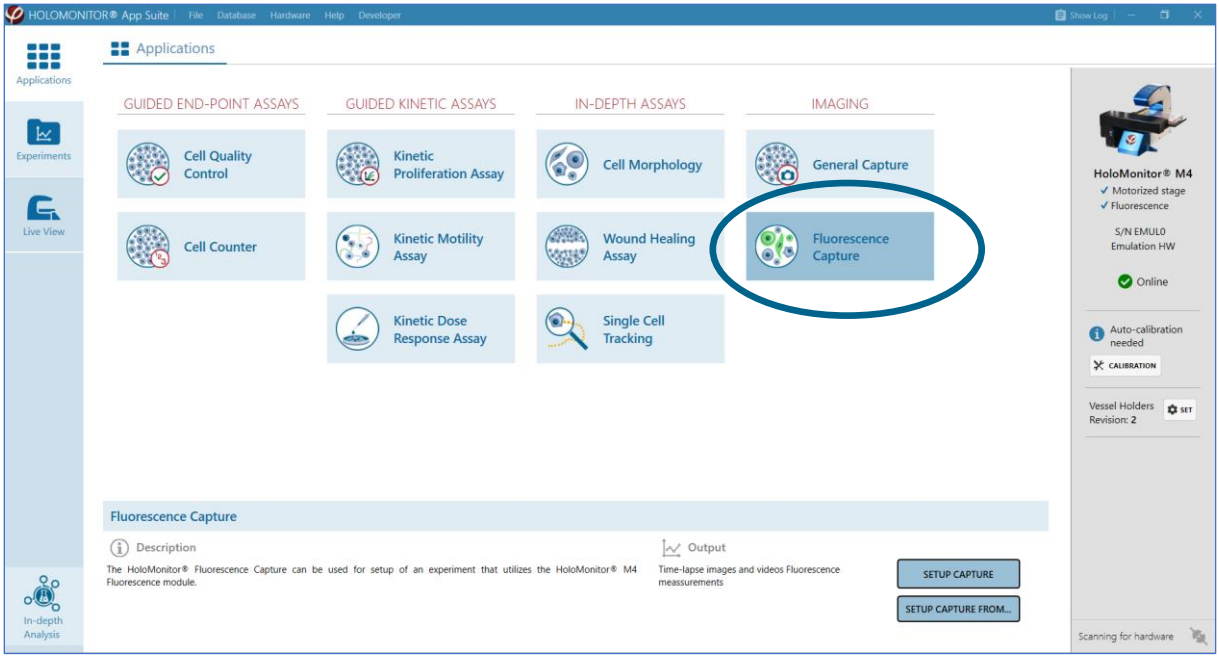


13. Press Save calibration when the fluorescence focus and alignment are as good as possible. The z position (from step 4) will be saved with the calibration and used as the default z position during Capture Setup.
14. Stop live capture (from step 8).

Fluorescence capture

The Fluorescence Capture module can be used as a flexible setup for an experiment where both holographic and fluorescence images are captured. The resulting holographic image databases can be used for analysis by any licensed applications, and the fluorescence images can be analyzed using the In-Depth Single Cell Tracking and Cell Morphology assays. The possible output comprises time-lapse images and videos, as well as all tracking and morphological analysis results.

Note that the fluorescence image focus must be freshly calibrated before every experiment. The calibration is performed in Live View.



Setting up an experiment

Please follow the procedures as explained in the Fluorescence Capture Protocol.

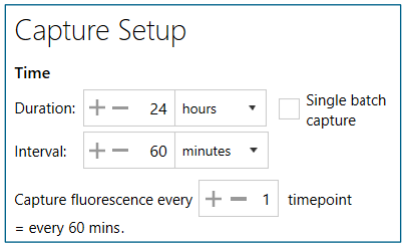
Basic setup options are identical to the standard App Suite assays, and so are Start capture options. Capture Setup contains additional fluorescence options.

Capture setup

In this window, the frequency of capture for both holographic and fluorescence images as well as the duration of the experiment are set. The holographic images can be validated to ensure good capture quality.

Below the live image are tabs for Validation, Stage Controls, Holography and Fluorescence. Validations, Stage controls and Holography are identical to the standard App Suite software.

Capture duration and interval



In Capture Setup it is possible to set the interval and duration of holographic image capture, as well as the frequency of fluorescence capture.

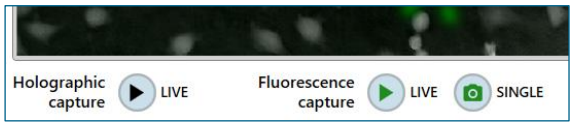
Note! To prevent cell damage, be careful how often your cells are exposed to fluorescence. This interval depends on cell type, fluorescence labels, exposure time, cell treatment, and cell density.

Fluorescence image display

Use the toggle button to display either a holographic image, an overlay image, or the fluorescence image.

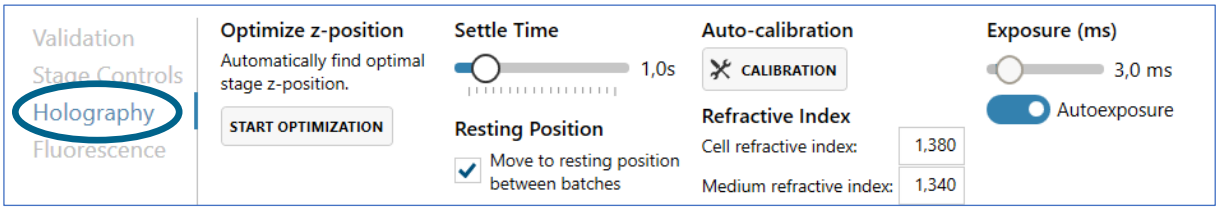
Live image capture

When Holographic Capture is activated, the holographic images are continuously updated.



Fluorescence capture Live updates the fluorescence image continuously, while Single gives a single image. To avoid cell damage, use Single as often as possible.

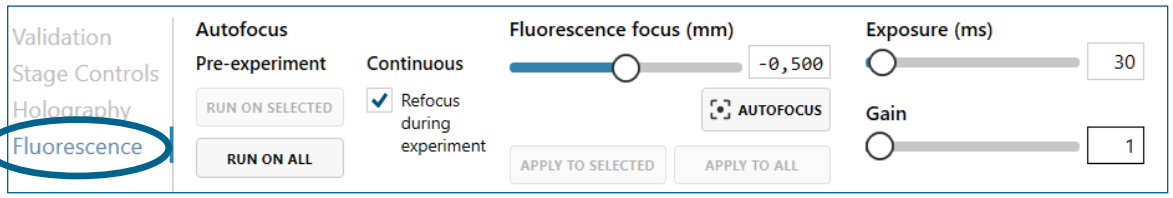
Holography tab



To prepare for fluorescence capture, select the Holography tab and click Start optimization. This will allow the software to automatically optimize the holographic focus to 1300±5 for each capture position, which is optimal for fluorescence focus during capture. In addition, this function validates the image quality. Values for the inspected positions are displayed in the Positions list. If the automatically set Z-value does not lead to a software focus of 1300±5, change the Z-value manually using the Stage controls.

Fluorescence tab

In the Fluorescence tab, there are functions for autofocusing the fluorescence, for refocusing during the experiment, refocusing the fluorescence, adjusting exposure time, and adjusting gain.

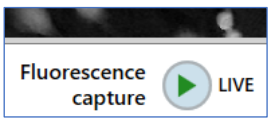


Autofocus adjustment pre-experiment

Before the experiment, to automatically adjust the fluorescence focus for selected positions or all positions, press Run on Selected or Run on All.

Manually adjust focus pre-experiment

To manually adjust the Fluorescence focus before the experiment, start Fluorescence capture Live. Use the Fluorescence Focus slider to achieve the optimally focused fluorescence image. The Fluorescence focus slider represents how much the fluorescence focus is offset from the holographic focus, thus allowing the fluorescent images to be captured in focus based on the holography autofocus. Once satisfied, press Apply to Selected or Apply to All. Repeat with other positions if needed.



Autofocus during experiment

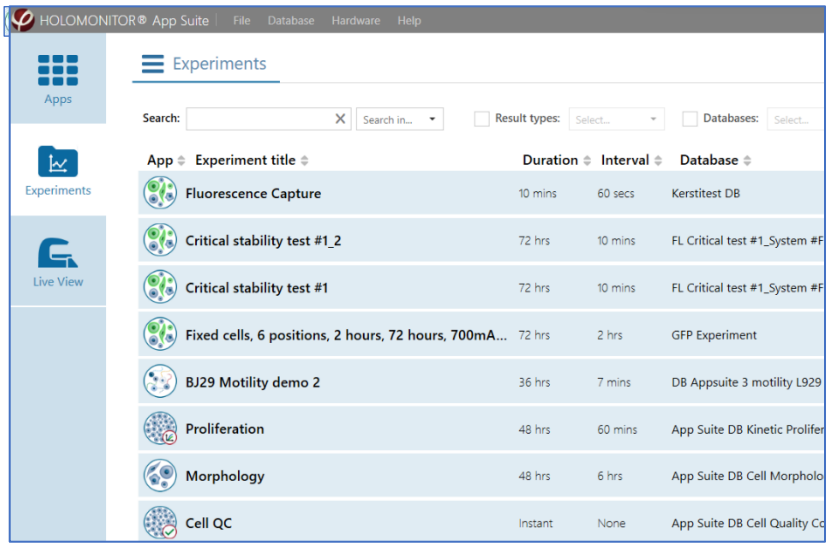
Check Continuous: Refocus during experiment for the software to adjust the fluorescence focus during the capture.

Exposure time and gain adjustment pre-experiment

Adjust the fluorescence Exposure time and Gain if needed for optimal fluorescence imaging. Exposure time and gain cannot be adjusted during the experiment.

Results

The fluorescence experiments are found together with all other experiments in the Experiment list in the Experiment tab.



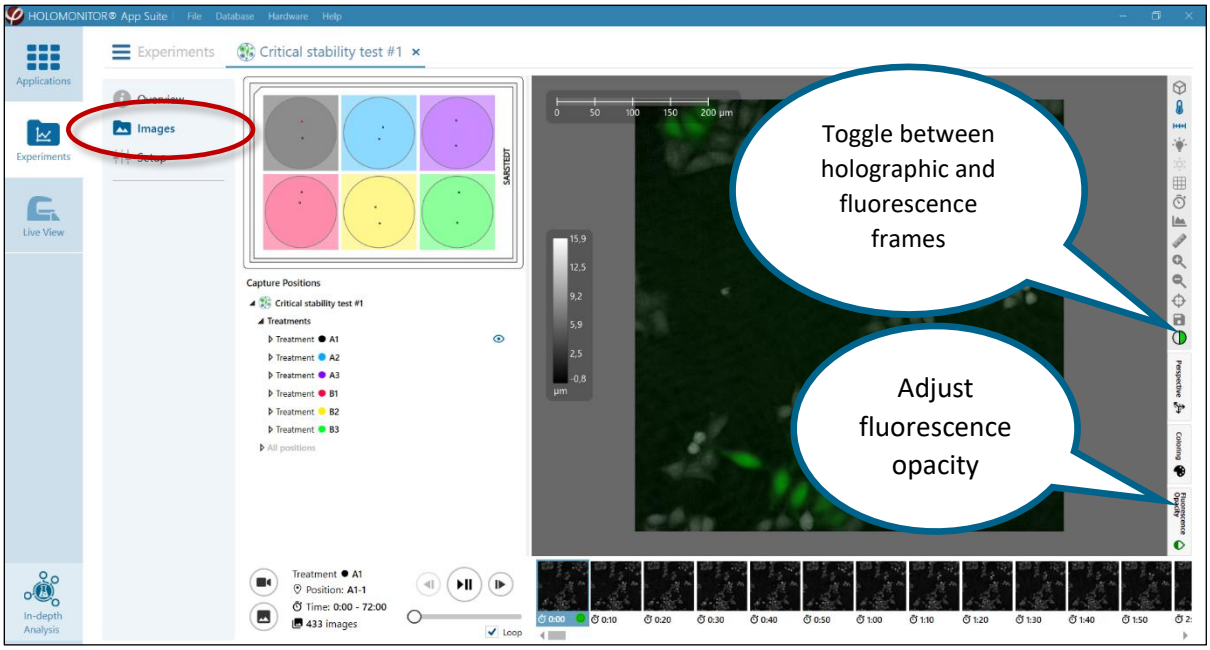
To make a full analysis of an experiment, including the fluorescence data, use In Depth analysis. The holographic images of fluorescence experiments can be analyzed with Guided assay analyses. The fluorescence images can be viewed in the Experiment Images but cannot be analyzed within the guided assays.

Viewing fluorescent images

Holographic images with fluorescent overlay can be viewed in the Images tab in the Experiment overview.

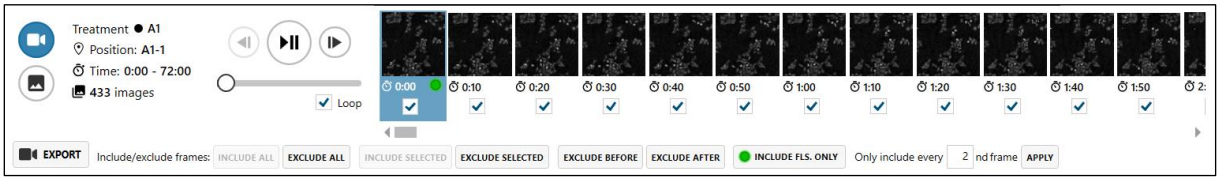
Use the fluorescence toggle button  to shift between a holographic image, an overlay image, or only the fluorescence image in the viewing window. There is no way to adjust the fluorescence presentation in this view, but the holographic presentation options can all be applied as in other App Suite versions.

Fluorescence Opacity can be adjusted using the slide bar in the Fluorescence Opacity tab.



Creating fluorescence movies and export fluorescence images

The movie and image makers have the same functions as in the standard App Suite software, but there is also an option to include only the fluorescence images. Use the play function ⏯ to preview the movies.



In-depth analysis

The fluorescence content in each image can be analyzed using Cell Morphology or Single Cell Tracking. The holographic part of each image can be analyzed as in other versions of App Suite. In order to perform any analysis, the cells must first be identified, based on the holographic images. Green dots in the image frame list indicate that the image also contains fluorescence information.

Identify cells

Identify Cells based on the holography images 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. In the identify tab, the fluorescence overlay can be adjusted. For further detail regarding the holographic image cell identification, please see the Identify Cell information in the In-Depth analysis section in this manual.

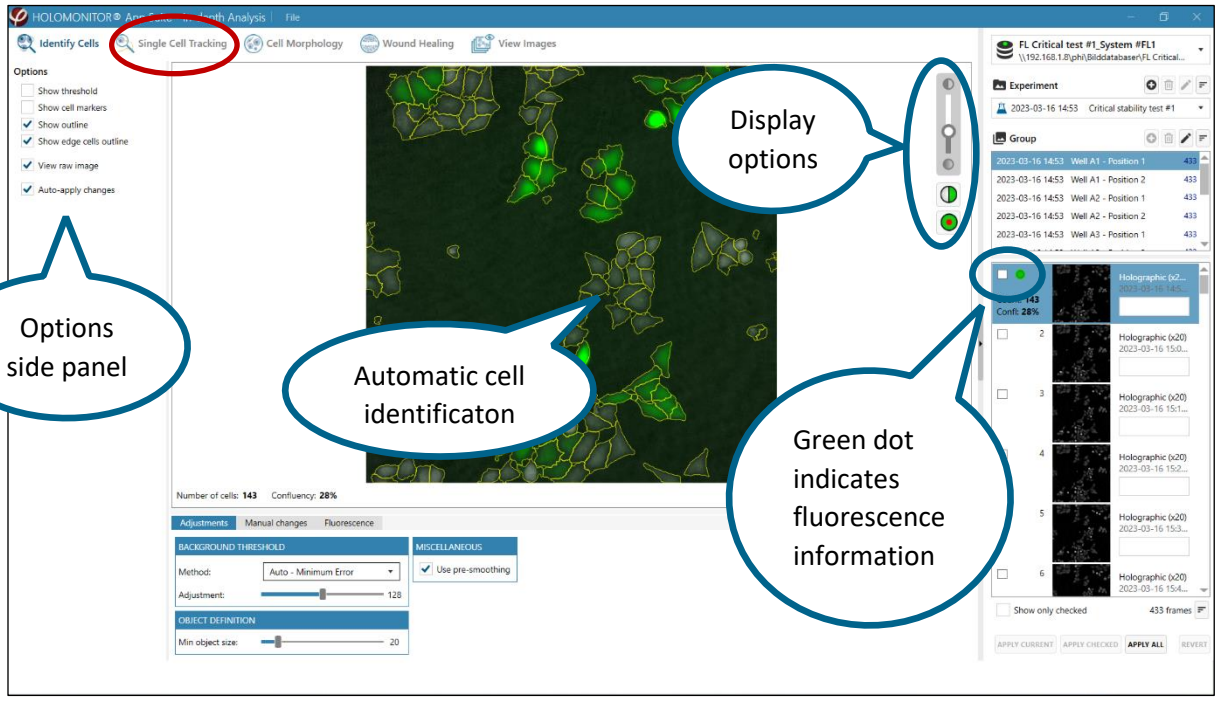
Image display options

Image display options become available to the right of the image window when hovering over the cell image with the mouse cursor.

Use the slide bar to adjust the hologram image contrast.

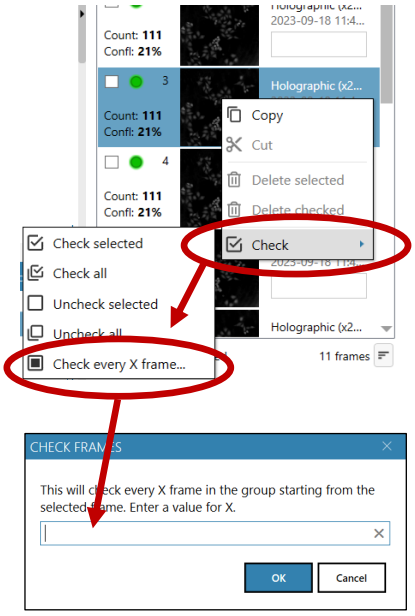
Toggle  button to display the fluorescence image.

Use the  button to highlight saturated areas.



Use check to sort the image frame list

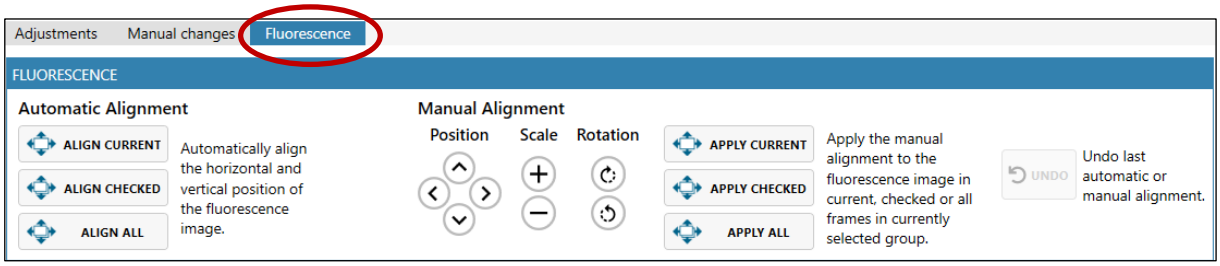
In order to work with only the fluorescence images, right click in the Image Frame list to access the Check options. As an example, if holography images were captured every 10 minutes, while fluorescence images were captured every 2 hours, every 12th holography image has an accompanying fluorescence image. Use Check every X to check every 12 images. Then use the Show only Checked function which is found below the Image Frame list. Use the toggle button described above to show only the holography images, overlay images, or only the fluorescence images.



Adjusting the fluorescence overlay

If the fluorescent overlay needs to be adjusted, there are both automated and manual functions that can be applied. For the automated functions, either only the current image or a subset of checked images or all images in the currently selected group can be adjusted.

For the manual overlay adjustment, use the Manual Alignment functions to move position, scale, and rotate the fluorescence image until it aligns with the holographic image. Then apply the adjustments to either only the current image or a subset of checked images or to all images in the currently selected group.



Single cell tracking

Based on the holographic images, cells can be tracked through a time-lapse sequence and analyzed for movement and morphological changes over time as well as for fluorescent content. The software also notes cell divisions and supplies cell family trees.

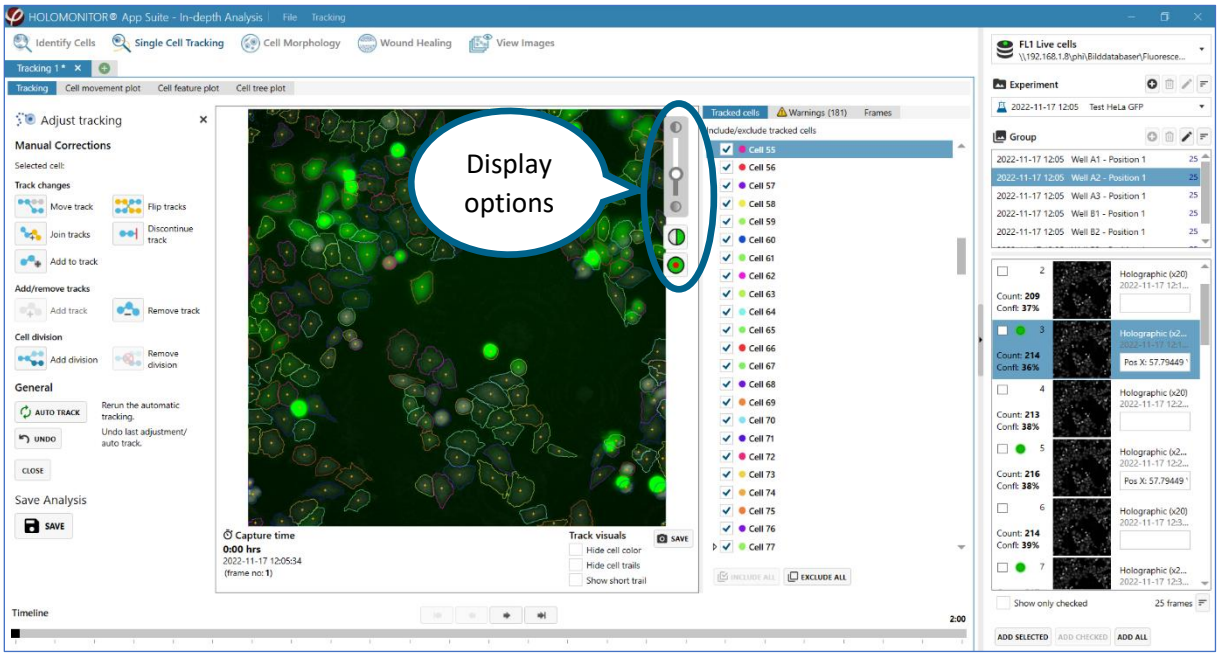
Note that the cells must be identified based on the holographic images in the Identify cells tab before using the tracking functions.

Note that the holographic images must be captured with adequate 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.

All tracking functions are identical to the previous version of App Suite, but the cell features include the fluorescence information.

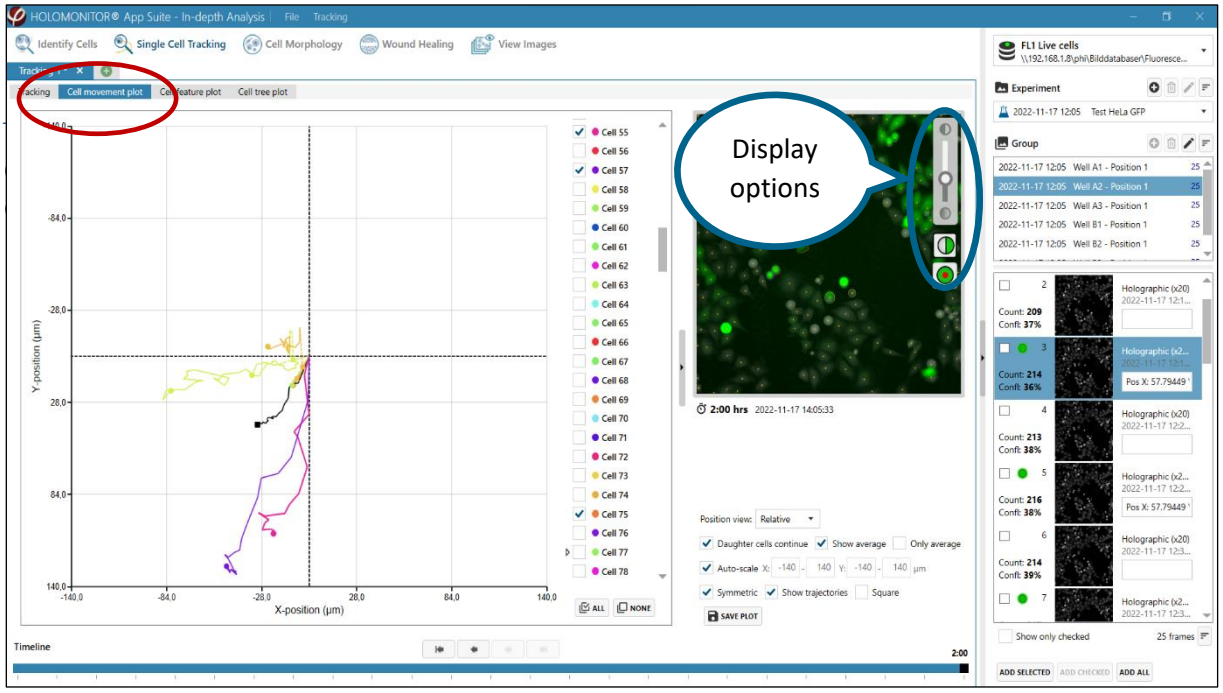
Image display options become available when hovering over the cell image with the mouse cursor.

- Use the slide bar to adjust the hologram image contrast.
- Toggle button  to display the fluorescence image.
- Use the button  to highlight saturated areas.



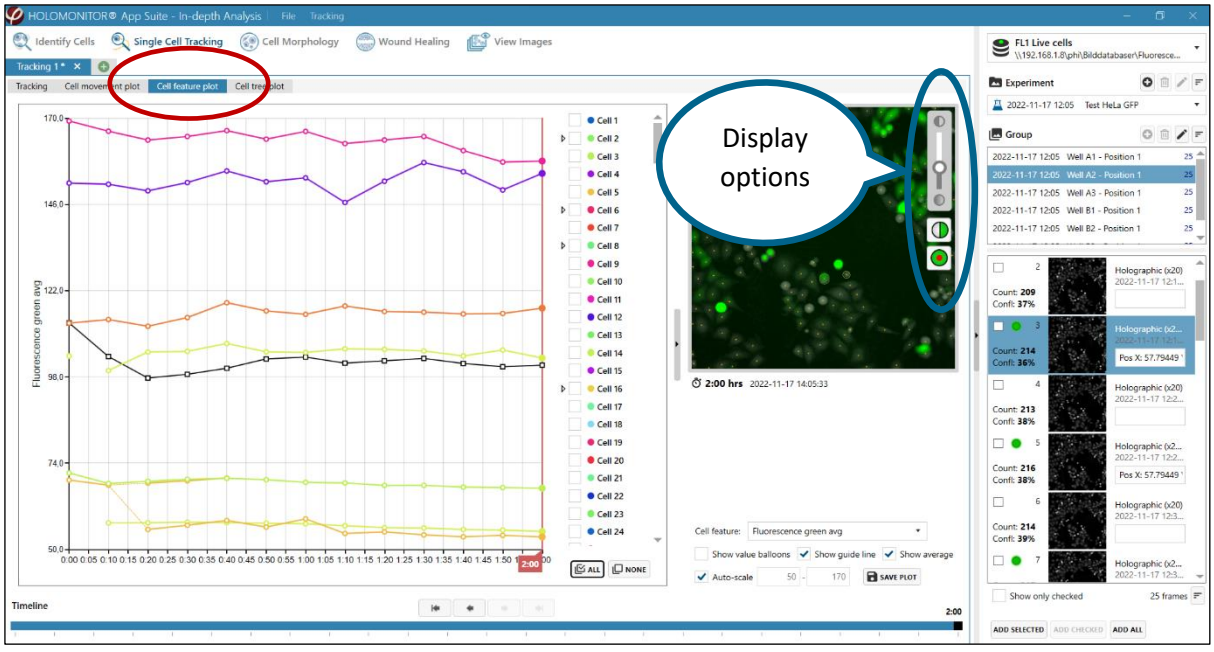
Cell movement plot

The cell motility is displayed only for selected cells. In order to select only cells that contain fluorescence, hover with the mouse cursor over the image to activate the use fluorescence overlay button. Then click the fluorescent cells in the image to display their data in the cell movement plot.



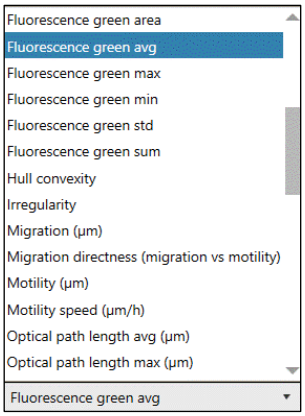
Cell feature plot

In order to select only cells that contain fluorescence, hover with the mouse cursor over the image and use fluorescence overlay. By clicking the fluorescent cells in the image, they are selected to display their data in the graph. Any feature that is selected will be displayed only for the selected cells. In the example below, GFP-expressing cells display variation in fluorescence.



Fluorescence cell features

Select one of the fluorescence options in the Cell Feature drop down list.



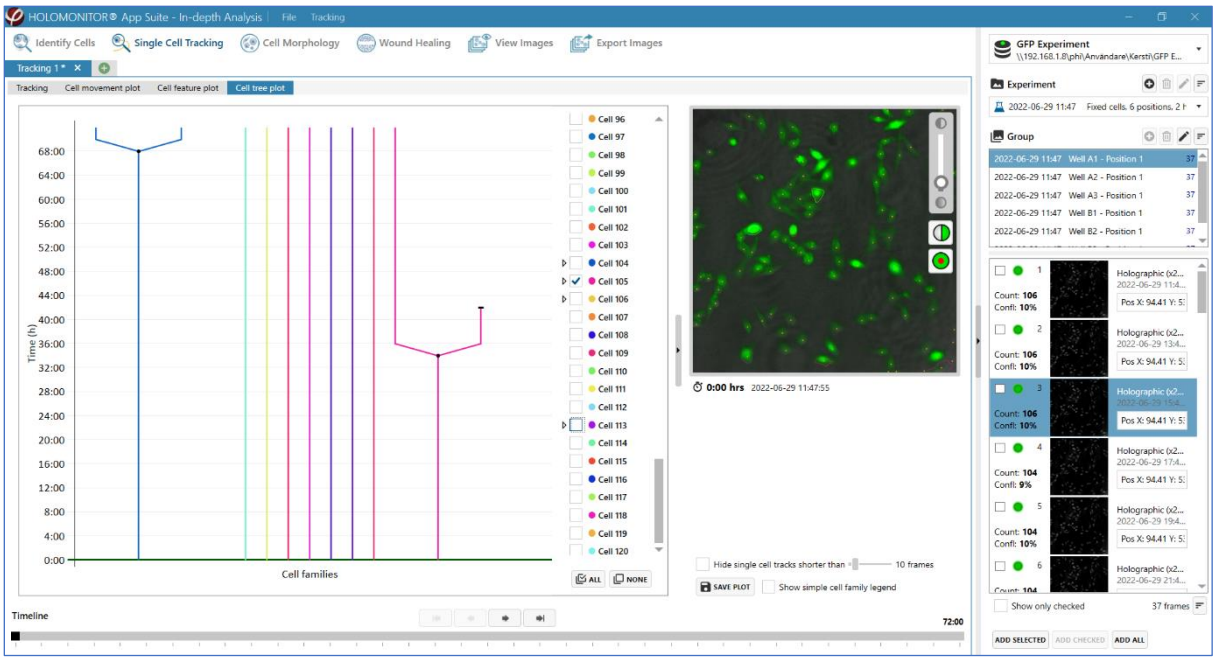
- Green area-indicates the size of cell area that is green.
- Green avg - indicates the average green intensity in each pixel of the identified cell.
- Green max - indicates the maximum green intensity of any pixel of the identified cell.
- Green min - indicates the minimum green intensity of any pixel of the identified cell.
- Green std – indicates the standard deviation of the green intensity in all pixels of the identified cell.
- Green sum – indicates the sum of the green intensity of all pixels in the identified cell.

Note that the fluorescence intensity is a relative value, depending both on the exposure time and the gain but also on the fluorophore.

Cell tree analysis

In the cell tree plot tab, cell trees are constructed based on the tracking of cell divisions. Other occasions, such as cells traveling out of the field of view, are noted as well.

In order to select only cells that contain fluorescence, hover with the mouse cursor over the image and use fluorescence overlay. By clicking the fluorescent cells in the image, they are selected to display their cell tree data.

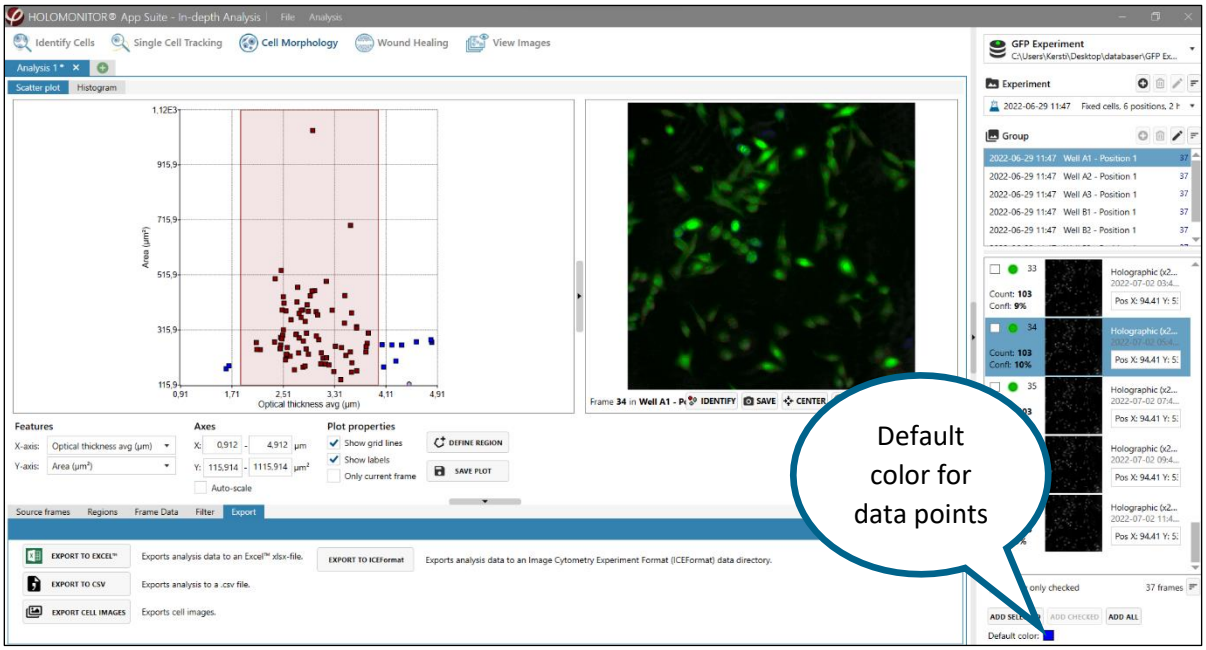


Cell morphology

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

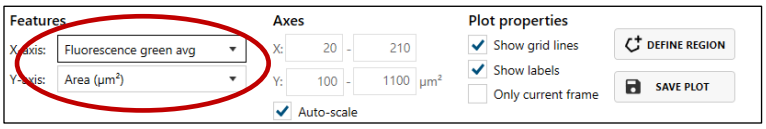
Note that the cells must be identified in the Identify cells tab before using the analysis functions.

All functions are identical to the holography version of App Suite, but for fluorescence, the cell features include the fluorescence information.

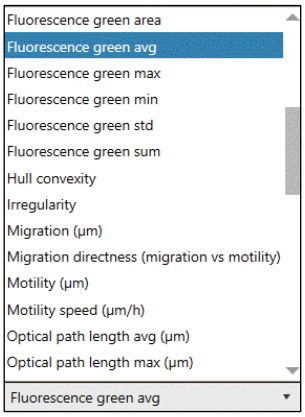


Change the scatter plot axis features

The fluorescence features are found in the list of x- and y-axis features situated below the scatter plot.



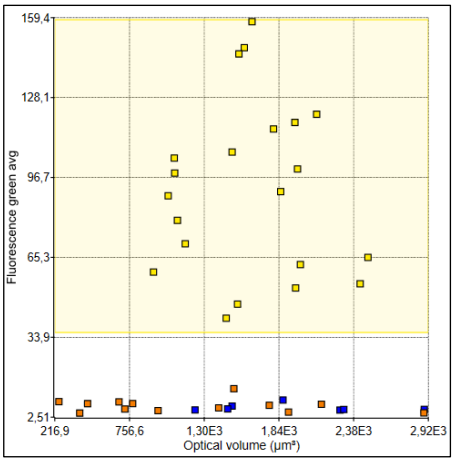
- Green area-indicates the size of cell area that is green.
- Green avg - indicates the average green intensity in each pixel of the identified cell.
- Green max - indicates the maximum green intensity of any pixel of the identified cell.
- Green min - indicates the minimum green intensity of any pixel of the identified cell.
- Green std – indicates the standard deviation of the green intensity in all pixels of the identified cell.
- Green sum – indicates the sum of the green intensity of all pixels in the identified cell.

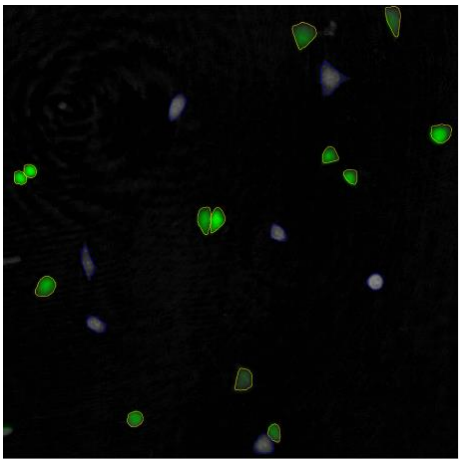


Plot regions

Use the regions to define which cells contain fluorescence and which cells are non-labelled.

The example shows a Y-axis region in yellow with a cutoff for cells with only a small amount of fluorescence. In the cell image, fluorescent cells are outlined in yellow.

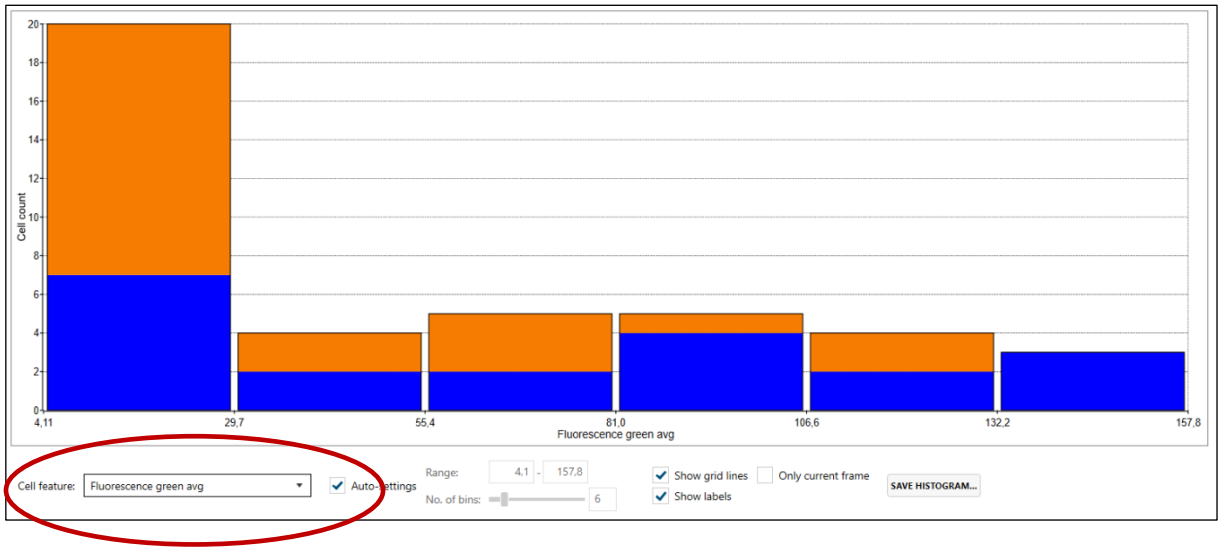




Display results as histograms

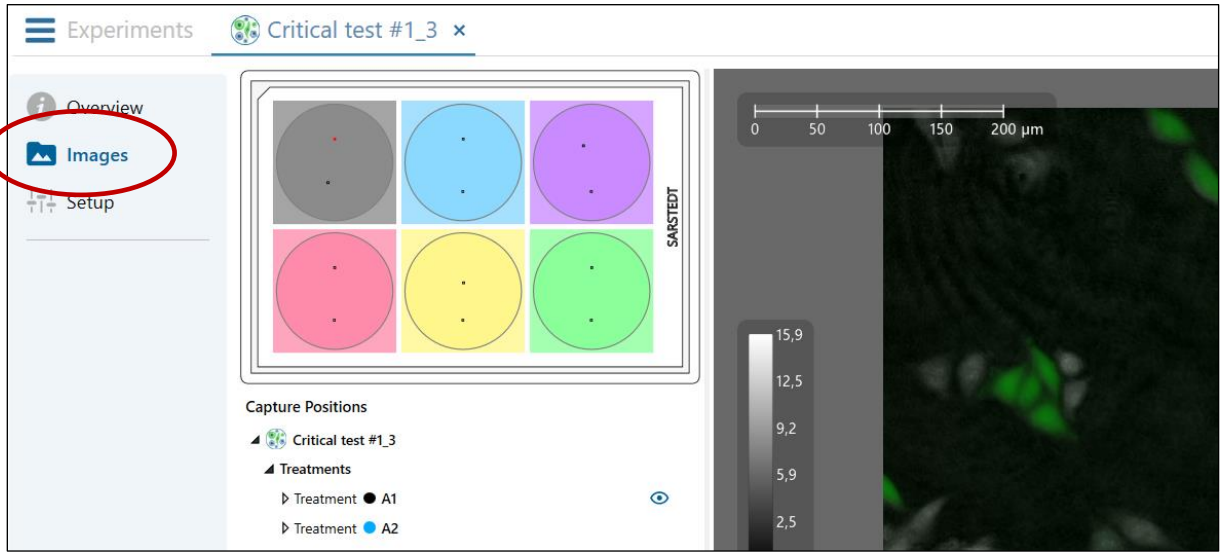
In the Histogram tab, the results are displayed as histograms, where one cell parameter is offset against the cell count. If several regions have been added to the scatter plot, or several frames with different colors, they will show up with the appropriate color in the histogram as well.

Fluorescence data is shown in the same way as all other features.



View fluorescence images

Fluorescence images can only be viewed in the Image tab in the Experiment, not in the In Depth: View images.



TROUBLESHOOTING

Startup

The software cannot find 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.

Windows have deleted/corrupted USB drive (PHI_DEV01)

Contact PHI support for further assistance.

App Suite cannot find HoloMonitor after upgrading

Open Windows "device manager". Go to "other devices" where you will see two "unknow devices" listed. Right click and choose "update device" for both. Then restart the software.

The software license is not activated or has expired

Contact your sales representative or support@phiab.com-

If the software still is unable to find HoloMonitor, contact support@phiab.com or a local representative.

The software cannot connect to the motorized stage

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 connect to the motorized stage, contact support@phiab.com

The software cannot connect to the camera

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 connect to the camera, contact support@phiab.com

The software cannot connect to the fluorescence unit

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.

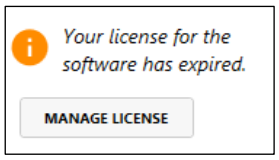
App Suite needs to be restarted.

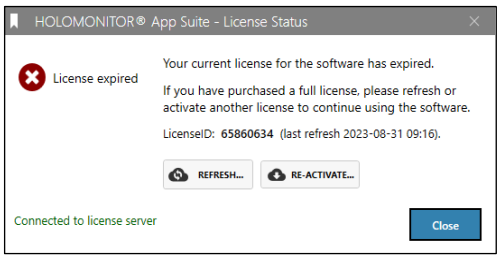
Restart the software.

If the software still is unable to connect to the fluorescence unit, contact support@phiab.com

The license has expired

If warnings come up that the license is expired, your time-limited license is not active anymore. If you want to continue using App Suite, please contact a local distributor or support@phiab.com.





The auto-calibration does not pass

All calibrations values are in the red

If the Calibration wizard cannot 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
- Electricity cable is not connected to the laser hub
- Dirty optics: objective, top window
- Dirty laser tip or fiber connector
- Laser requires periodic recalibration
- Dirt/condensation inside HoloMonitor

Check the laser

- Is the red light shining on the objective?
- If there is red light, is there AC power connected to the laser and the green light on the laser module is blinking?
- Is the laser dot focused? Check with laser template that was included in shipment. If missing, contact support@phiab.com.
- 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 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, 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 aligned properly. The guiding metal ridge on the laser connection must slip into the guiding notch in the connector socket at the back of the HoloMonitor (see the section for Hardware installation for pictures). Connections must be finger tight.

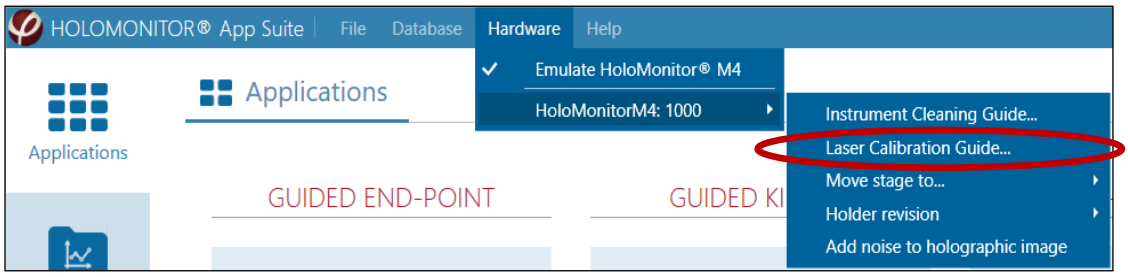
After checking the laser, recalibrate

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

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 indicates that the laser needs periodic recalibration.

- Go to the top menu bar, 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 and stable.



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 bar, select Hardware, and then the current HoloMonitor.
- 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 of the values (black arrow heads) should be in the green range and stable.

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.com or the local distributor for further assistance.

Experiment setup

There is no vessel map for petri dishes

The HoloMonitor has black cell culture vessel holders

HoloMonitor 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 HoloMonitor are delivered with the blue holders.

For Revision 1, there are Vessel Maps matching the microscope slide holder and the standard multiwell plate holder. For Revision 2, there are Vessel maps matching the microscope slide holder, the standard multiwell 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 local distributor or support@phiab.com.

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 at 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 the software.

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 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.

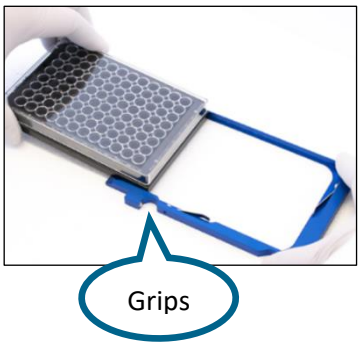
All standard well plates are not the same. They are all slightly different. If a different vessel than the recommended is used, the Vessel Maps will not correlate with the actual wells.

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 accidently placed the wrong way in the vessel holder.

Ensure that well A1 (Sarstedt, ibidi) or well 1 (Eppendorf) of the plate is placed according to the table below.



Stage has moved (lost its calibration)

The stage needs to be recalibrated. Contact your local distributor or support@phiab.com for further assistance.

Vessel	Vendor cat. number	HoloLid™	Final working volume	Growth area, cm^2/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
ibidi® 24-multiwell plate	82426	71131	1.7 mL/well	1.54	top left

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 appears disturbed

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.

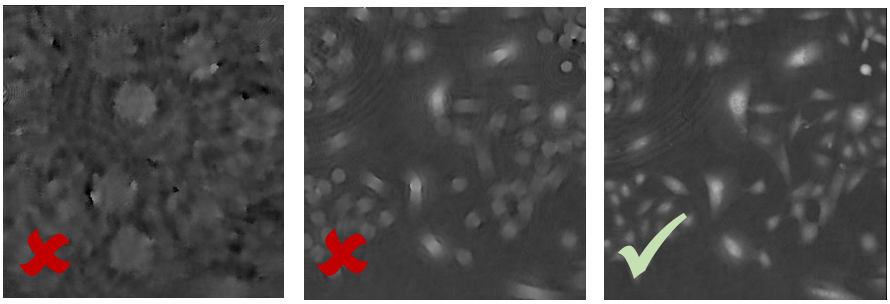
Clean the instrument as described in the Cleaning instructions in the software.

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™.

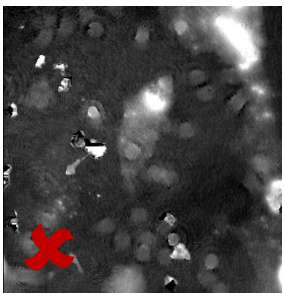
Re-calibrate according to the Calibration procedure.

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

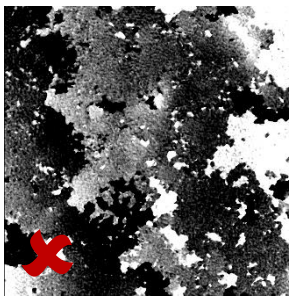
Wrong focus



Condensation

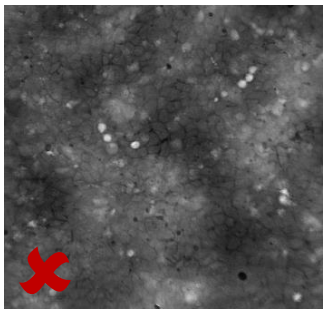


Some condensation

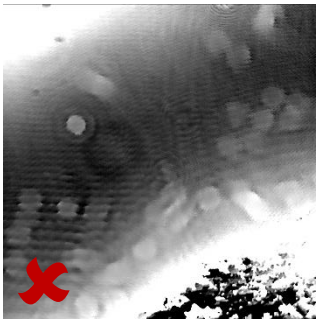


Full condensation

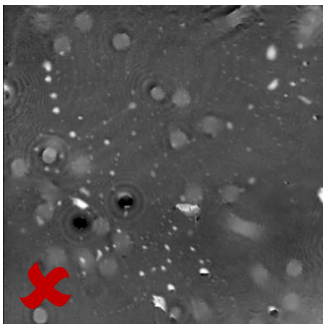
Cells in many layers



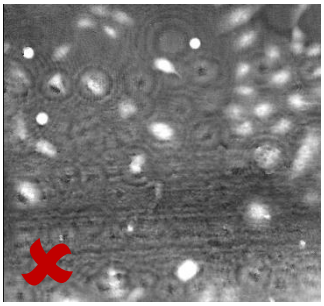
Air bubble



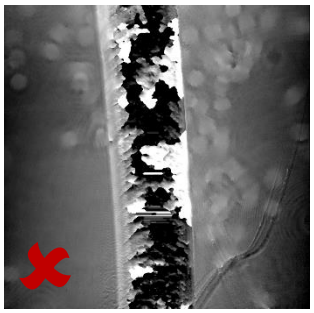
Dirt & Particles

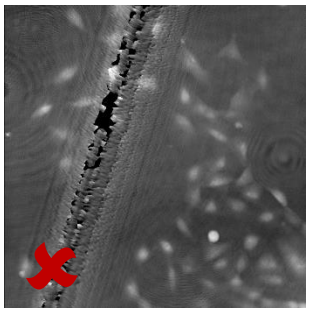


Smear

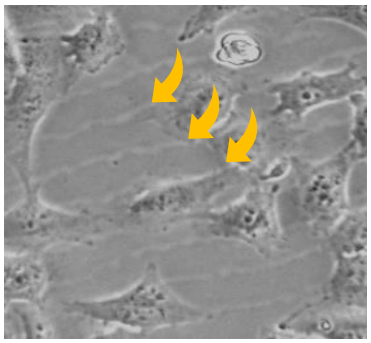


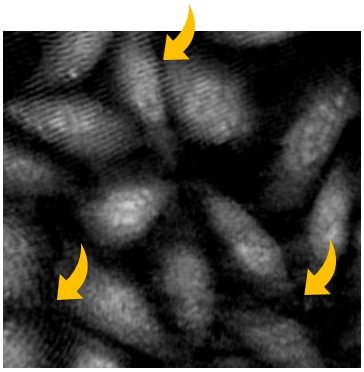
Small and big scratches





Poor quality 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.com for advice.

Condensation has formed inside HoloMonitor

HoloMonitor 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 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.com 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.com

Color settings are not optimal

Try resetting the color scale.

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.

My databases

There are no databases in My Databases

All databases have been removed from My Databases.

Browse to locate and open databases.

There are no databases on the computer.

Create a new database for the experiment.

The databases in My Databases 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.

New database cannot be added

Make sure the new database is created in an empty folder.

Experiment capture

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

The cell culture vessel may not be properly placed in the vessel 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.com for further assistance.

The motorized stage needs to serviced

Contact your local distributor or support@phiab.com 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 the internet. If available, use flight mode.

HoloMonitor loosing connection to computer

Bad USB cable, change to new cable.

Bad USB contact in the computer. Try a different contact.

Cells are dying during the experiment

Cells were seeded in standard 24 or 96 well plates

For longer term experiments, cells may require multiwell plates with gas permeable bottom surfaces, such as Sarstedt Lumox, as the HoloLids disturbs regular gas exchange through the medium surface.

The laser intensity is quite low and does not cause cell death.

Results

Guided results

There are no databases in My Databases

All databases have been removed from My Databases

Browse to locate and open databases.

The databases in My Databases cannot be accessed

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

Remove the database name from the My Databasesand then browse to access the database again at the new location.

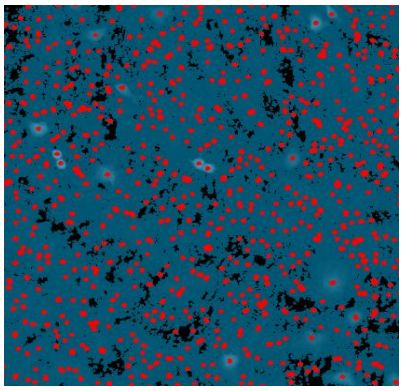
If the database is not on the computer hard drive, the connection may have failed, and the database cannot be accessed anymore, the name will be inactive and there will be a red dot

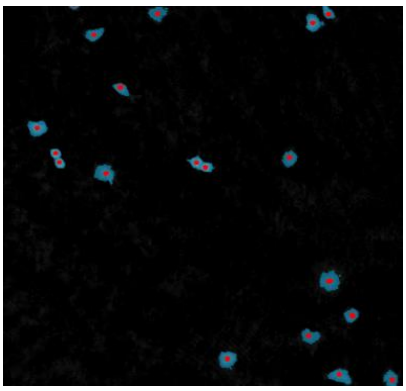
External drive USB connection or internet connection were interrupted.

Image analysis is not optimal

The automatic cell identification looks strange

The threshold setting may not suit the cells. Use the Sensitivity slider 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 slider 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.

Cells are stretched long and narrow and are counted as several cells

Stretched out, narrow cells with uneven 3D structure are very difficult for the software to identify properly. If possible, switch cell type.

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 experiment and group have been selected

Select an Experiment 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.

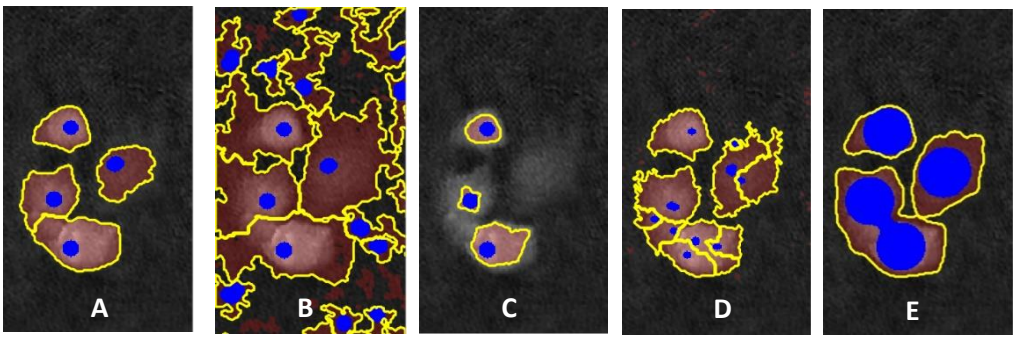
Seed the cells at a lower density.

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

Ensure that Auto-calibration has been performed with acceptable values 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 trees have abnormally many or short branches

Tracking is not performed optimally, fix warnings, check that tracks are correct.

Cell morphology

No image frames are visible in the Image Frame list

No Experiment and group have been selected

Select an Experiment 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.

Wound healing

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.

Graphs look “spiky”

Adjust masks for single frames

View images

No image frames are visible in the Image Frame list

No Experiment and group have been selected

Select an Experiment 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 Experiment and group have been selected

Select an Experiment 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.

TECHNOLOGY

Holographic microscopy

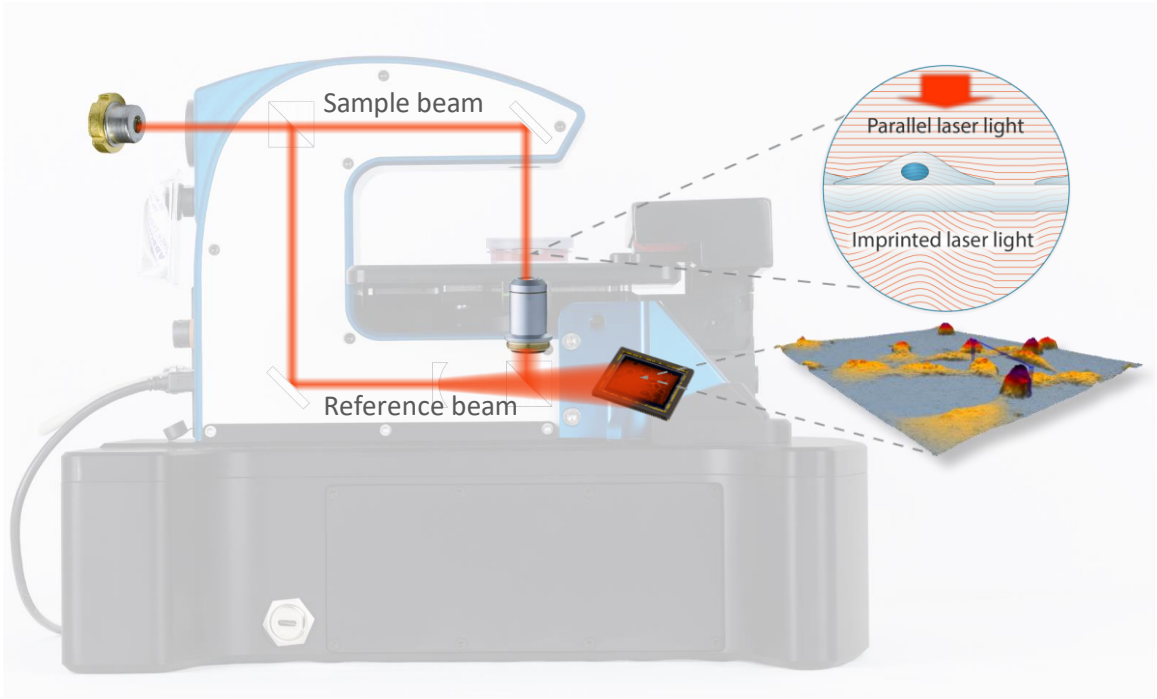
To properly understand the output variables that App Suite presents, it is important to understand digital holography. Below is a crash course. For more information, see Alm et al. 2011 and2013; Kim 2010.

In short

The delay of the light caused by cells when the light passes through them is called the phase shift. This is the actual difference that is measured with digital holography. The phase shift can be used to calculate a wide variety of cell features such as area, thickness, volume, and different cell shapes. These features can be used to trace changes in the cells such as growth, division and death. This non-invasive technology makes holography ideal for long term live cell imaging.

How does it work?

The light source in HoloMonitor is a low intensity laser LED. The original laser beamis first divided into a reference beam 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 into a sample beam and a reference beam. To the right, the laser wave front passes through the cells and the phase delay is imprinted upon the light waves.

Calculations

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. A phase image displays the phase delay, *i.e.* the phase shift of the light, caused by the sample. The quantified phase shift is the basic raw image data of holographic microscopy. Below is an equation that describes cell morphology features as calculated by the App Suite software, based on the phase image. The estimated cell optical thickness *(T)* of a cell in pixel *i* is obtained from the phase shift *(φ)*, the wavelength of the laser light *(λ)*, the refractive index of the cells *(n_c)* and the refractive index of the surrounding medium *(n_m):*

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

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 optical thickness or by a refractive index change in the cellular area imaged by a pixel.

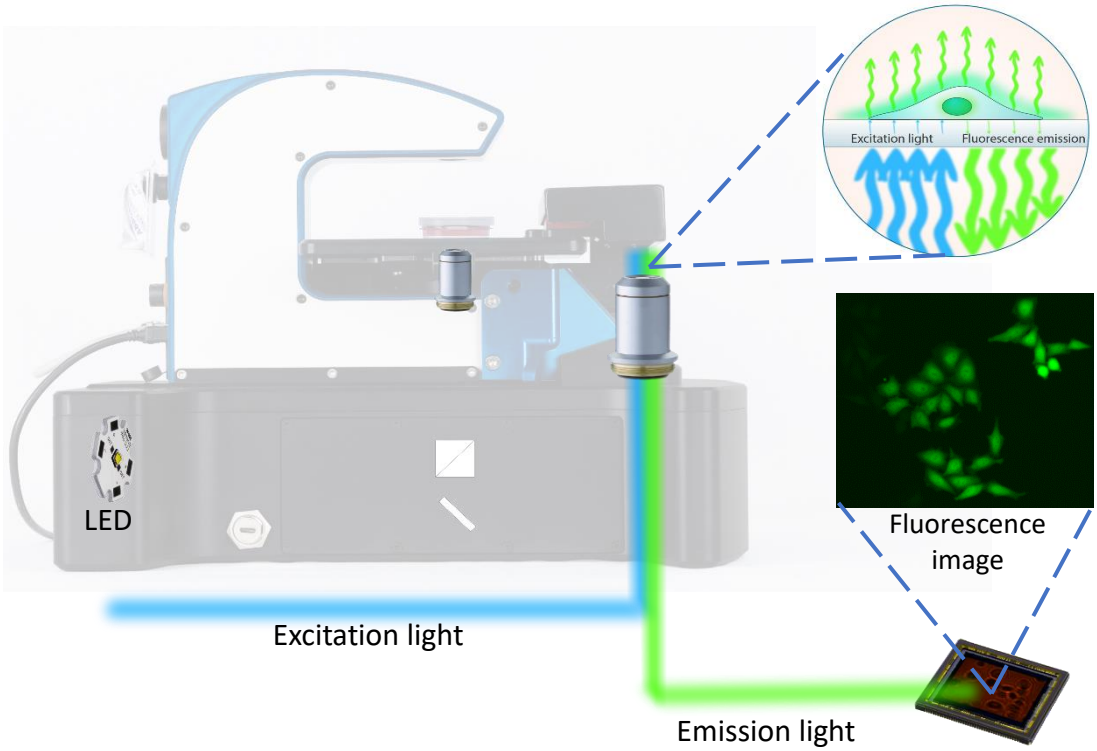
Fluorescence microscopy

Fluorescence microscopy is a well-established well-documented technology described in numerous sources.

In short

When some molecules receive energy from light, they will send out a flash of light. The incoming light is called excitation light and the outgoing signal is called the emission light. The molecules are called fluorophores. HoloMonitorFL combines holographic and fluorescence capabilities, thus adding another dimension to cell analysis.

Fluorescence labeling, using fluorophores to mark specific proteins, is a powerful tool to detect intracellular processes, but the technology usually does not allow for long term live cell imaging.

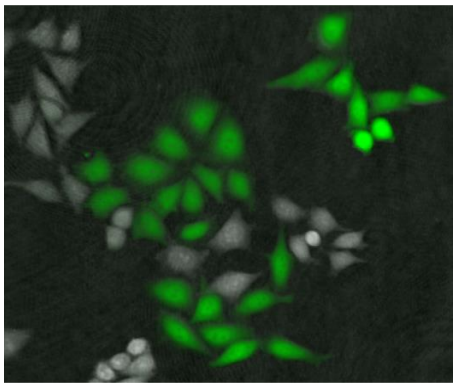


The fluorescence setup, showing how the excitation light is exciting the fluorophores in the cells. The emitted light is directed onto the image sensor.

Holography and fluorescence combined

Holographic images contribute with data on cell morphology, but cannot give any detail regarding molecular biology, e.g., protein expression. By combining holography and fluorescence, live cells can both be monitored over time, and intracellular processes can be detected.

The image shows a holographic image overlayed by a fluorescence image.



ANALYSIS FEATURES

Output variables for guided results

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 (CV). 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 (µm²)

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:

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

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/cm²)

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 (µm)

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 optical 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 (µm/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.

Mean cell diameter (µm)

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

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

where *k* is the number of single cells in the frame, and *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 (µm^3)

The estimated optical 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 optical volume is independent from the cell shape.

Output variables for in-depth analysis

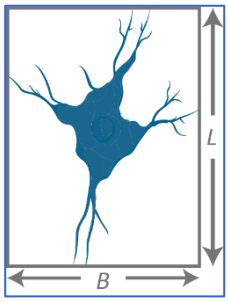
Area (µm²)

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.

Boxed length and breadth (µm)



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. This is also known as an area-minimized (aligned) bounding box.

Boxed center position x and y

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

Centroid position x and y

The centroid position (C_x) is the arithmetic mean of a cell's shape, weighted by the optical thickness of every pixel. This corresponds to the center of mass using the optical thickness as the weight distribution.

$$C_x = \frac{\sum_{x,y} T_{xy} \cdot x}{\sum_{x,y} T_{xy}} \qquad C_y = \frac{\sum_{x,y} T_{xy} \cdot y}{\sum_{x,y} T_{xy}}$$

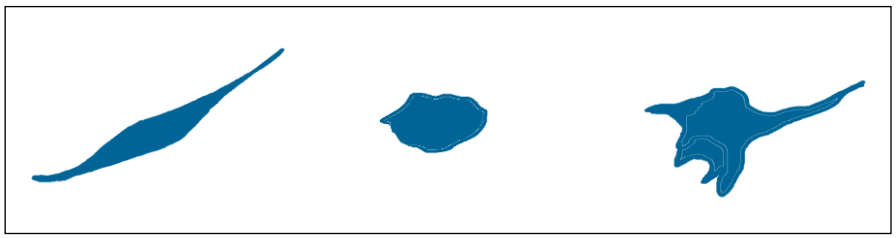
Where T_{xy} is the optical thickness in a specific point.

Eccentricity

Eccentricity (*E*) describes 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.

$$E = \sqrt{1 - \frac{B^2}{L^2}}$$

Where *B* is the boxed breadth and *L* is the boxed Length



High eccentricity Circular High irregularity

Fluorescence green area (µm^2)

Green Area indicates the size of cell area that is green.

Fluorescence green avg

Green Avg indicates the average green intensity in each pixel of the identified cell.

Fluorescence green max

Green max indicates the maximum green intensity of any pixel of the identified cell.

Fluorescence green min

Green min - indicates the minimum green intensity of any pixel of the identified cell.

Fluorescence green std

Green STD indicates the standard deviation of the green intensity in all pixels of the identified cell.

Fluorescence green sum

Green sum indicates the sum of the green intensity of all pixels in the identified cell.

Hull convexity

Hull convexity (*HC*) is a measure of how much the 3D cell shape deviates from a fully convex shape. A higher value means less indentations in the cell's thickness i.e., a more convex shape.

$$HC = \frac{V}{V_c}$$

Where *V* is the optical volume and V_c is the (morphologically) closed volume, using a circular kernel with an adaptive size.

Irregularity

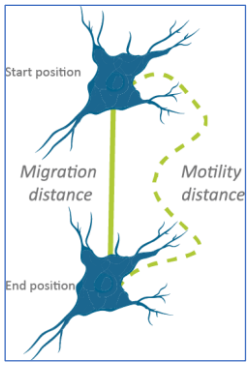
Irregularity (*Irr*) is a measure of how much the circumference of the cell deviates from the circumference of a perfect circle. A value of 0 means the cell is circular and higher values mean a longer, more irregular outline. The calculations are based on a threshold setting that distinguishes background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

$$Irr = 1 - \frac{4\pi A}{p^2}$$

Where *A* is the cell area and *p* is the cell perimeter length.

Migration distance (µm)

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



Motility distance (µm)

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 (µm/h)

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.

Optical volume (µm^3)

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

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

Where λ is the wavelength of the light, s_{xy}^2 is the calibrated size of the area imaged by a single pixel, φ is the phase shift in the same pixel, n_c is the refractive index of the cell, and n_m is the refractive index of the cell culture medium.

Optical thickness (µm)

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

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

Where *V* is the optical cell volume and A is the cell area, λ is the wavelength of the light, φ is the phase shift in the same pixel, n_c is the refractive index of the cell, n_m is the refractive index of the cell culture medium, and N is the number of pixels which image the cell area.

Optical thickness max (µm)

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

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

Where λ is the wavelength of the light, φ is the phase shift in the same pixel, n_c is the refractive index of the cell, n_m is the refractive index of the cell culture medium, and N is the number of pixels which image the cell area.

Optical path length (µm)

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.

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

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

Where T_{avg} is the average cell optical thickness, n_c is the refractive index of the cell, n_m is the refractive index of the cell culture medium, λ is the wavelength of the light, j is the phase shift in the same pixel, and N is the number of pixels which image the cell area.

Note that contrary to cell optical thickness, OPD is independent of the refractive index of the cells and the cell culture medium.

Peak position

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

Perimeter length (µm)

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	The phase shift sum of a cell (φ_{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 and φ_i is the average phase shift in radians created by the area imaged by object pixel i divided by 2π.

Roughness

Roughness (I_{rough}) 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 segmented 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.

$$I_{rough} = |I - I_{smooth}|$$

I is the original image and I_{smooth} is the original image smoothed with a gaussian kernel of an adaptive size.

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

Shape convexity (*SC*) is a measure on how convex the outline of the cell is. If the cell shape is concave, this value is zero, but the larger concave indentations, the smaller value.

$$SC = \frac{A}{A_{ch}}$$

Where *A* is the area of the cell, and A_{ch} is the area of the cells convex hull.

Texture

Texture is a measure of the variation of the cell structure and may differ depending on where the cell is in its life cycle or depending on different treatments. Texture describes smoothness, coarseness and regularity. For HoloMonitor images the texture depends on the phase shift values in every pixel of a cell. 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. The calculations can be found 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.
Texture correlation info1	A different way of calculating the texture correlation.
Texture correlation info2	A different way of calculating the texture correlation.
Texture energy	Provides the sum of squared elements in the gray-level co-occurrence matrix. Also known as uniformity or the angular second moment.
Texture entropy	Entropy is a statistical measure of randomness that can be used to characterize the texture of the cell image.
Texture homogeneity	Measures how many different gray levels the image holds.
Texture maxprob	High values occur if one combination of pixels dominates the pixel pairs in the window.

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