



phi

PHASE
HOLOGRAPHIC
IMAGING

holo monitor[®]

App Suite

App Suite 3.5

Setup and Operation Manual

Revision 3

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INTRODUCTION TO HOLOMONITOR® M4

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

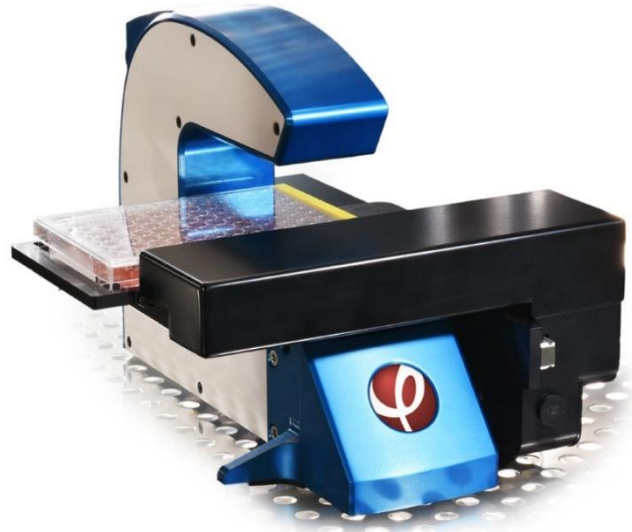
- Cell count
- Cell culture quality assessment
- Cell proliferation
- Cell motility
- Cell migration
- Cell morphology
- Wound healing
- Cell division/mitosis
- Toxicology/dose-response

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. All images are holographic

reconstructions, based on the hologram captured by the HoloMonitor. 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.

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





THIS MANUAL

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

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

The **third part** is a troubleshooting guide.

The **fourth part** is appendixes.

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

INSTRUMENT SAFETY

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

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

and referring to the harmonized standards

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

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

Important information



Risk for physical damage to person or property.



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



Risk for laser light causing damage to person or property.



WARRANTY

The manufacturer guarantees that the instrument has no material or production defects when delivered, according to EU directive 99/44/EC sale of consumer goods and associated guarantees. If the manufacturer is informed within 6 months after delivery, the manufacturer is obliged to rectify such defects. It is the manufacturer's decision whether to repair the instrument or replace the instrument with an instrument free of defects.

No guarantee is provided for defects caused by natural wear (wearing parts in particular) or improper use.

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



Apart from the procedures specified in this manual, no maintenance or repair of HoloMonitor M4 may be performed by users. Repairs may only be performed by authorized personnel.

Warranty

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

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

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

Limited Warranty

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

COMPUTER REQUIREMENTS

HoloMonitor M4 is controlled by the proprietary HoloMonitor App Suite software (App Suite) running on an external computer. App Suite needs to be installed prior to use of HoloMonitor M4. These are the recommended computer specifications:

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

INCUBATOR REQUIREMENTS

The HoloMonitor M4 is designed to work in an incubator with 95% humidity and 5% CO₂ at 37°C but will work from 10-40°C and at ambient humidity and in any combination of the ambient gases (CO₂, O₂, N₂ etc.).

The incubator needs to have sufficient space for the HoloMonitor M4 motorized stage to move during the experiments.



When sterilizing the incubator with heat or chemicals other than ethanol, HoloMonitor M4 and all cables need to be removed from the incubator.

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

SOFTWARE

Software installation

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

Software activation

The activation step usually requires internet access but may be performed without internet access by using another, internet-connected, computer.

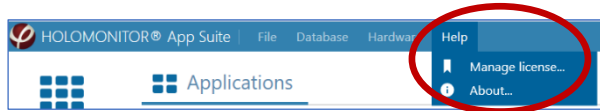
A request for activation will appear automatically the first time someone uses the software.

One software license is valid for two computers.

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.

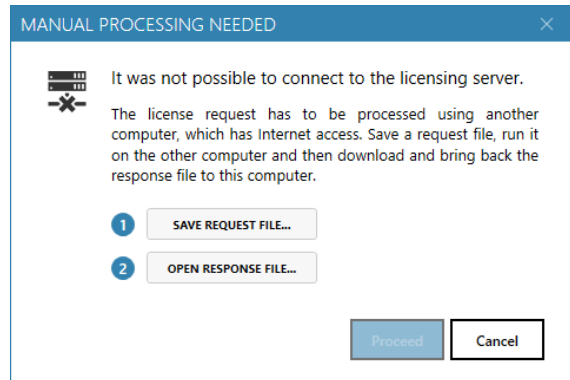


Activating license offline

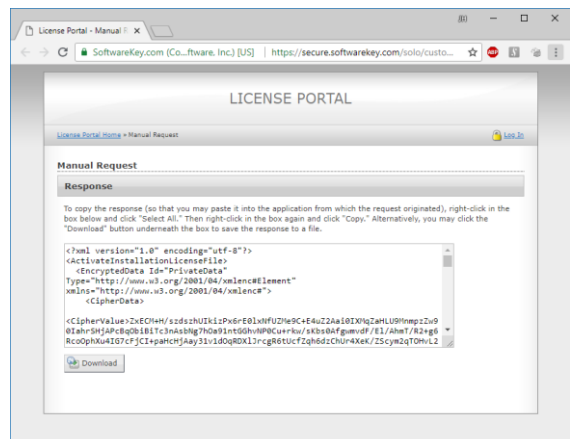
When Internet is not available on the computer it is still possible to activate, refresh and return a license using another computer with Internet access.

The entry to all actions is the same as when Internet is available, i.e., through buttons on the license dialog. The **Manual Processing dialog** is shown to the user when the action is attempted.

Now the user must save a file, transfer it to another computer via e.g., 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

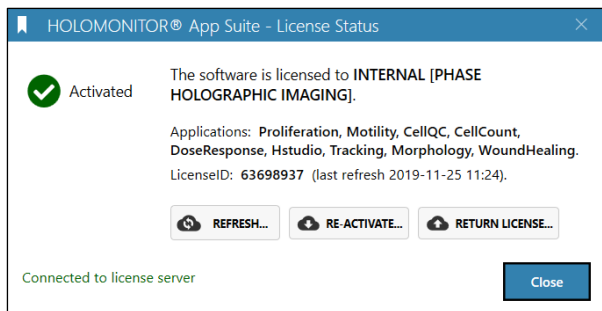
The current license status can always be accessed via the **Help tab** in the top menu bar.

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

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

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

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



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 recommend scheduling updates to a time when the computer is not used or disabling internet connection or enabling Airplane 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 INSTALLATION

The HoloMonitor M4 should be delivered and assembled by trained personnel. They will ensure that the instrument is correctly assembled and calibrated.

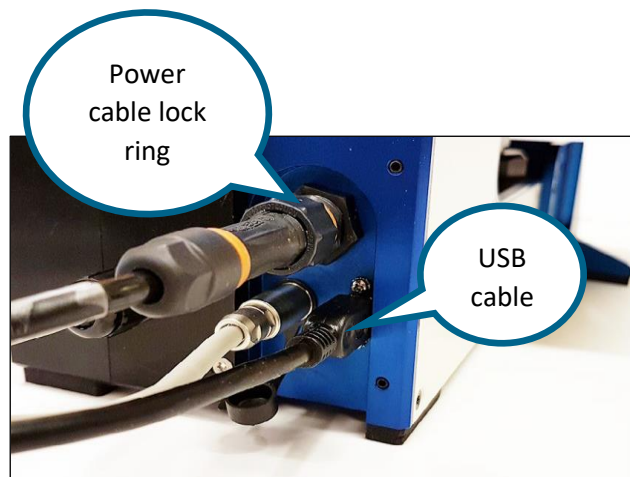
Instrument Assembly inside incubator

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

2. Connect the USB-cable to the computer.

3. Connect the computer power cable to the wall outlet.

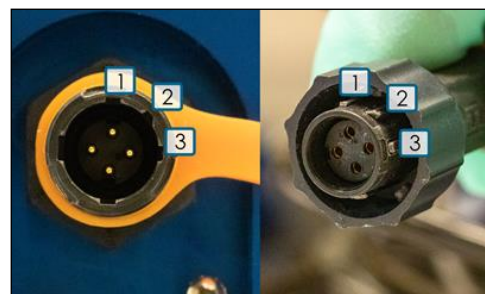
4. Clean the instrument exterior. The instrument can be wiped down with a paper towel dampened with 70 % ethanol. Cleaning must be performed so that no liquid enters the interior of the instrument.



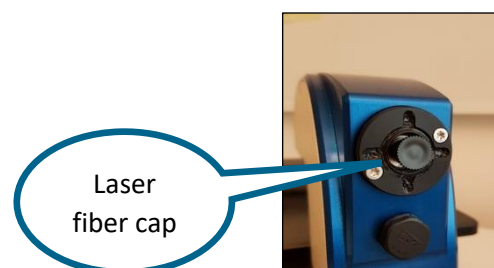
5. Place HoloMonitor M4 inside the cell culture incubator. Make sure that there is space for the motorized stage to move.

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

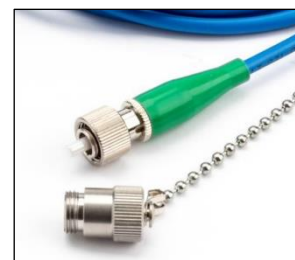
7. Wipe down the power cable with ethanol and connect it to the instrument. Ensure that it is properly aligned before tightening the lock ring. The contact has ridges in a pattern that will fit slots in the connector. When properly aligned, the lock ring will be guided into place when tightened.



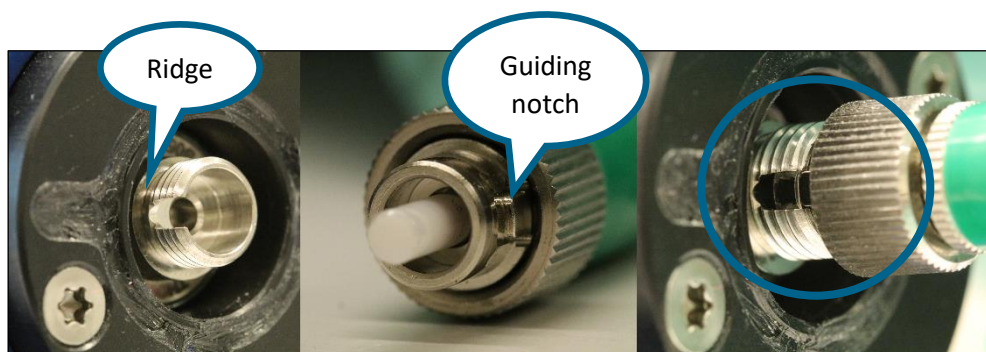
8. Remove the black cap covering the laser fiber connection point. Save the protective cap for future use.



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 M4 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 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 bend the fiber. Do not touch the fiber tip.

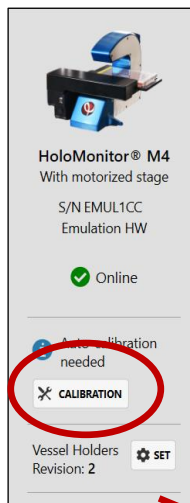


To ensure optimal laser function, the surrounding temperature for the laser unit must be maximum 24 C. Do not place the laser unit in direct sun light.



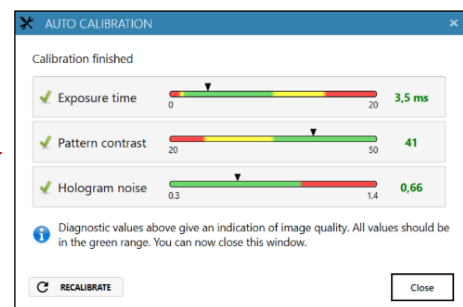
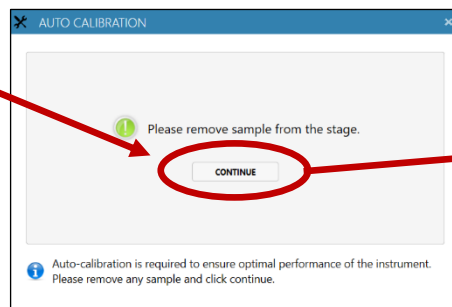
The HoloMonitor laser unit is a Class 2M laser. A Class 2M laser is safe because of the aversion response, if viewed by a naked eye. However, to avoid any unnecessary discomfort when handling the disconnected optical fiber, make sure that the laser beam emerging from the fiber does not radiate directly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

AUTO CALIBRATION



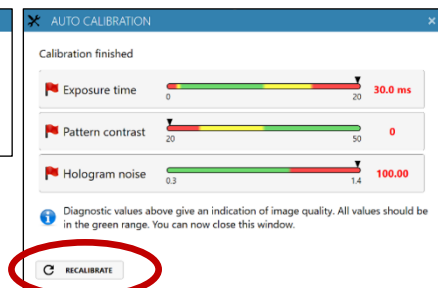
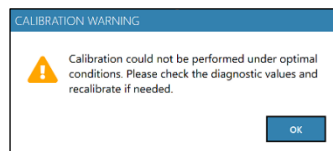
Perform **Auto-calibration** before each use, especially when setting up an experiment. **Auto-calibration** gives a background image that is used by App Suite to lower background noise and increase image quality. The calibration procedure takes approximately one minute. A **Calibration** button can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

1. Start **App Suite** and wait for the HoloMonitor M4 to initialize.
2. When HoloMonitor M4 is ready, click the **Calibration** button below the instrument information.
3. Follow the instructions and ensure that there is no sample on the stage.
4. 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. After verifying that HoloMonitor M4 is properly installed and cleaned, click **Recalibrate**.



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

HOLODRY™

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

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

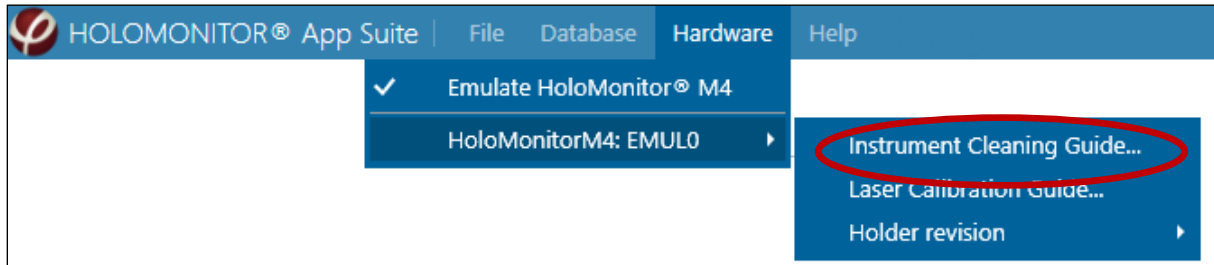
For further information, see the HoloDry protocol (<https://phiab.com/resources/manuals-protocols/>).



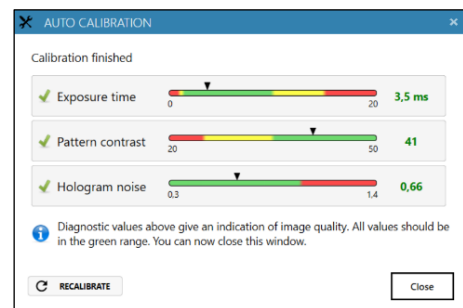
CLEANING INSTRUCTIONS

Cleaning optical surfaces

Go to the **title bar**, select **Hardware menu**, current HoloMonitor M4 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 (black arrow heads) should be in the green range.



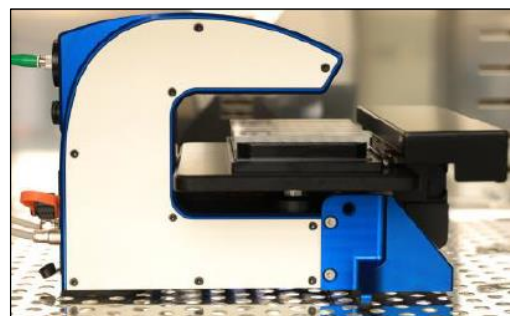
If any of the Diagnostic values remain outside the green range after several cleaning attempts, contact the technical support at support@phiab.se or your local distributor for further assistance.

Cleaning instrument exterior

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



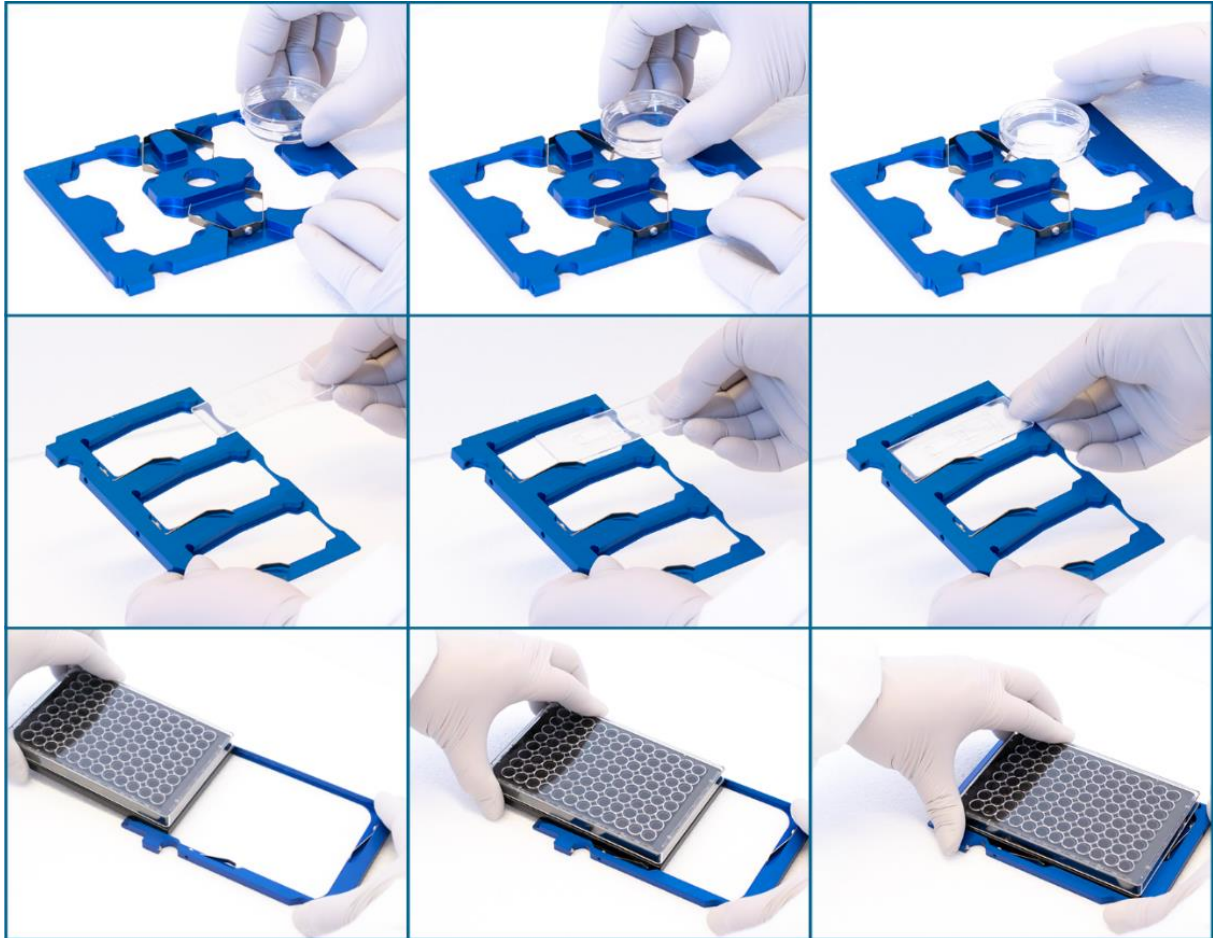
Avoid having any liquid entering the interior of the instrument.



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

VESSEL HOLDERS

HoloMonitor M4 is equipped with a set of blue cell culture vessel holders called *revision 2*. There is a standard \varnothing 35 mm Petri dish holder, a microscope slide holder, and a standard 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.

HoloMonitor® M4
With motorized stage
S/N EMUL1CC
Emulation HW
Online
Auto-calibration needed
CALIBRATION
Vessel Holders Revision: 2 SET

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.

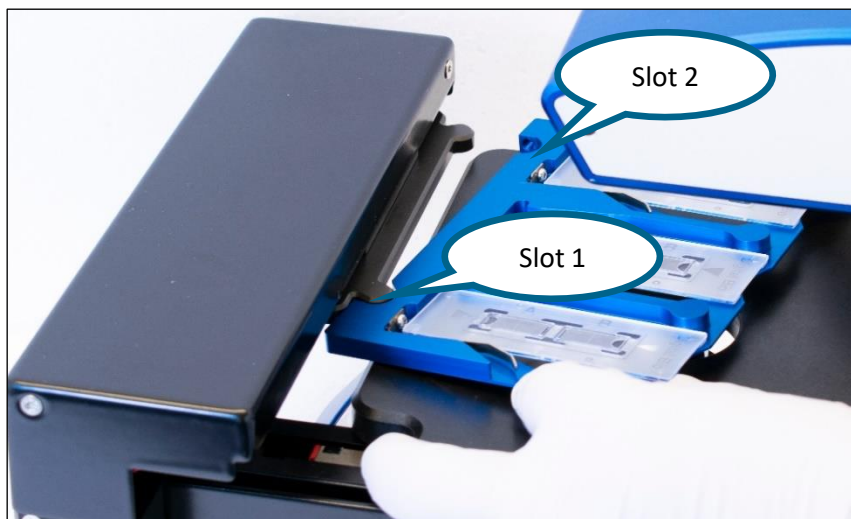
1. Place the culture vessels on to the holder. Place the vessel as shown above and ensure that they are correctly fitted on to the holder. For multiwell plates, slide them sideways along the tracks on to the vessel holder with the bevel/s according to the table below. There is a spring that holds the 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 M4 stage, click slot 1 in place, and thereafter slot 2.



LONG-TERM STORAGE

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

The HoloMonitor M4 should not be taken out of and placed back into the incubator repeatedly, as temperature fluctuations can cause disturbances. Once the HoloMonitor M4 is removed for long term storage, preferably, leave at room temperature at least two days.

All 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 M4.



To extend laser unit working hours, it is recommended to disconnect it from AC power when HoloMonitor M4 is not in use.



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

SAMPLE REQUIREMENTS

Cells

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

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

Cell culture vessels

For the best imaging quality, use:

- ibidi® plastic ware (channel slides), chemotaxis slides, 35 mm culture dishes or 24-well plates with wound healing inserts
- Sarstedt 6-well plates with HoloLid™
- Sarstedt Lumox® 24 or 96 well plates with HoloLid™
- Glass-bottom Petri dishes are highly recommended

Recommended vessels	Vendor cat. number	Growth area, cm ² /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 unscratched

It is very important to keep the cell culture vessels perfectly clean and unscratched. Even wiping the cell culture vessel clean with ethanol will probably not help much as traces of dirt and tissue fiber will persist. Instead, avoid touching the top and bottom surfaces of the cell culture vessel when handling it, even when wearing gloves. Avoid anything that might scratch the surfaces. Cell culture plastic is very easily scratched.



Smudges and scratches will affect image quality.



Non-recommended vessels may cause problems such as

- HoloLid does not fit
- Volume recommended for HoloLid is not correct
- Vessel pattern does not fit
- Image quality may be affected

HOLOLIDS™

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

HoloLids have an optically neutral window which dips into the cell culture medium. This eliminates image disturbances caused by vibrating medium surfaces or by the transient condensation which often occur inside cell culture vessels. 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 cm ² /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



Optically neutral
window in contact
with cell culture
medium

HOLOMONITOR® APP SUITE SOFTWARE

Introduction

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

The imaging procedure is simple and does not affect the cells in any measurable way. To simply count cells takes less than a minute. Confluence measurements and morphology measurements are performed simultaneously. To further analyze the cell morphology, either the built-in analysis functions can be used, or data can be exported as .xlsx files for further work in Excel or similar software. Cell movement and morphology can be tracked over time through a time-lapse sequence for both individual cells and cell populations. The raw data will remain intact through all analyses, as all changes made by the user only concern how the results are displayed.

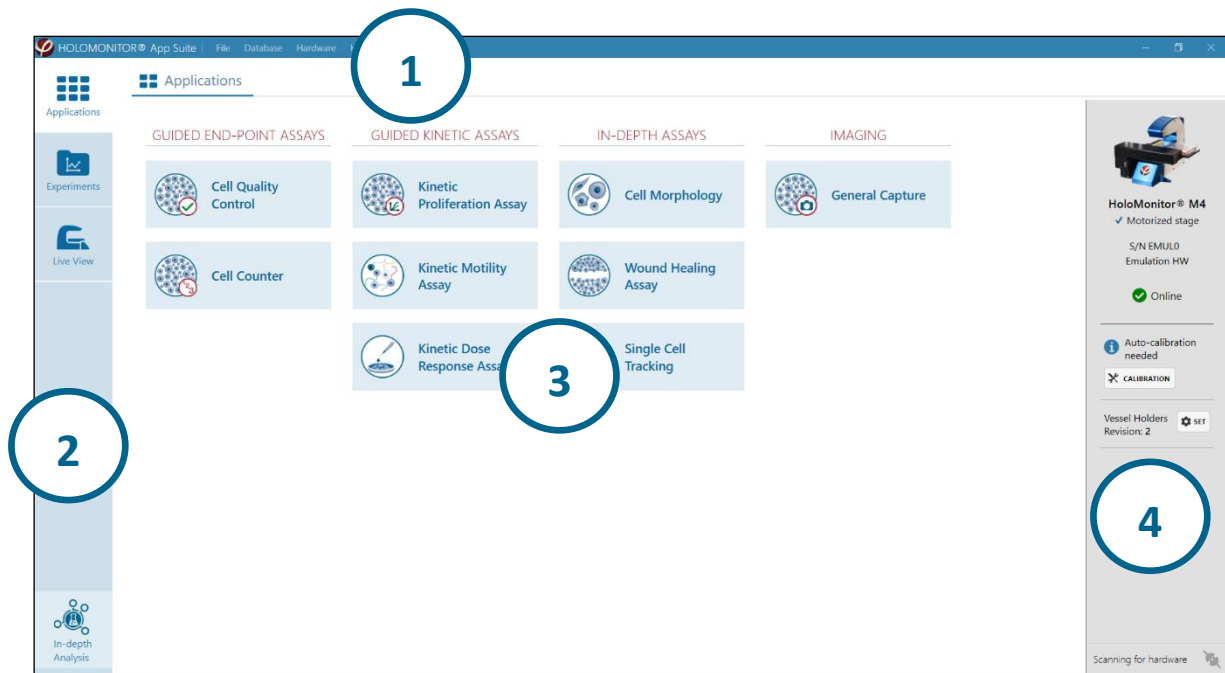
HoloMonitor App Suite software comprises applications that perform assays such as [Cell Counting](#), [Cell Quality Control](#), [Kinetic Proliferation](#), [Kinetic Motility](#), [Kinetic Dose Response](#), [Cell Morphology](#), [Wound Healing](#), and [Single Cell Tracking](#). Each assay has a specific 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.

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



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

Overview

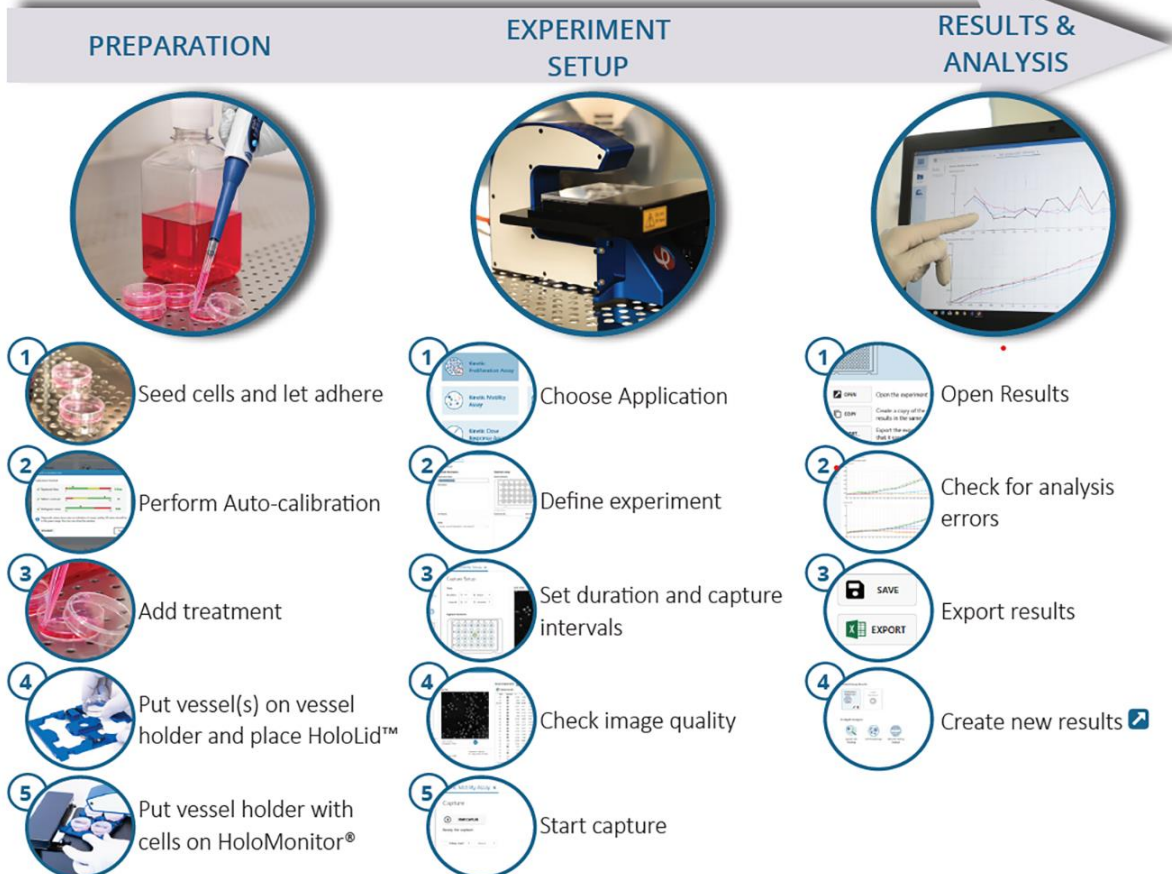


1. In the top menu bar, the Database Handler, the Instrument Cleaning Guide, the Laser Calibration Guide and the HoloMonitor M4 Emulator can be accessed. The Database Handler allows for copying, editing, transferring, and deleting data. The HoloMonitor Emulator is a virtual HoloMonitor M4. When activated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

Workflow for guided applications

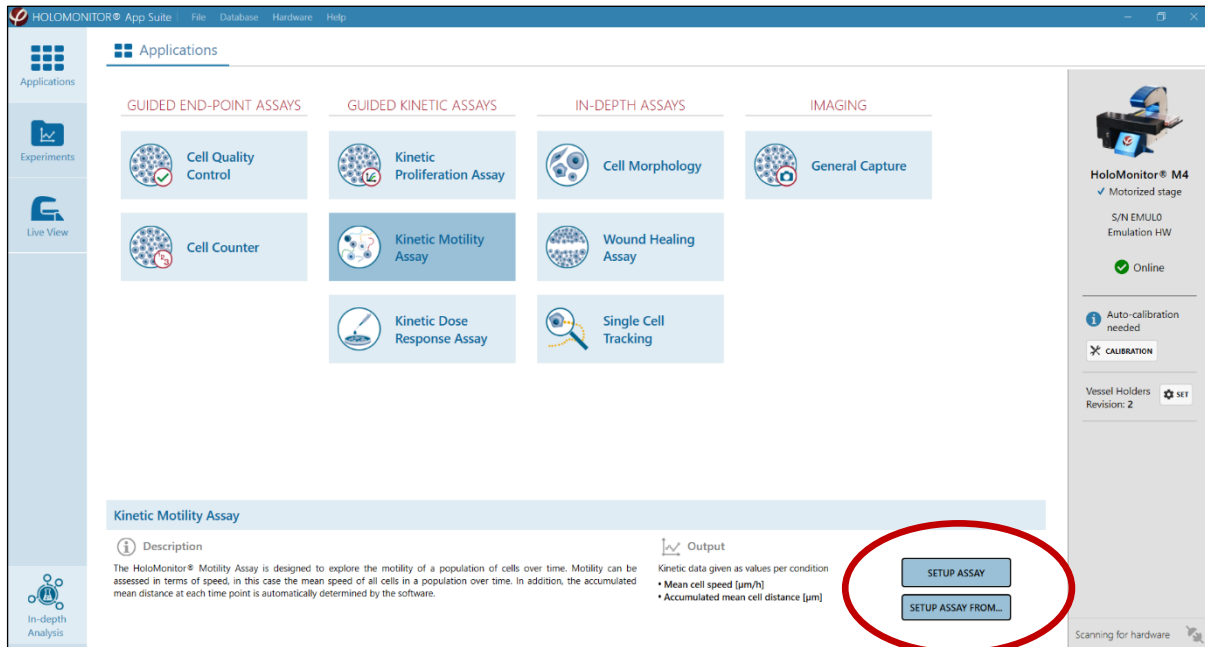
All applications have a similar workflow that guides the user through setup to image acquisition. The **Guided assays** provide results immediately during the experiment while the results for the **In-Depth applications** can be extracted after analyses. The exact procedure for each application is described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

WORK FLOW:



Applications

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



Assays in short

Cell Quality Control

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

Kinetic Proliferation Assay

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

Kinetic Motility Assay

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

Kinetic Dose Response Assay

The **Kinetic Dose Response assay** is designed for automated 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 Assay

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 Assay

Using **Wound Healing assay** 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

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

Output Variables

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

Setting up an experiment

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

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

Setup assay

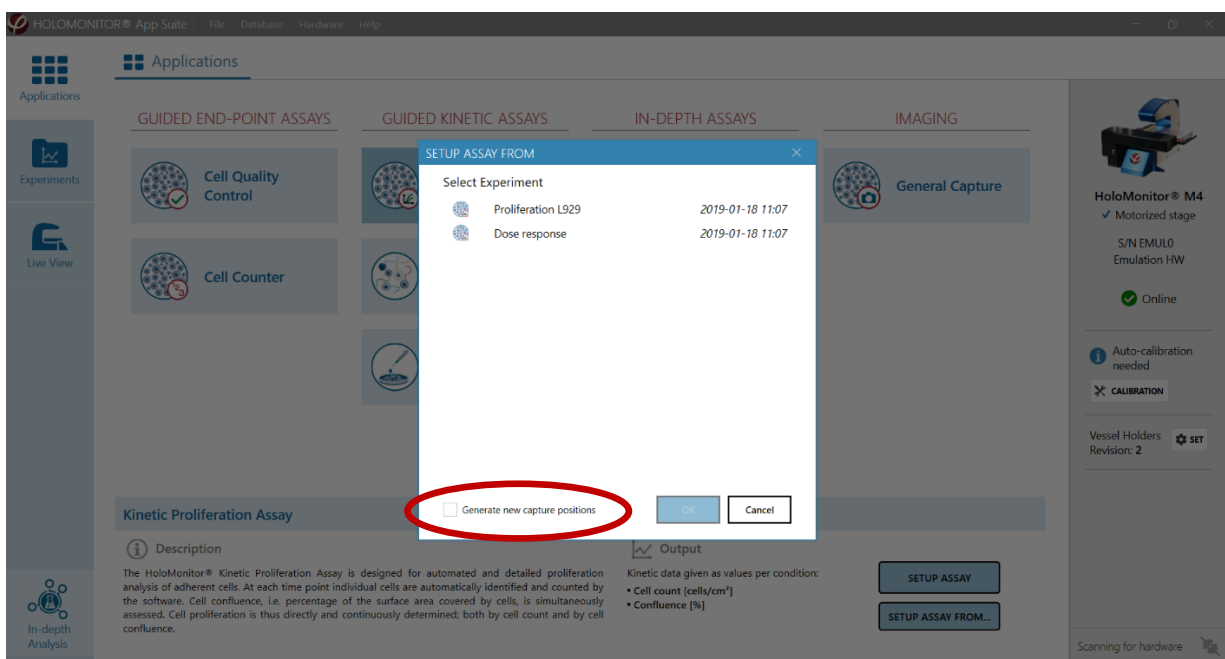
Click the [Setup Assay](#) button to begin experiment set up.

Setup Assay From

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

All settings from the selected experiment will be applied by default for the new experimental setup, including the capturing positions.

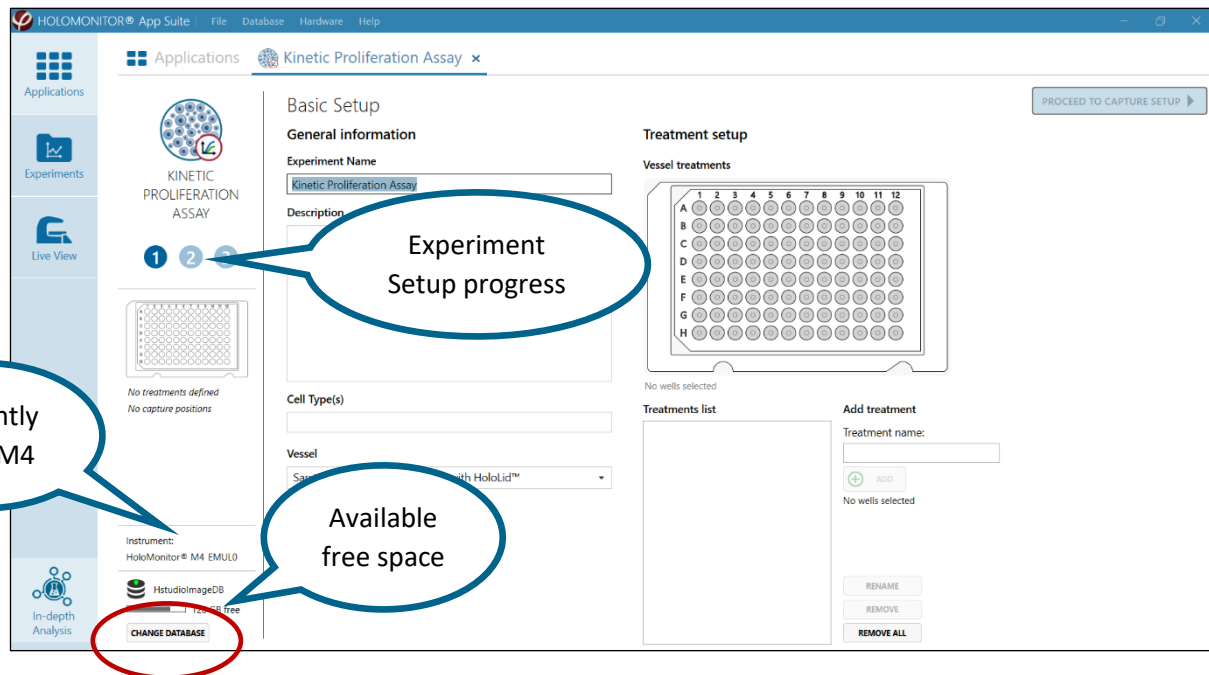
To create new capturing positions for the new experiment, check [Generate New Capture Positions](#).



Experiment Progress

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

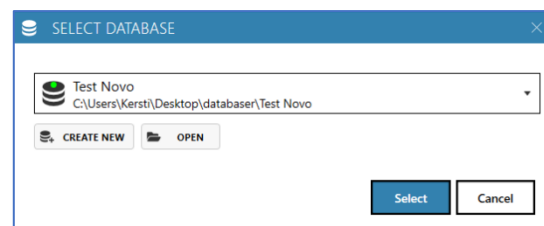
As the experiment setup proceeds, more details will be displayed below the setup progress indicators, such as cell culture vessels used, number of treatments added, the duration of the experiment and the storage space required.



Switching Database

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

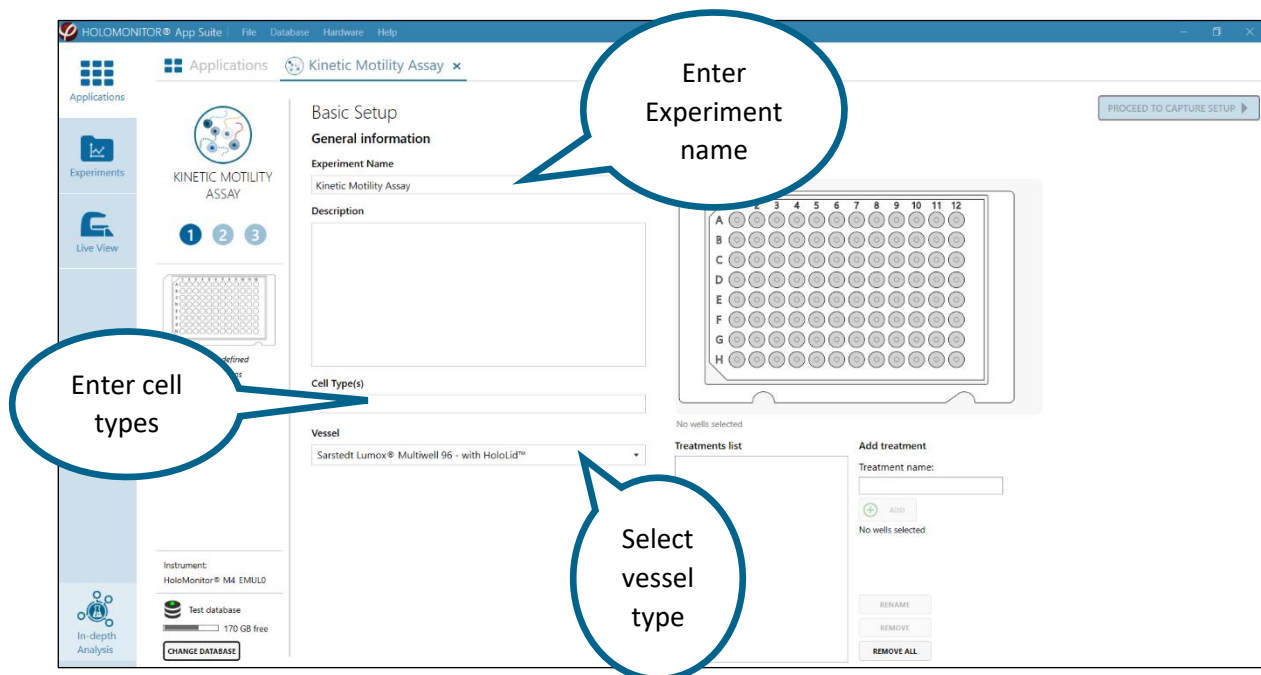
Click the **Change Database** button to switch databases. The currently used database is shown in the **Select Database** dialog box. Use the drop-down list to select another database and then set it as default (see images below). If the database is not found in the drop-down list, click the **Open**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



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, 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 black and blue vessel holder configurations:

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

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

Treatments or Conditions Setup

Here, 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.

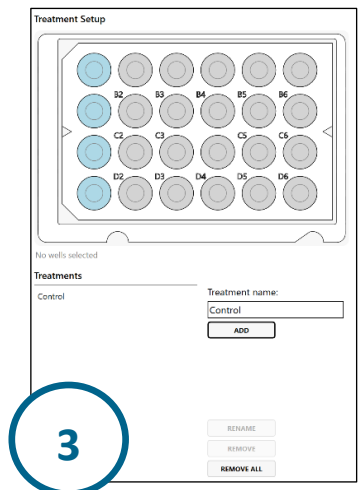
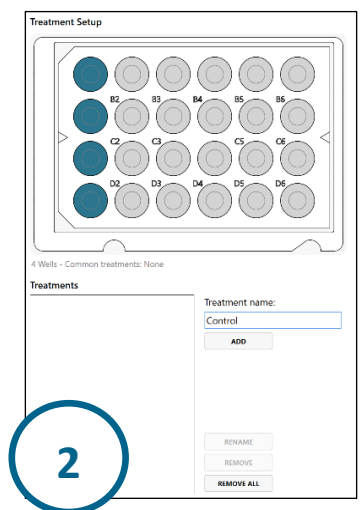
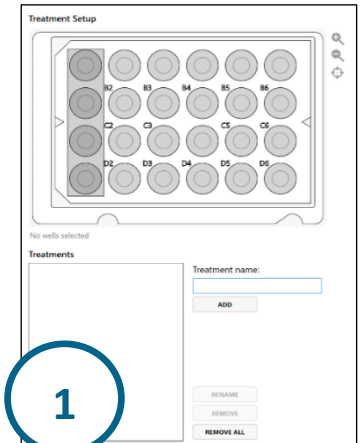
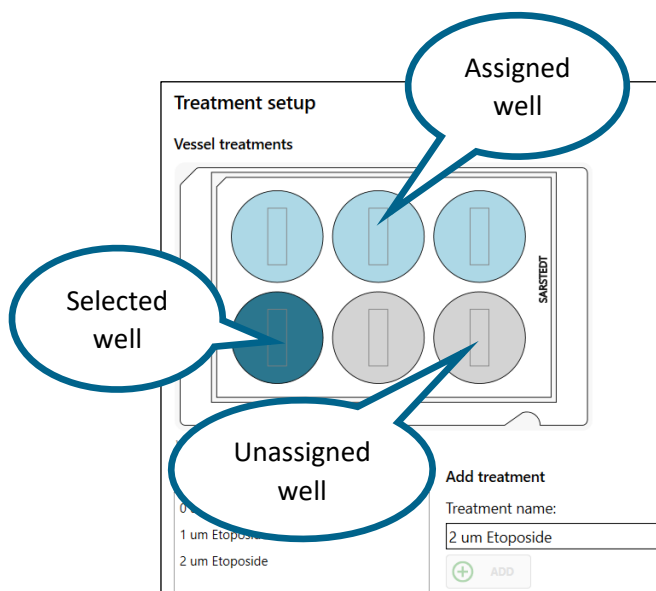
Add treatments

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.

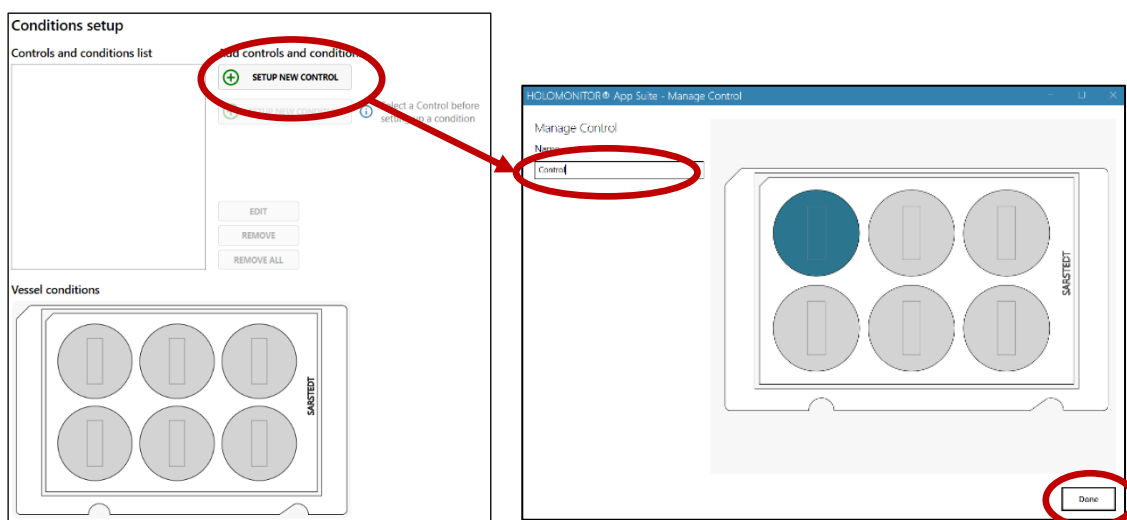


Set Dose-response conditions

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.

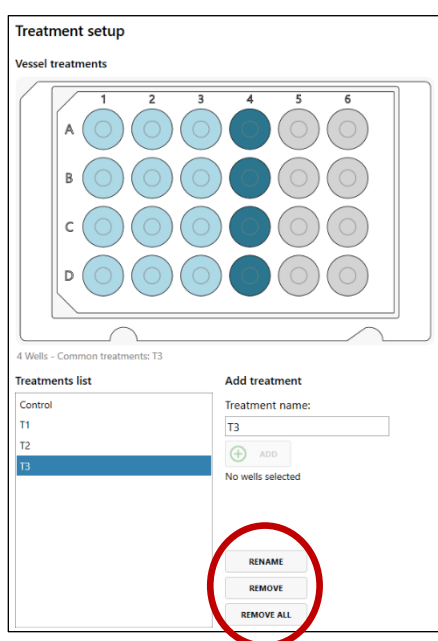
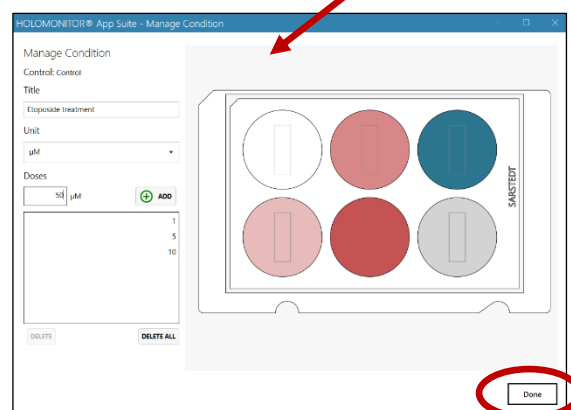


An experiment can have more than one control, e.g. if there are two different cell lines. Use [Setup New Control](#) to add more controls. Note that the controls must have unique names, otherwise it will not be possible to save them.



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.

The screenshot shows the 'Capture Setup' window in the HOLOMONITOR App Suite. The interface is divided into several sections:

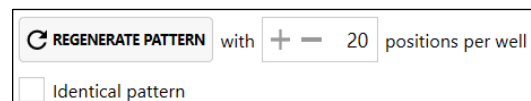
- Left Sidebar:** Contains 'Experiments' and 'Live View' buttons. Below them, a 'KINETIC PROLIFERATION ASSAY' is selected, showing a progress indicator with steps 1, 2, and 3.
- Setup Progress:** A callout box points to the progress indicator, stating 'Setup progress'. Below it, details are shown: '3 treatments (24 wells)', '120 capture positions', 'Duration: 48 hrs', 'Interval: 60 mins', 'Est. batch time: 3 mins', '49 batches', and '7.6 GB storage required'. A callout box labeled 'Storage requirement' points to the storage information.
- Time Settings:** A callout box labeled 'Capture duration and interval' points to the 'Duration: 48 hours' and 'Interval: 60 minutes' fields.
- Vessel Map:** A 4x6 grid of wells is shown, with a callout box labeled 'Vessel map' pointing to it.
- Regenerate Pattern:** A callout box labeled 'Positions list' points to the 'REGENERATE PATTERN' button and the 'Identical pattern' checkbox.
- Live View:** A callout box labeled 'Image of current position' points to the 'Live View' window, which displays a dark image of a well with white spots.

Set capture duration and interval

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

Set capture positions automatically

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



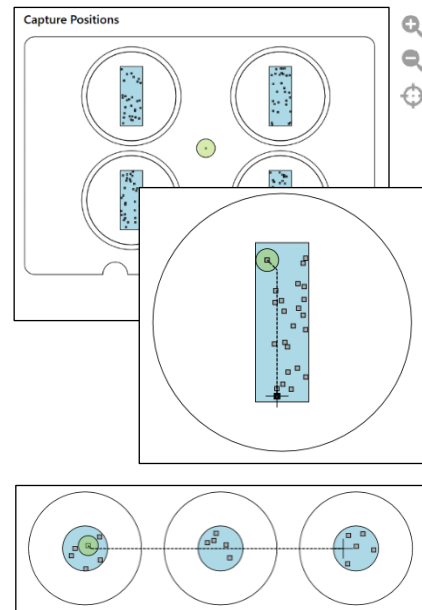
To generate an identical capture pattern in each well, check **Identical Pattern** before clicking **Regenerate Pattern**.

Moving the stage to the capture positions

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.

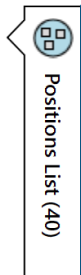


WELL	PREVIEW	X	Y	FOCUS
A1	N/A	-1.737	0.272	-1,450
A1	N/A	-0.929	-0.714	-1,450
A1	N/A	-0.697	1.742	-1,450
A1	N/A	0.945	-0.686	-1,450
A1	N/A	-0.105	-1.669	-1,450
A2	N/A	1.359	-2.224	-1,450
A2	N/A	0.011	1.707	-1,450
A2	N/A	1.481	1.219	-1,450
A2	N/A	0.289	-0.715	-1,450
A2	N/A	0.771	2.323	-1,450
B1	N/A	-0.207	1.383	-1,450
B1	N/A	0.581	0.701	-1,450
B1	N/A	1.504	-1.538	-1,450
B1	N/A	-1.417	-1.274	-1,450
B1	N/A	-0.691	-0.443	-1,450

Go to the selected position
 Set the current focus to all selected positions
 Create a capture position at the current location
 Move the selected position to a new random location in the same well
 Replace the selected position with the current stage position
 Copy the selected position to all wells

Positions list

Each capture position that is marked in the [Vessel map](#) is also listed in the [Positions list](#). 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.

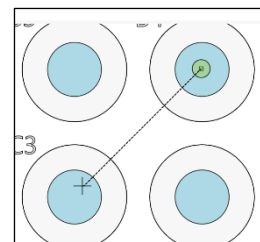


It is possible to set positions manually, and to edit existing positions.

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 (see image to the right, green marker corresponds to objective, “+” corresponds to new position).
- Inspect the image quality in the [Live View](#) window.
- Click the [Add Current Location](#) button at the bottom of the [Positions list](#) to set the current position.



Edit capture positions

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

[Delete](#) will delete all selected positions.

[Select All](#) will select all positions in the list.

[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.

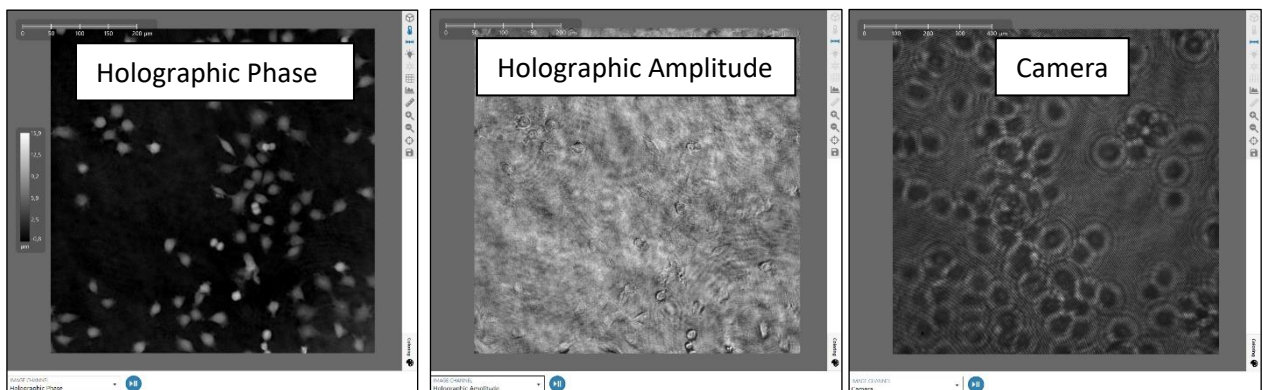
[Regenerate Position](#) will automatically replace the selected position with a random new position.

[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 blue button  to pause the showing of live images.



Play/pause function does not turn on and off 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.



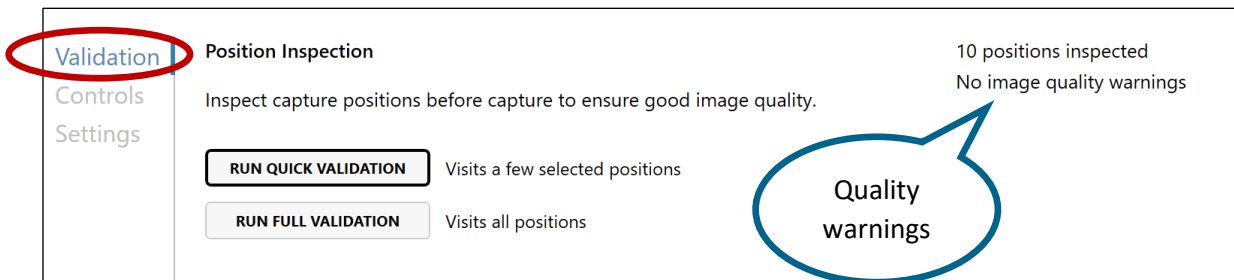
These actions will only pan and zoom the image. The sample and the motorized stage will not be moved.

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.

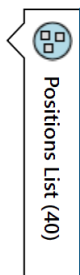
After validation, the software will either indicate that no image quality warnings were found, or that there were warnings.

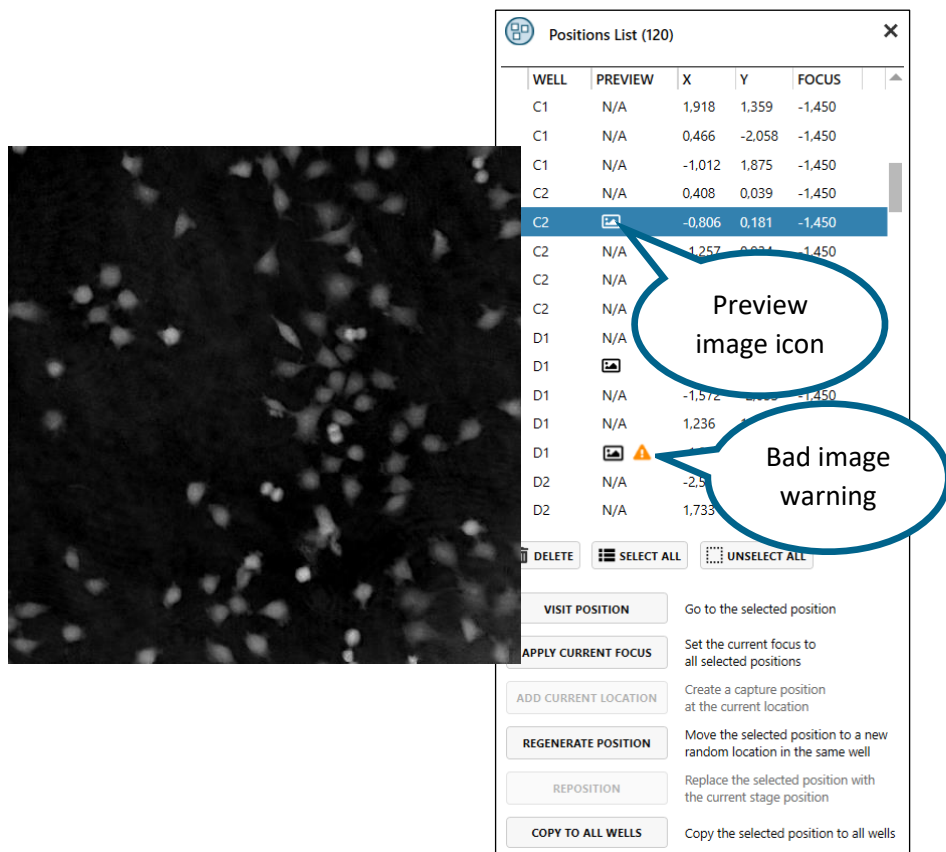
Viewing validated positions

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.

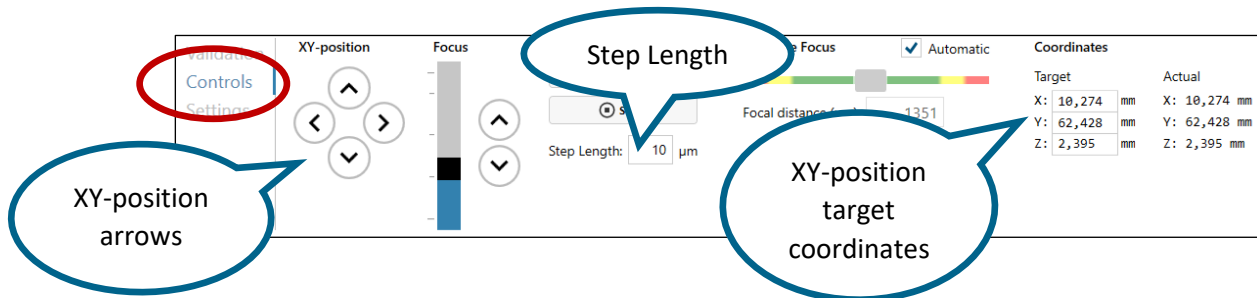
Motorized stage controls

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 **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.



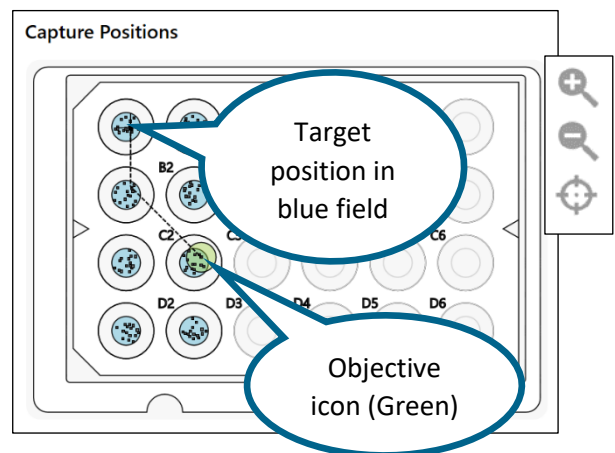
Stage movement

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.

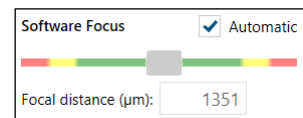
To better see the Vessel map, the window can be moved by click-and-drag, and it can be zoomed in or out by click-and-scroll. The window can also be zoomed in and out as well as reset to original size with the buttons found to the right of the Vessel map or double-clicking left mouse button.

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 M4 was installed, but sometimes focus needs to be adjusted by the user, e.g. if the cell culture vessel has a coating that makes autofocusing difficult. HoloMonitor M4 has both a coarse mechanical and a fine software focus. They need to cooperate for the image to be in focus.

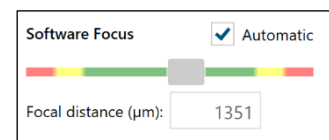
The software can only find focus when the cell culture vessel is at the approximately correct distance from the objective. Use the mechanical focus, *i.e.* move the motorized stage, to move the cells to the approximately correct distance from the objective. The software focus then finds and calculates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



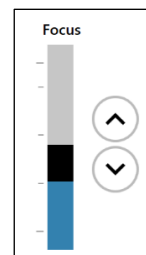
Focusing in practice - semi automatic focusing

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 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 center.

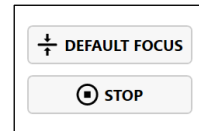


The black box in the [Focus](#) bar represents the motorized stage. Move the black box up or down to move the motorized stage up or down. Click the two arrows for controlled stage movements up or down. The arrows move with the step length entered in the [Step length](#) text box.



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.

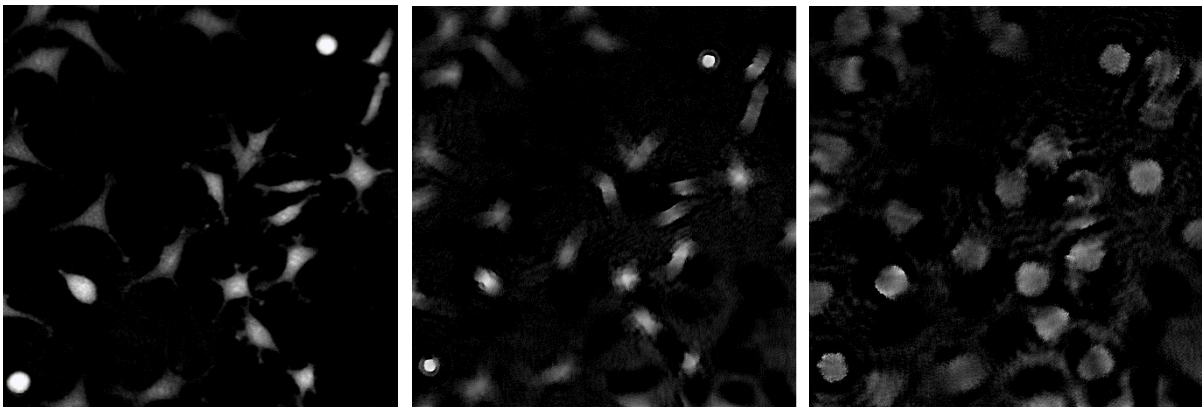
Manual focusing

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.

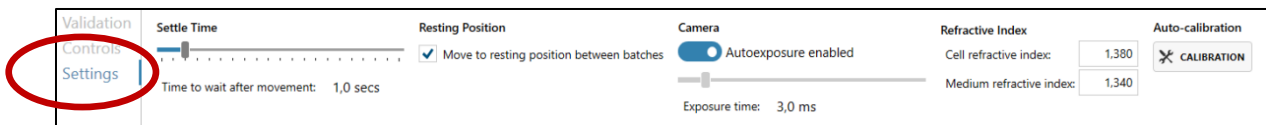
Images in and out of focus

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



Settings

In the [Settings tab](#), the stage movement [settling time](#) and [resting position](#) can be handled, as well as the [Exposure time](#) of the camera. The [Calibration function](#) can be accessed here. Note that [Auto-calibration](#) must be performed before the start of each experiment.



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

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

[Camera exposure time](#) is set to auto exposure by default. It is possible to set the exposure time manually, as may be needed with extremely difficult samples.

[Refractive Index](#) is used by App Suite to calculate cell 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.

Auto-Calibration

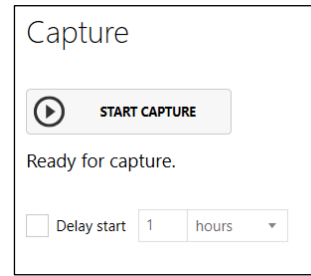
[Auto-calibration](#) gives a background image that is used by App Suite to lower background noise and increase image quality. There is a more detailed description in the Image calibration section in the HoloMonitor Installation chapter.

Capture

In this page, **Image capture** is started and monitored.

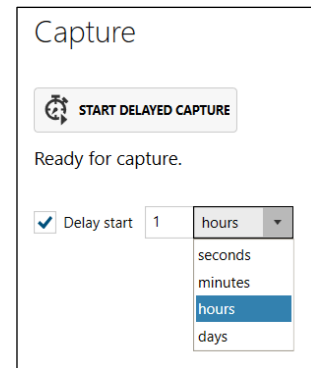
Start capture

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



Delaying Start Capture

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

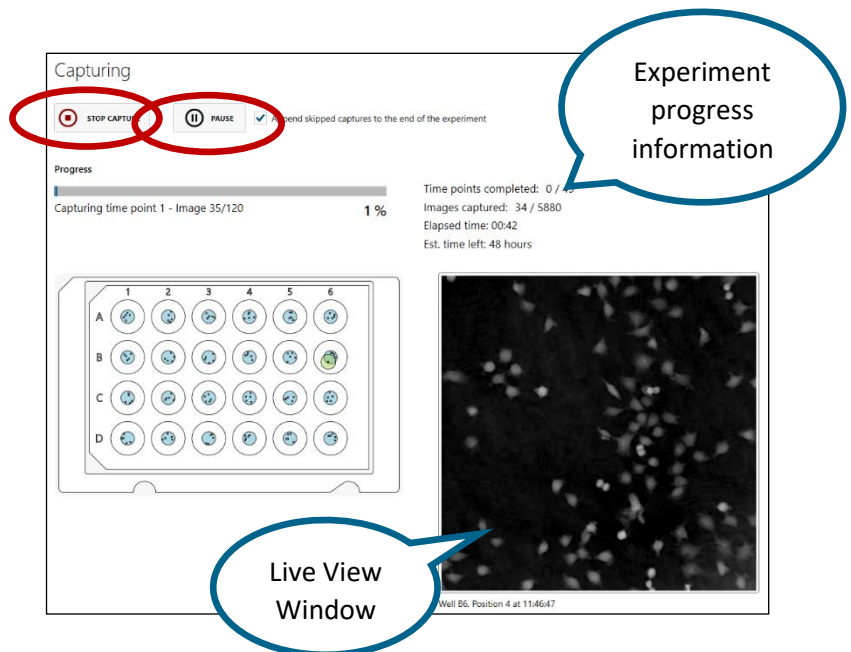


During capture

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

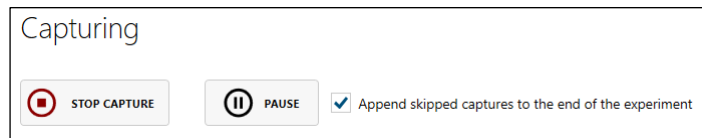


For **Guided Assays**, results are calculated during the experiment and can be viewed while the experiment is running (See Results for Guided Assays, next section).

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

Paus Capture

The capture can be paused by clicking the [Paus Capture](#) button. The same number of images that are skipped during the paus can be used to prolong the experiment once it is restarted.



Stop capture

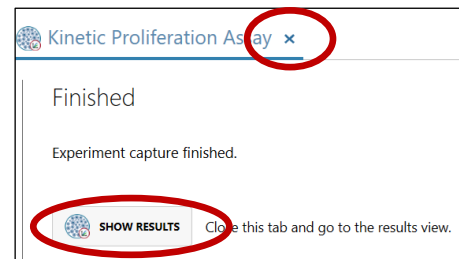
The capture can be interrupted by clicking the [Stop Capture](#) button in the upper left corner. The dialog box will appear to confirm the cancellation. The experiment will then be interrupted and closed.

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 Assays

The analysis of a guided experiment is automatic and starts when image capture begins. Hence, the results are ready for viewing even during capture. Experiment results are mainly presented as graphs and/or tables, 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.

The screenshot shows the HOLOMONITOR interface. On the left, the 'Experiments' tab is selected. A table lists experiments with columns for 'Experiment title', 'Database', and 'Time started'. One experiment, 'New Experiment', has a spinning red cog icon next to its title, indicating it is in progress. On the right, the 'EXPERIMENT SUMMARY' panel is visible for the selected experiment 'KA1 Motility L929 etoposide'. Below the summary, there are four buttons: 'OPEN', 'COPY', 'EXPORT...', and 'DELETE'. Callouts provide additional context: 'New Experiment in progress' points to the spinning cog, 'The Experiments tab' points to the 'Experiments' tab in the sidebar, and 'Open, Export, Copy or Delete Experiments' points to the four action buttons.

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

EXPERIMENT SUMMARY
KA1 Motility L929 etoposide
Kinetic Motility Assay

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

Description: L929 cells treated with 0.50 or 100 mikromolar etoposide
Cell type: L929
Vessel map: Sarstedt TC 96

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

Open Experiment results

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

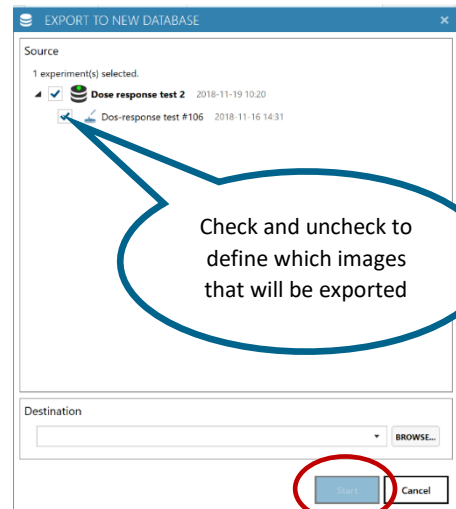
Copy an experiment

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

Export an experiment

Click **Export** (see image above) in order to 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. Right click the destination to create, name and thereafter select a new folder that will contain the exported experiment.
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

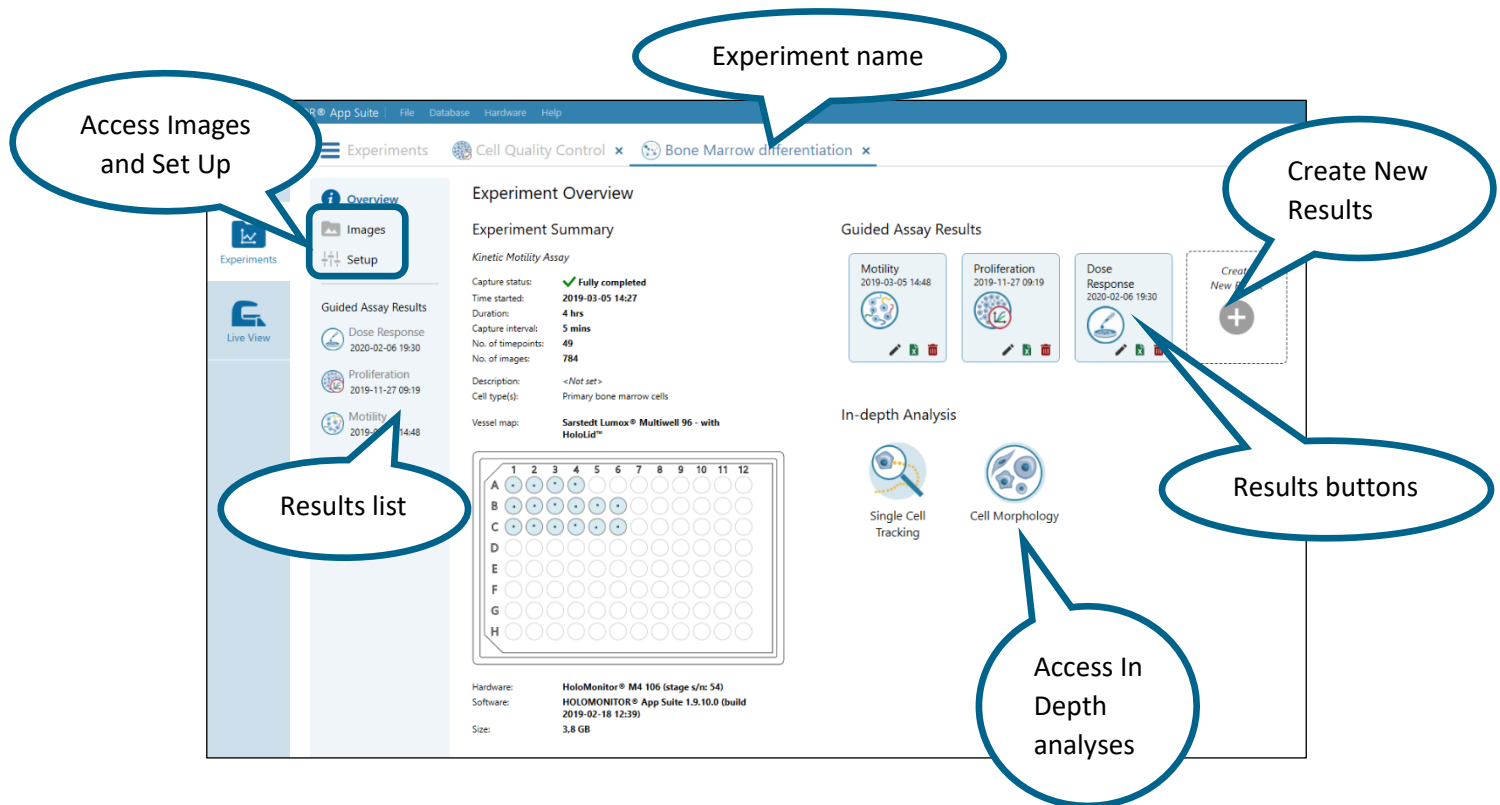
Click **Delete** (see image above) to delete the entire experiment with results, images and analyses.



This 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 Assay 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/>).

For further analysis, the results can be exported to Excel or similar software. There is a list of all presented variables in the **Analysis Features** section in the back of this manual.

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.



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.

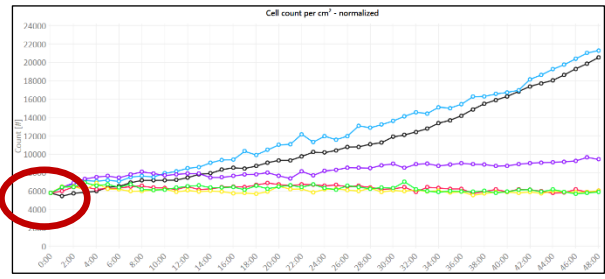
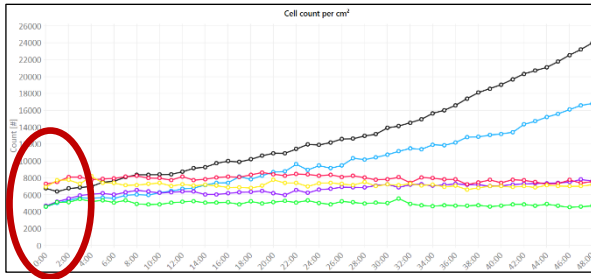


Image analysis

The basis for all results is the automatic image analysis, where cells are identified and counted. Also, the 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.

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

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

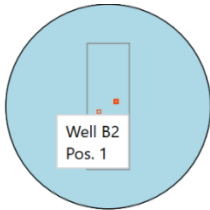
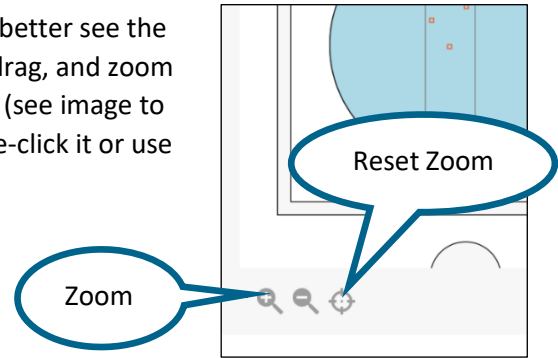
- Vessel Map:** A 4x6 grid of colored circles representing wells in a microplate.
- Capture Positions list:** A table listing individual image captures with columns for #, TIME, TREATMENT, CELL, POS., and SENS.
- Selected Image window:** A preview of a single image showing detected cells with blue and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

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

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.



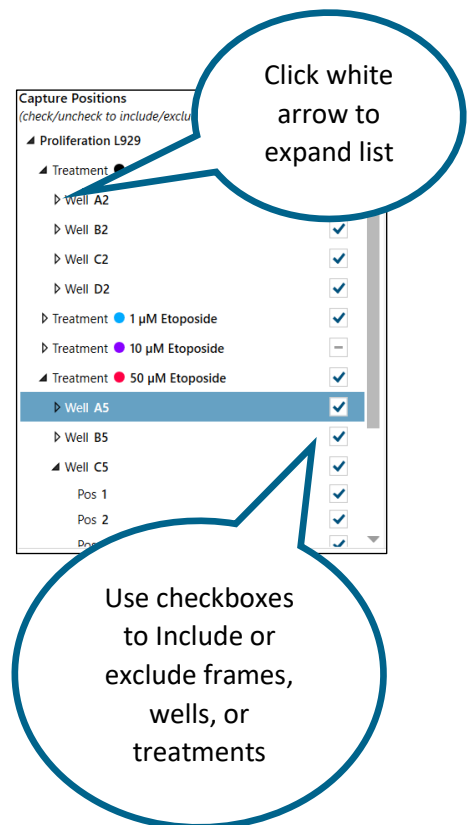
To see the position designation, hover the cursor above a position marker, in this case Well B2, Position 1 (see image to the left).

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.

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

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

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

405 images

The Position Images list

Images 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](#).

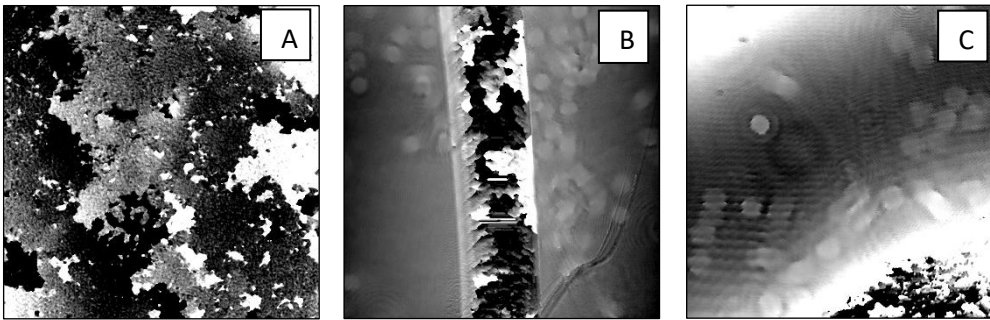
Image display functions are found below the [Image List](#).

- 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.

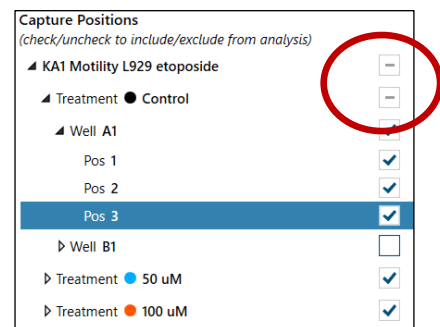


Sample examples of images with A. condensation, B. a scratch, and C. a corner of an air bubble, all of which cause insufficient image quality.

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).

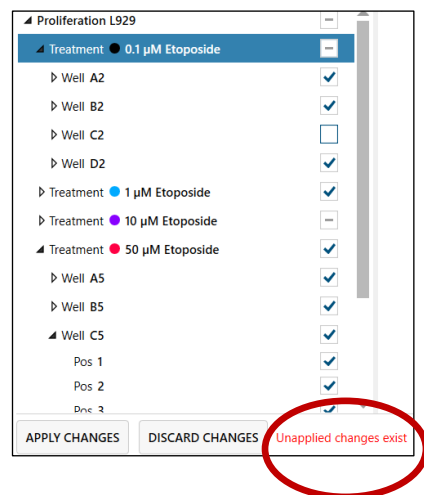


Apply changes

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.



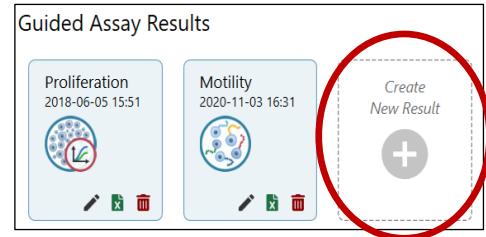
Changes made by checking and unchecking will not be saved and applied to the graphs and tables unless the [Apply Changes](#) button has been clicked.





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

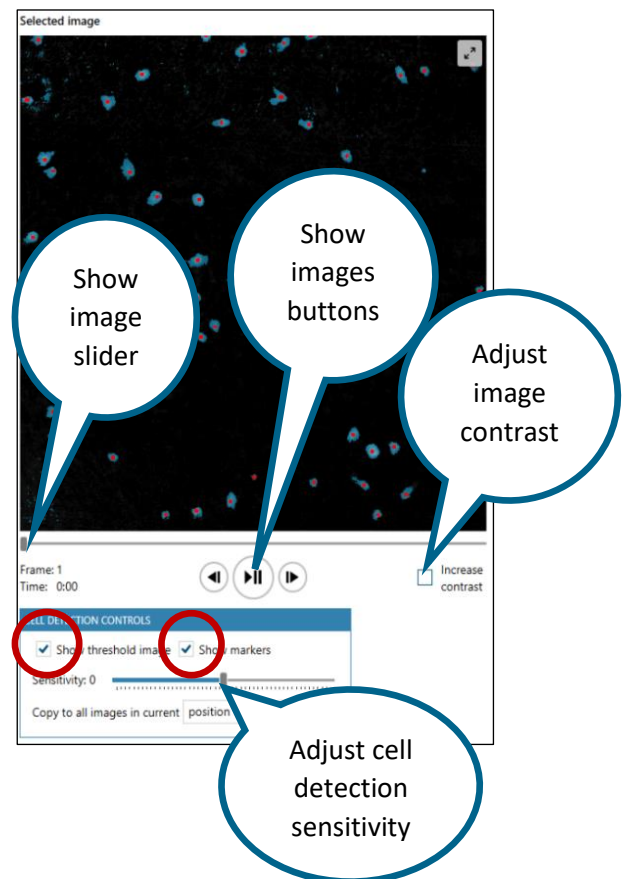
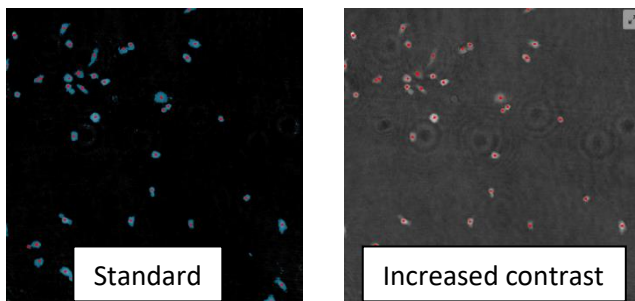
In order to keep the old results in parallel with new results, use the [Create New Results](#) button found in the [Experiment Overview](#) to create a parallel analysis (see [Reanalyze results](#) section for more information).



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).

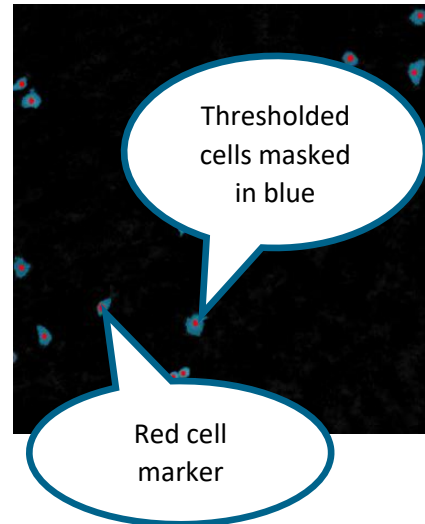
Checking [Increase Contrast](#) results in an image with a brighter background which sometimes allows the user to better distinguish the cells.



Automatic cell detection

The software automatically detects the cells in all images.

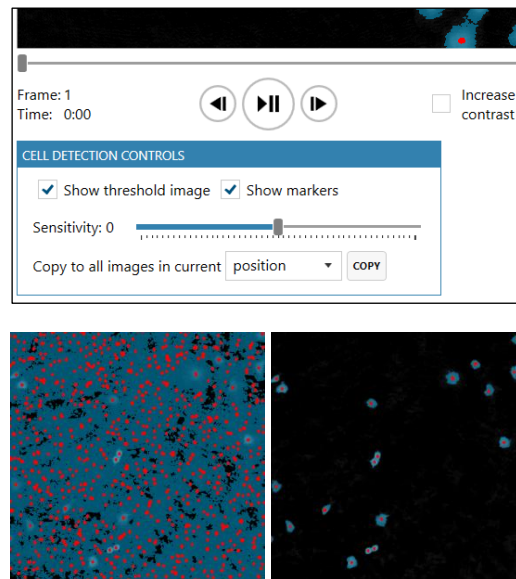
- The detection algorithm takes into consideration cell thickness, size and morphology to first separate the cells from the background, second split adjacent cells and third fine-tune the result.
- The algorithm identifies cells by setting a threshold that determines what is cell and what is background. 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 detection sensitivity

Usually, the cell detection algorithm identifies cells correctly, but if not, use the [Sensitivity slider](#) to perform a manual adjustment. To aid the adjustment, check [Show Threshold Image](#) and [Show Markers](#) in the [Cell Detection Controls](#) window. When a suitable sensitivity is found it is possible to copy the sensitivity value to all images in current:

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

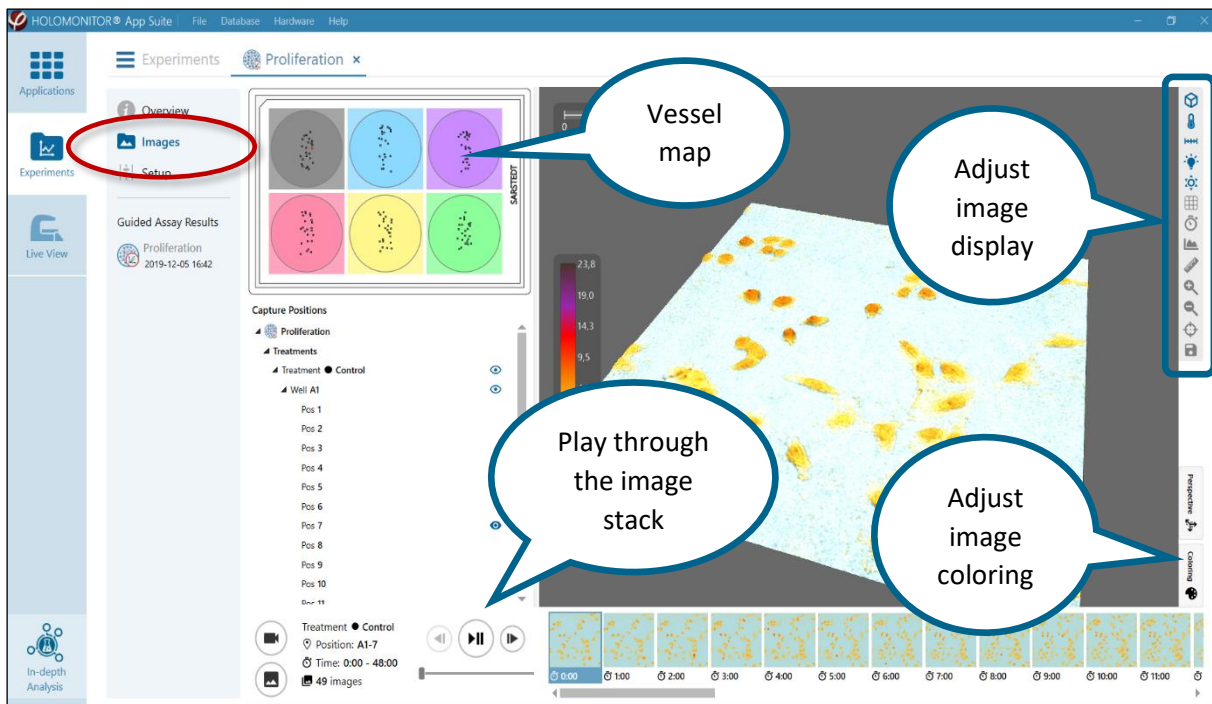


The examples show an image before (left) and after (right) threshold adjustment.

Images

View Images

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



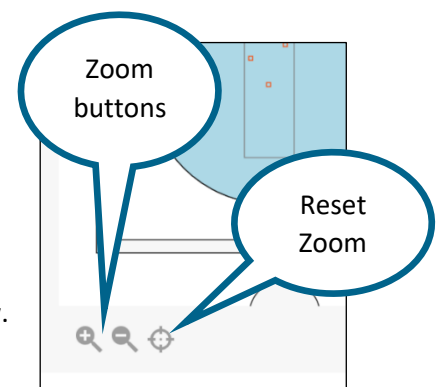
Move or zoom the captured image

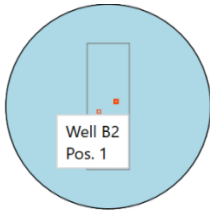
- To zoom the cell image, move the mouse cursor to the view area, click and scroll using the scroll button on the mouse.
- To move the cell image to a desired location, click, hold and drag the image using the left mouse button.
- To change viewing angle/perspective, click, hold and drag the image using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





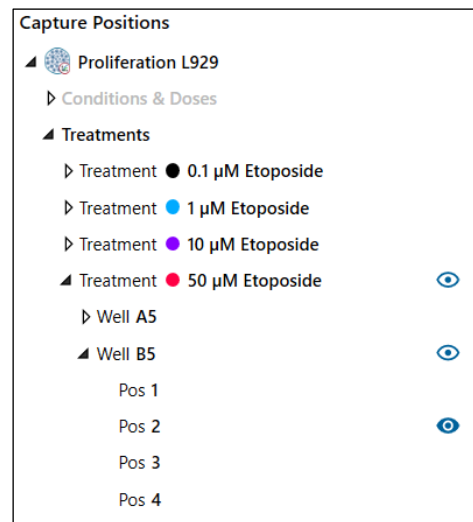
Hover the cursor above a position marker to see the designation of that position, in this case Well B2, Position 1 (see image to the left).


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 etc.



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

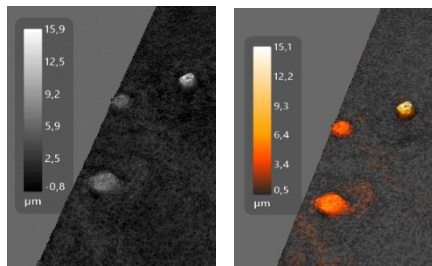
Images can be displayed by moving the [slider](#) below the selected images or by using the [play/pause](#) button or the [forwards](#) or [backwards](#) buttons below the selected image (see image to the right).

Adjusting image presentation

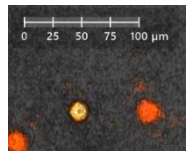
To the right of the 3D image view window there are several functions that adjust the presentation of the captured image (see image to the right). Blue functions are active while gray functions are inactive. All adjustments will be present in exported movies and images, when **As seen on screen** option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


















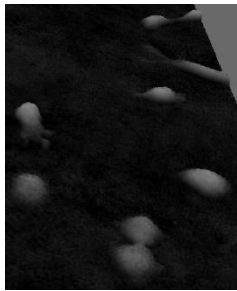
Show XY scale displays a horizontal scale bar representative of the distance in X and Y (See image to the right).



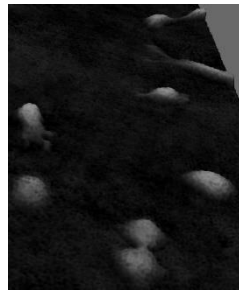
Enable Lighting applies an artificial light source to the image which may sometimes render an improved image (See images below).

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

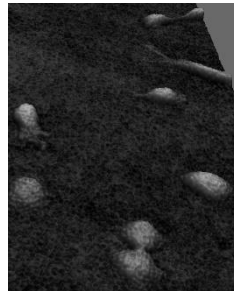
	View in 3D
	Show color bar
	Show XY scale
	Enable lighting
	Enable shiny surface
	Show background grid
	Show experiment time
	Show image histogram
	Line measure tool
	Zoom in
	Zoom out
	Reset image view
	Save view as image file
	Show perspective controls
	Show color controls



Standard image



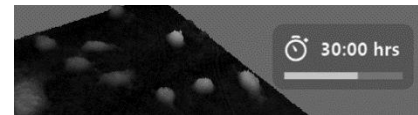
Enabled Lighting



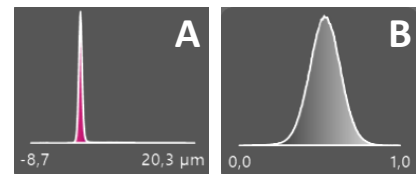
Enabled Lighting and Shiny surface

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).

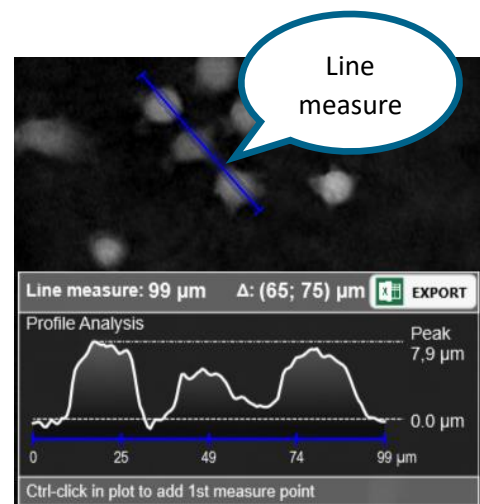


Show image histogram shows a histogram overlay of the pixel values in the image. For a phase image this equals a histogram of the optical thickness values (A) and for other image channels such as camera it shows a histogram of the pixel intensity values (B).



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.

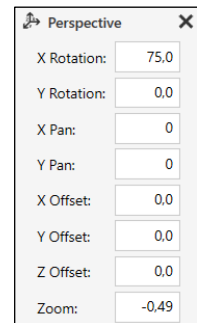


Zoom in and Zoom out will increase or decrease image view.

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.

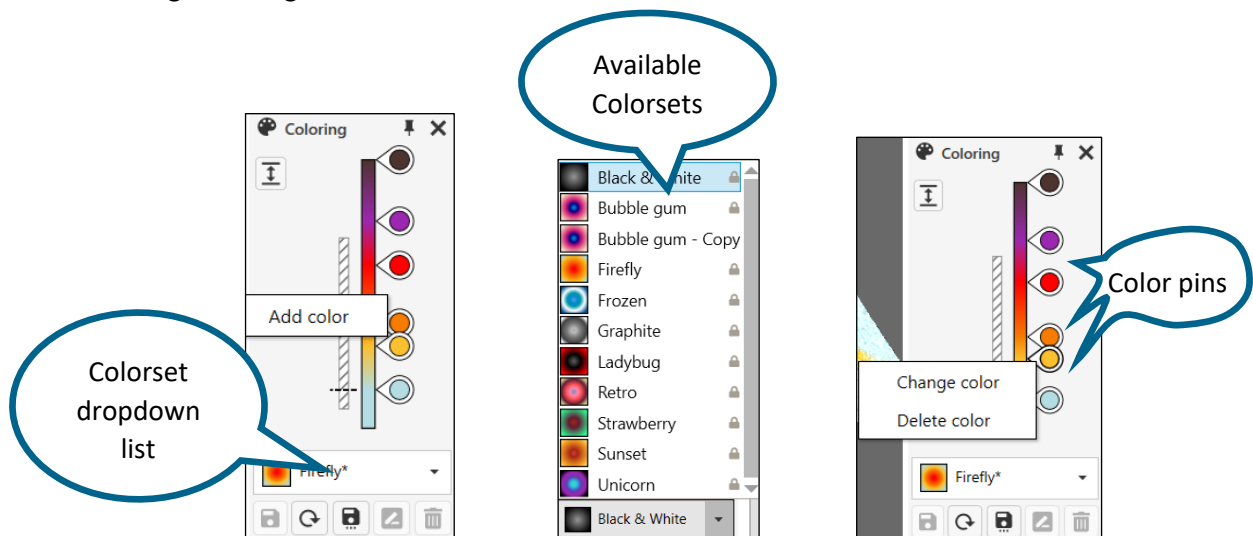


Coloring opens the **Coloring window**, which is used to adjust the color balance of the window and to color the image relatively to the cell optical thickness.

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:

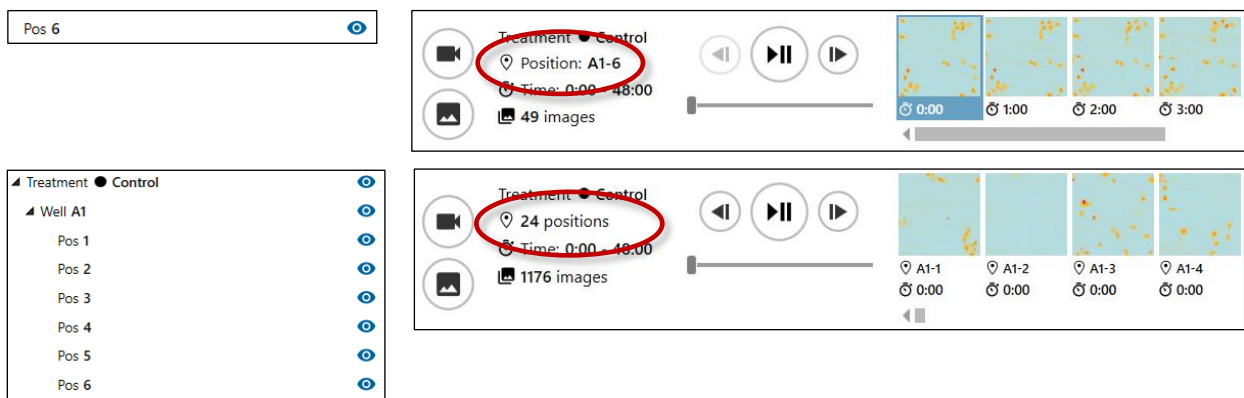
-  Save a new colorset
-  Go back to the last saved settings
-  Save the colorset to a new name
-  Rename the colorset
-  Delete the colorset

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.

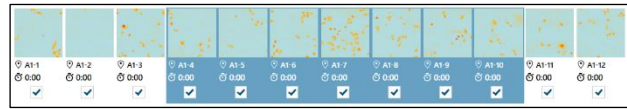


Check frames that should be included in the export. By default, all selected image frames are included. Unchecked frames will be excluded.

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



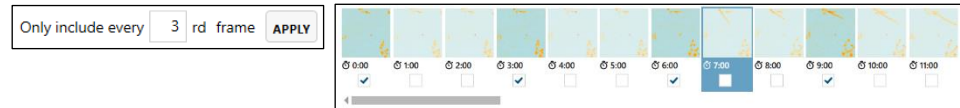
Remove selected frames will uncheck selected frames

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

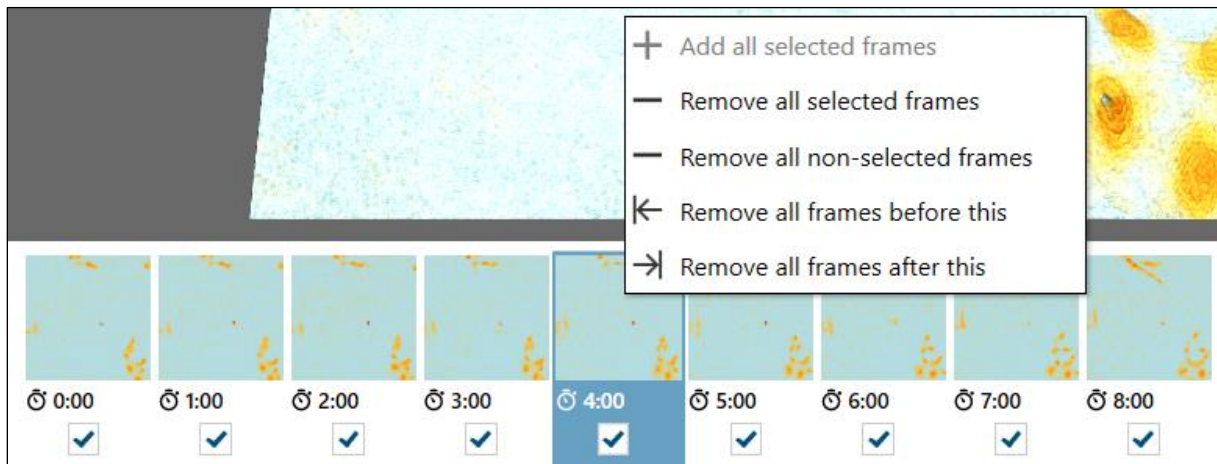
Remove all frames after will uncheck all frames after the currently selected frame



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



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



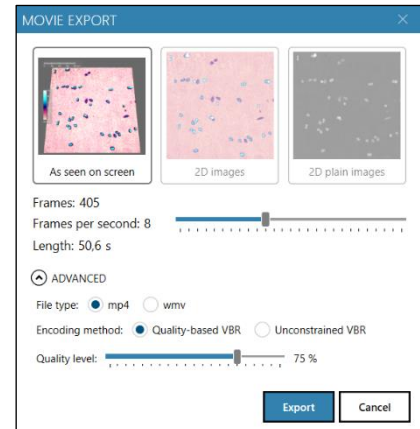
Export movie

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.

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.

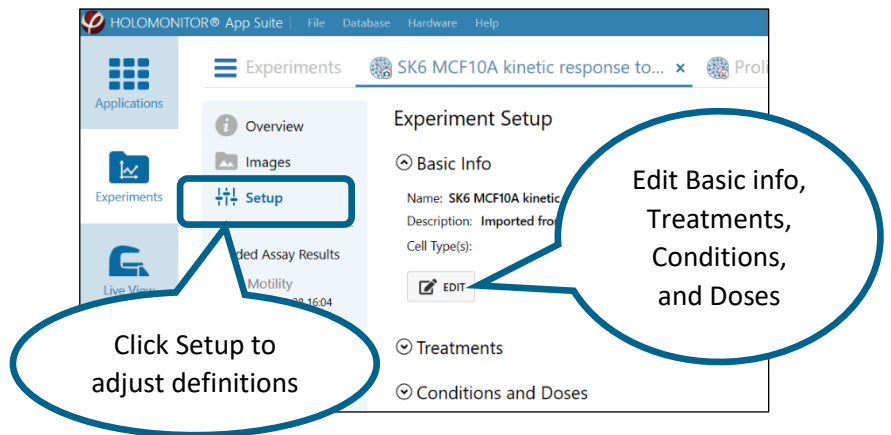
Selecting 2D Plain Images uses a 2D image with greyscale coloring adapted to each image.



Setup

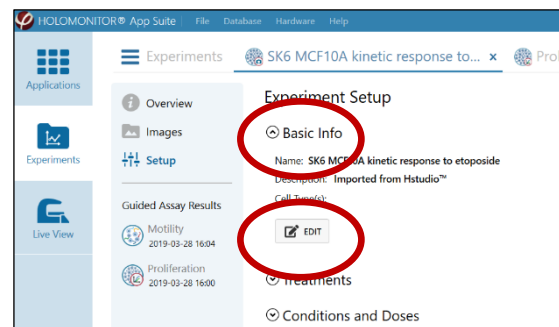
Viewing and Editing Experiment Setup, Treatments, Conditions, and Doses

All results are grouped and presented according to the treatments or conditions and doses that were defined when the experiment was set up. However, if needed, the definitions can be altered afterwards by clicking [Setup](#).



Basic Info

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

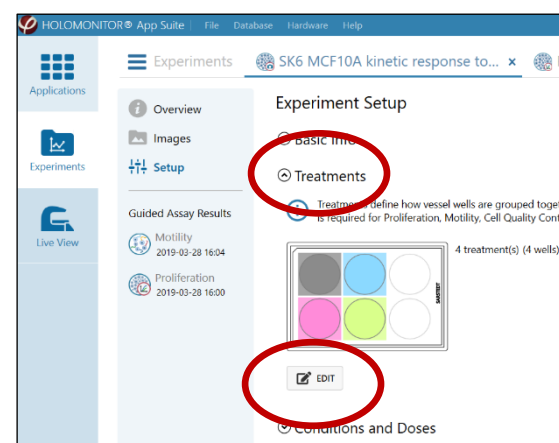


Treatments

To add or edit Treatments, click [Treatments](#) and then [Edit](#) to open the [Edit](#) window.

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

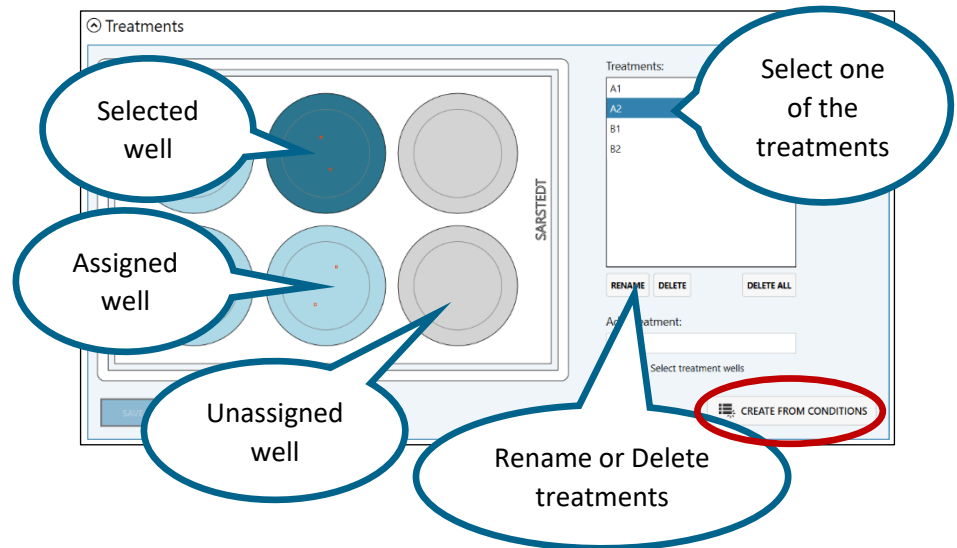
If e.g., a dose-response experiment is converted to a motility experiment, treatments can be added to the setup.



In order to **Add a new Treatment**, there must be unassigned wells (grey). Select 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.

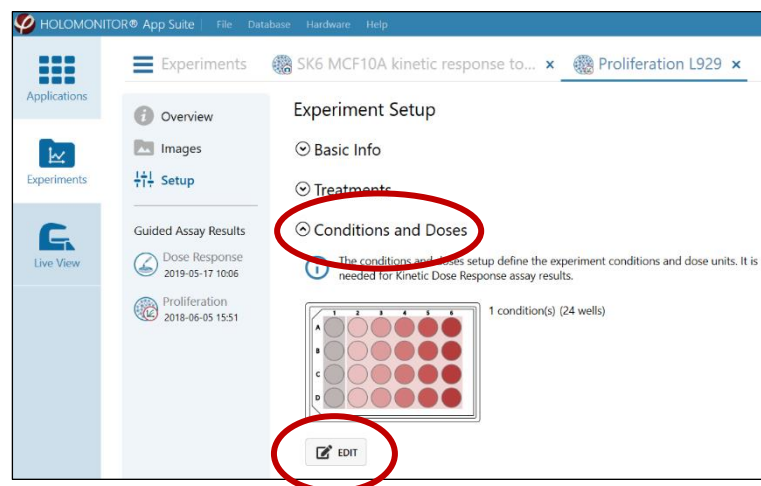


Editing treatments will cause previously saved settings and results to be deleted. In order to create parallel treatment set ups, first create a copy of the experiment.

Conditions and Doses

In **Conditions and Doses**, the conditions and doses for dose-response curves can be edited.

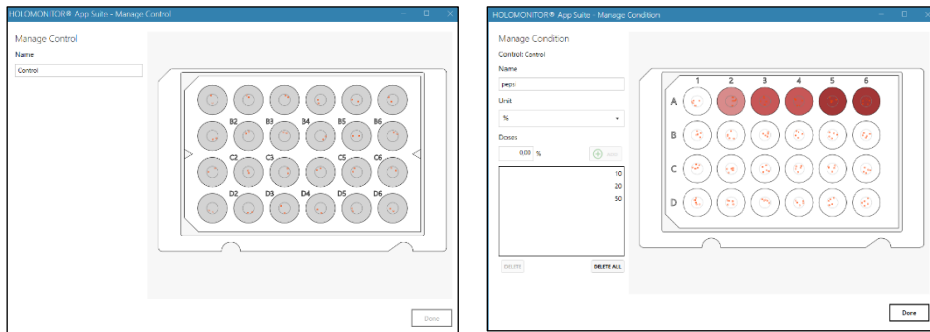
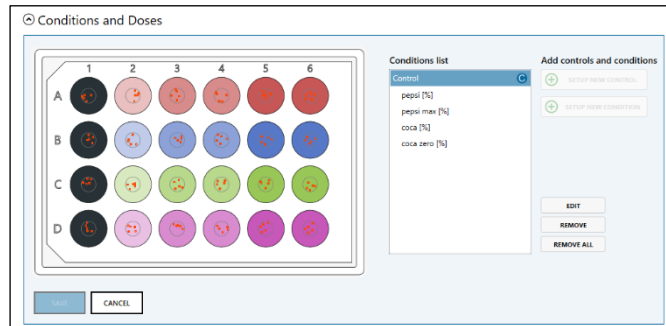
If an experiment was set up with treatments, e.g., a kinetic proliferation experiment, **Conditions and doses** can be added to create a dose response curve.



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.

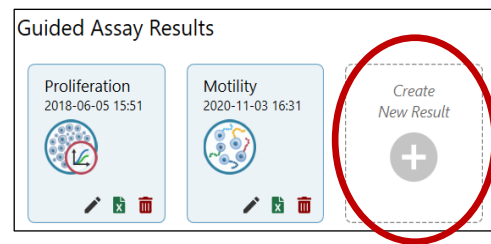


Editing conditions will cause previously saved settings and results to be deleted. To create parallel treatment setups, first create a copy of the experiment.

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

Create New Result

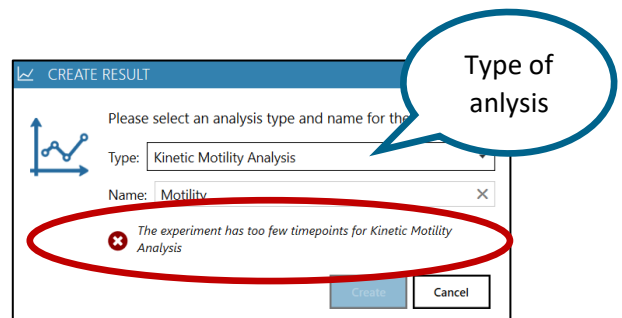
Experiments can be utilized for more than one analysis of the same type of results, or for more than one type of results. E.g. a proliferation assay can be reanalyzed for proliferation with results from certain wells excluded, or the proliferation data can be used to create a dose response curve if there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

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



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

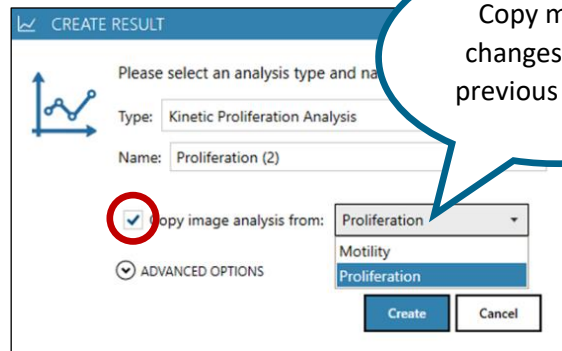


An experiment set up for dose response can be reanalyzed as e.g. a proliferation assay, but then conditions must be converted to treatments and vice versa. In that case, go to [Setup](#) and [Edit treatment](#) or [conditions](#).

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.



CREATE RESULT

Please select an analysis type and name for the new result.

Type: Kinetic Proliferation Analysis

Name: Proliferation (2)

Copy image analysis from: Proliferation

ADVANCED OPTIONS

Use results cache

Use image quality cache

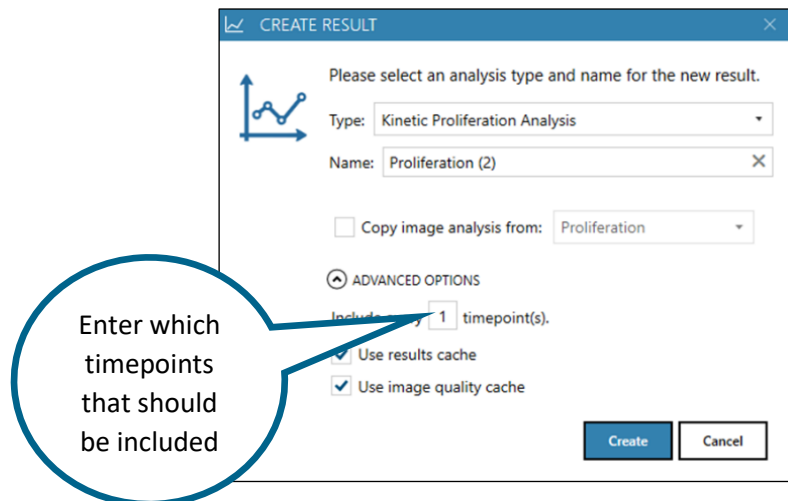
Create Cancel



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.



CREATE RESULT

Please select an analysis type and name for the new result.

Type: Kinetic Proliferation Analysis

Name: Proliferation (2)

Copy image analysis from: Proliferation

ADVANCED OPTIONS

Include: 1 timepoint(s).

Use results cache

Use image quality cache

Create Cancel

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.

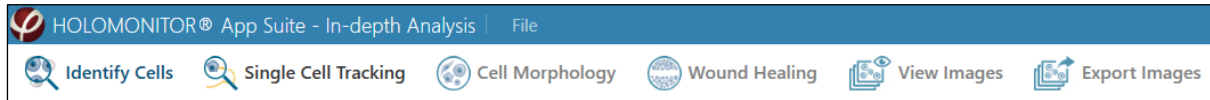


Not using the **Results Cache** and previous **Image Quality** calculations requires a lot of computing power and time. Depending on the experiment size and computer it can take from several minutes to several hours.

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.

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

View area and side panels

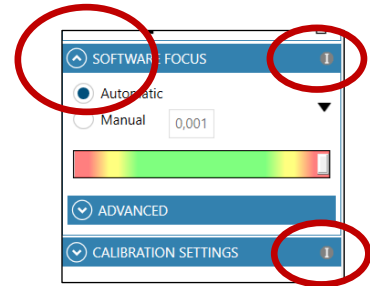
The **View area** is the main viewing area where cell images and cell data are shown. Basic functions controlling the **View area** are found in the **Side panels** to either side.



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.



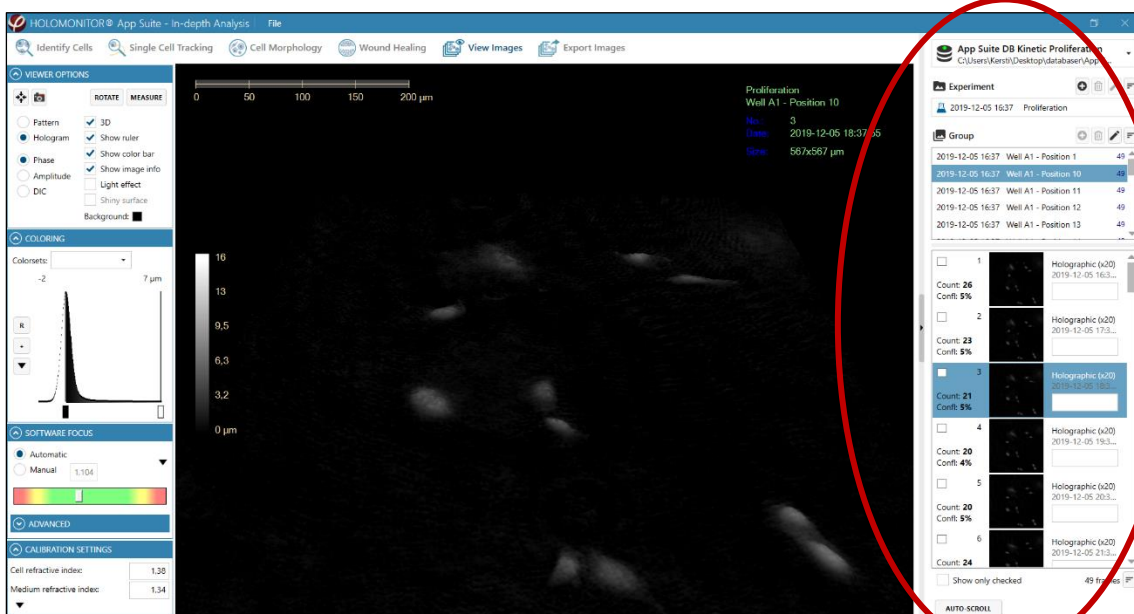
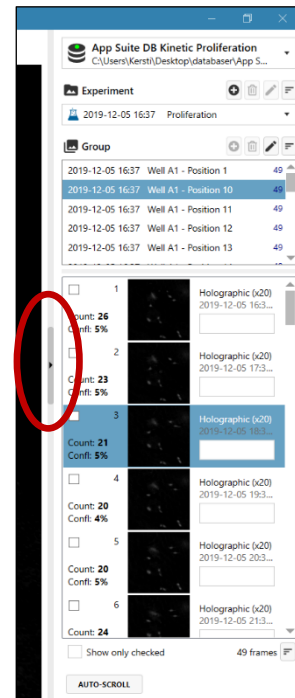
The right-side panel can be collapsed or expanded by clicking the little grey area with an arrowhead.

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.

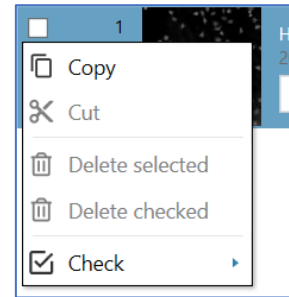
For data captured with App Suite, a Group comprises images from one position in a time-lapse sequence. For imported Hstudio data, a Group may also comprise images captured at various cell culture positions.



Copy, Cut or Delete frames



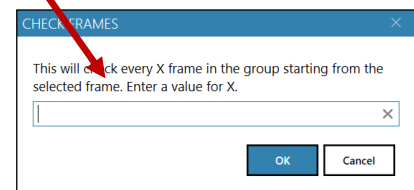
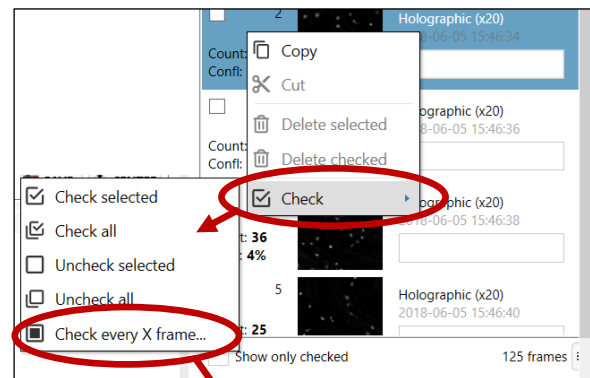
Copy, Cut or Delete Image Frames, work only with Hstudio data.



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.

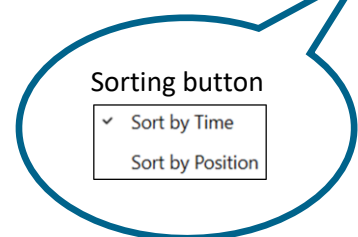
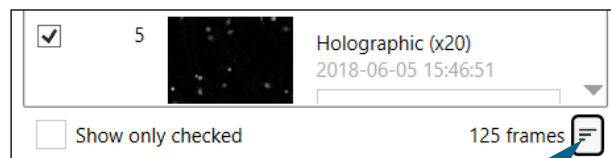
The Check every X frame is especially useful tool in combination with the Show only Checked function which is found below the Image Frame list.



The sorting button

The Image Frame list can be sorted either based on image frame number or on time of capture.

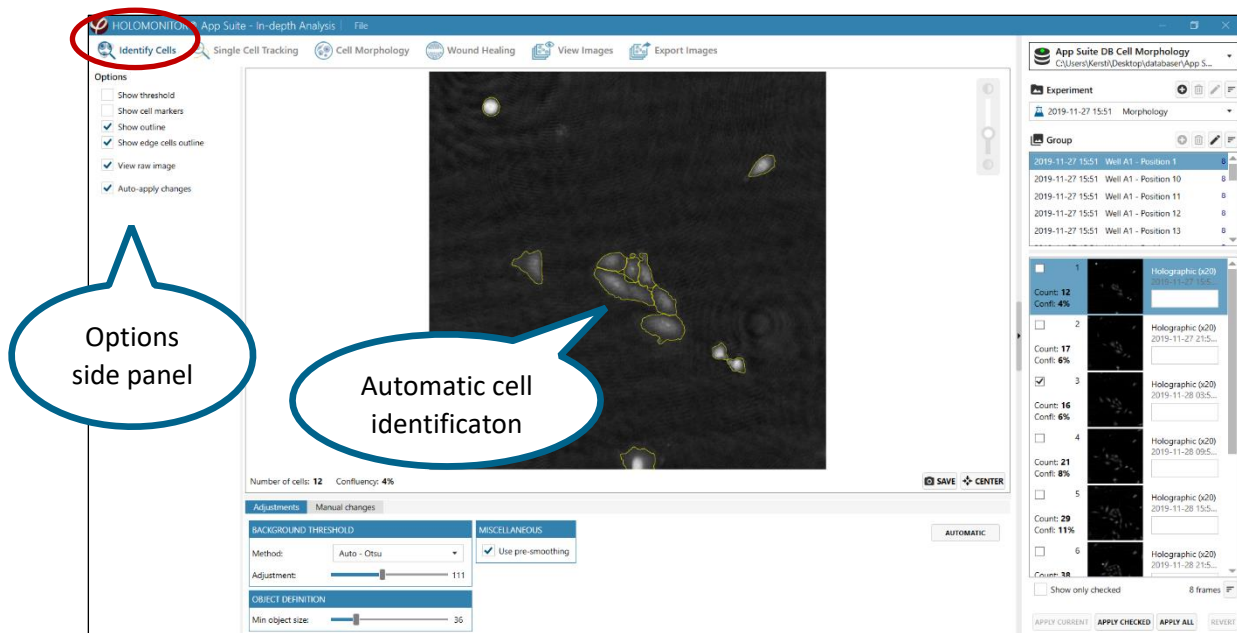
This function is mainly useful for Hstudio data, as App Suite data is already sorted by time and only shows one position.



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.

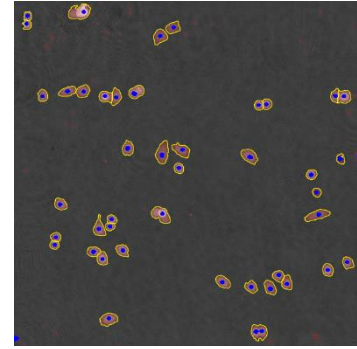
View raw image displays the image without any adjustments to the coloring.

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

Cell Identification Adjustments

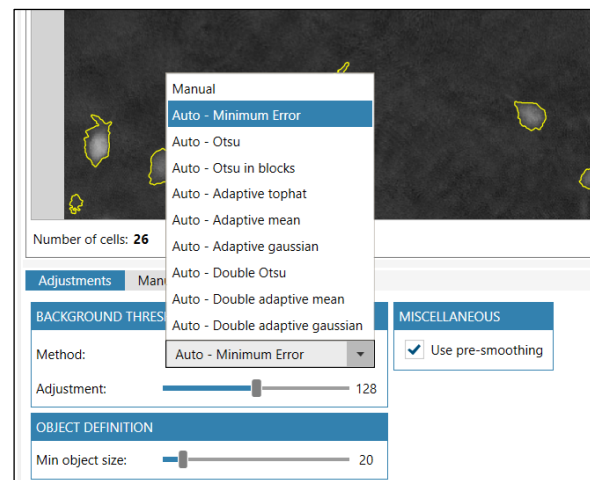
These adjustments encompass the entire frame and can be applied to other frames. The **cell identification adjustments** are performed in three steps:

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-like objects.

Adaptive mean sets an adaptive threshold using a mean filter.

Adaptive gaussian sets an adaptive threshold using a Gaussian filter.

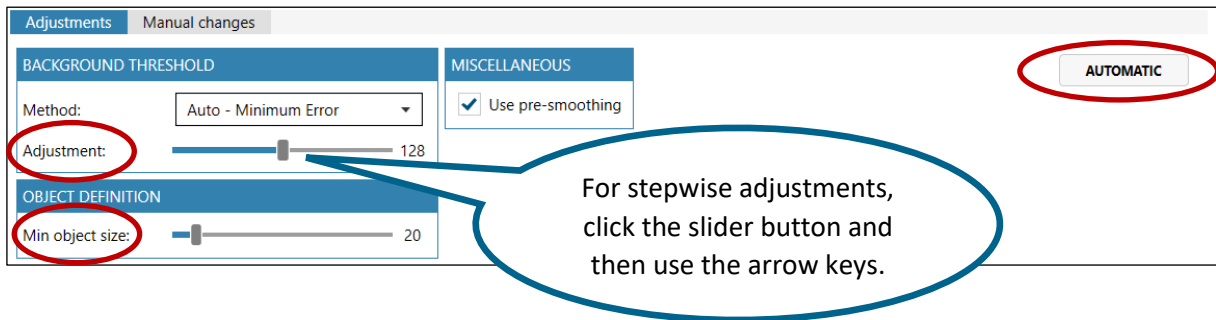
Double Otsu is a threshold method where both a wide and a narrow threshold mask are used. The narrow image is morphologically reconstructed under the wide image. The final image is used as threshold mask. The result is a cleaner threshold mask. Double Otsu uses double thresholding with Otsu global threshold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

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

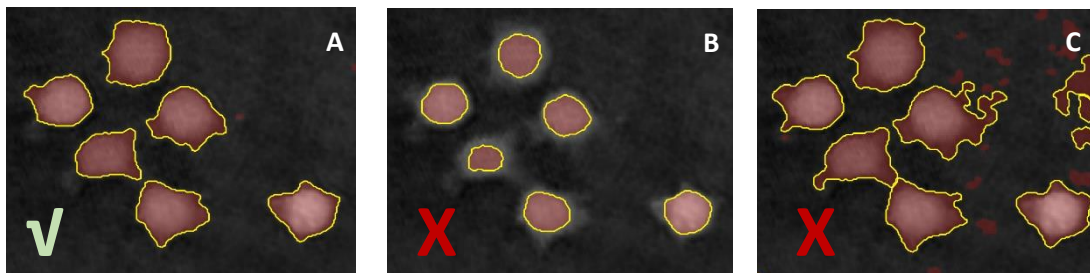
Double adaptive Gaussian is the same as Double Otsu, but with two adaptive Gaussian threshold masks.

2. Adjusting background threshold

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



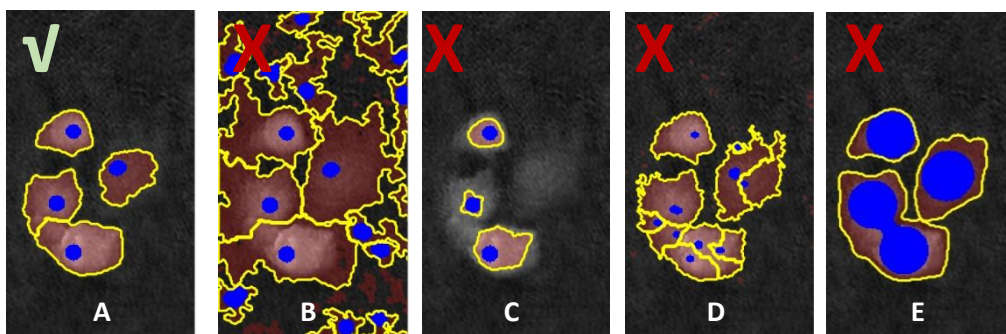
Adjusting the threshold between cell and background with the slider will adjust the minimum optical thickness of objects considered to be cells. Image A shows correctly thresholded cells, image B cells with a too high threshold, and Image C cells with a too low threshold.



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

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

Common cell identification problems



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

Pre-smoothing

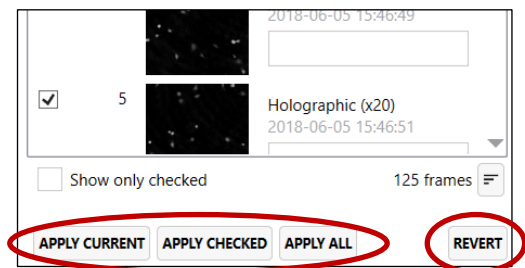
Activate [Use pre-smoothing](#) to smooth the cell edges.

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.



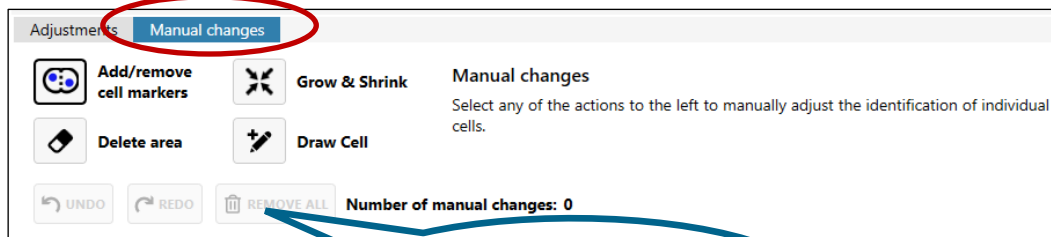
Revert Cell identification adjustments

Use the [Revert](#) button to revert any changes back to saved settings for the current frame.

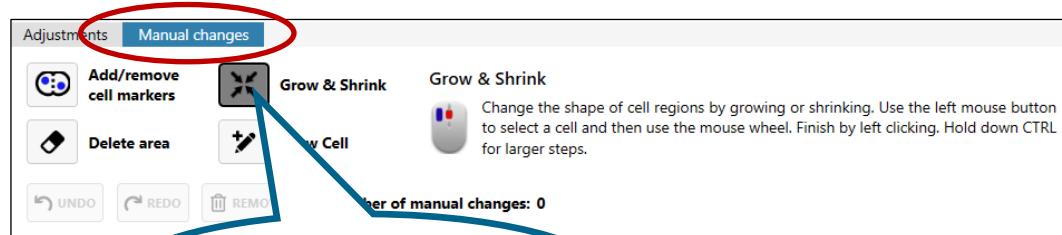
Manual identification adjustments for individual cells

Use the [Manual changes](#) tab, below the [View area](#), to manually adjust the 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.



Use these buttons to edit the manual changes



Activated function with corresponding description

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

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

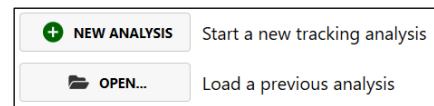
Start a new tracking

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.



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



Add image frames to the analysis

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

Image selection

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

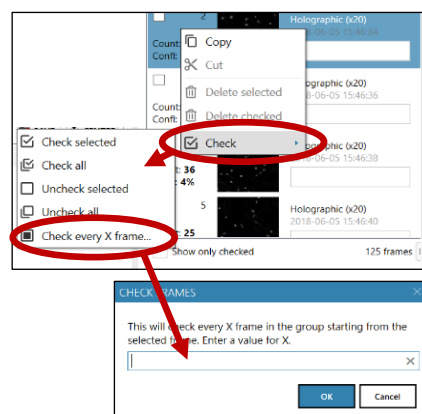
Image sorting

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

Check options

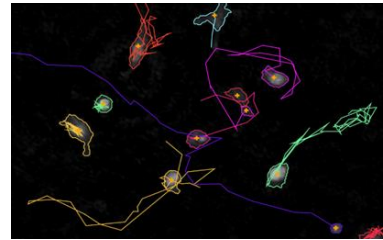
Access the **Check** options by right clicking in the **Image Frame list** (see image to the right).

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.

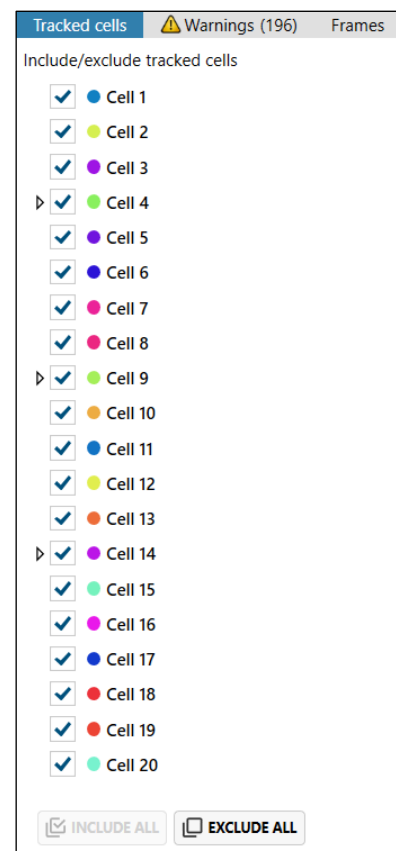
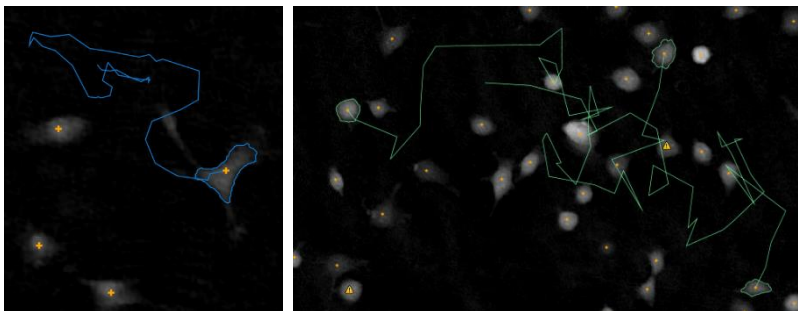
Use the **checkboxes** to **hide** or **shorten** the cell trails and to **hide the cell outlines**.

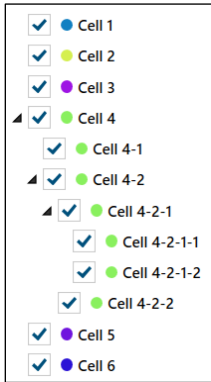


List of tracked cells

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, e.g., an individual cell or cell family can be shown.





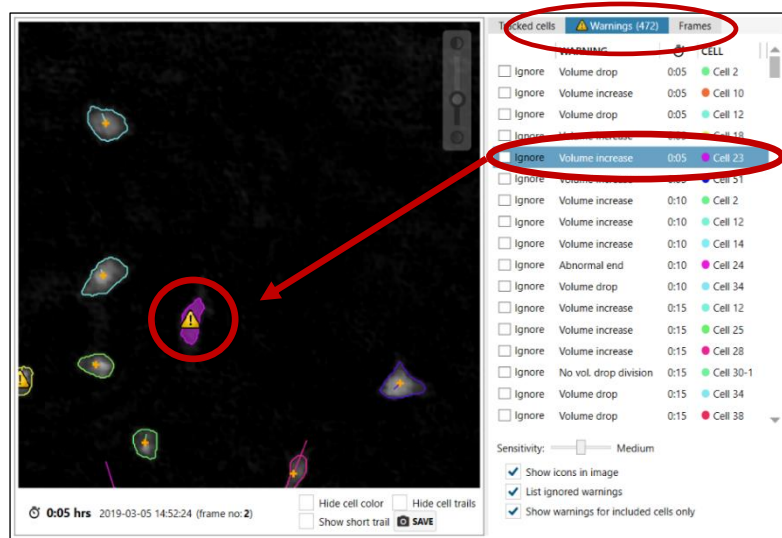
Cell families

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.

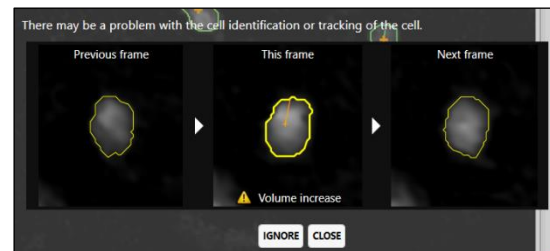
Cell tracking warnings

When the cell volume deviates 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 just do nothing with them. They will not e.g., 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**.

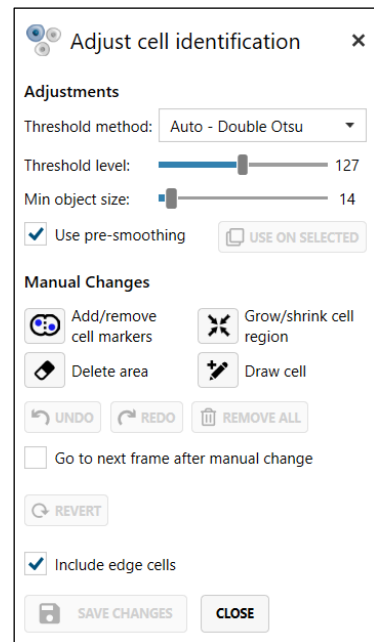
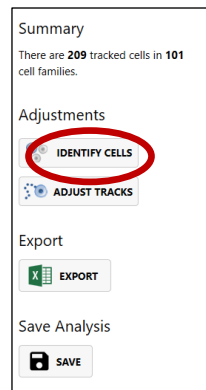
Adjust cell identification

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. Then click on [Move Track](#), and thereafter click the cell to which tracking should be transferred.

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

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

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

[Add to tracks](#) is used to add cells to an existing track. The cells are added frame by frame. Start by selecting a cell and click the [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.

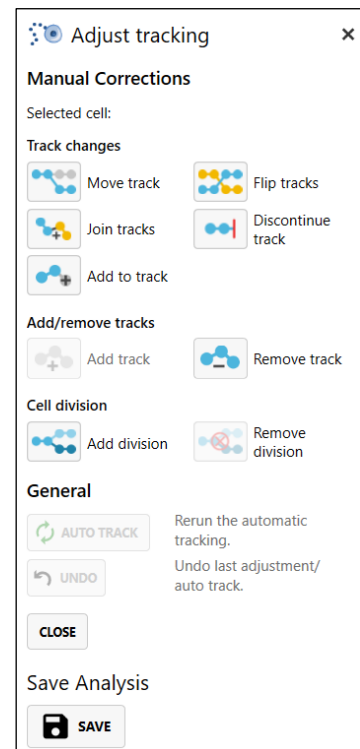
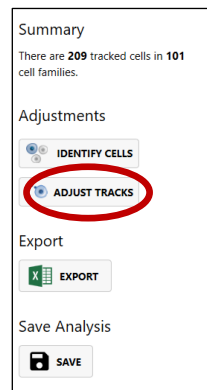
[Remove track](#) is used to remove an entire track. Select a cell and click the [Remove track](#) button.

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

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

[Auto track](#) is used to re-run the automatic tracking while incorporating all manual changes to, e.g., divisions and removals. 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.

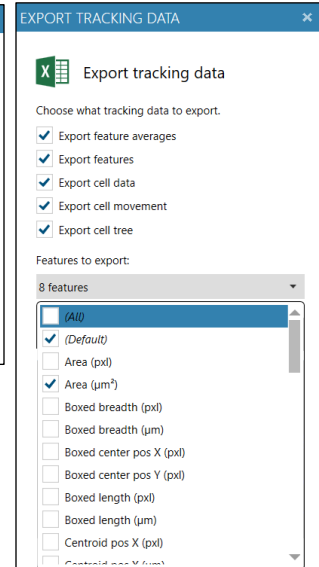
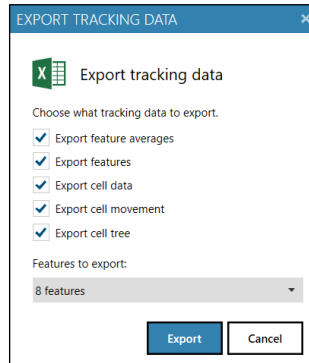


Export

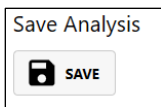
Click the **Export** button to export the tracking data.

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.

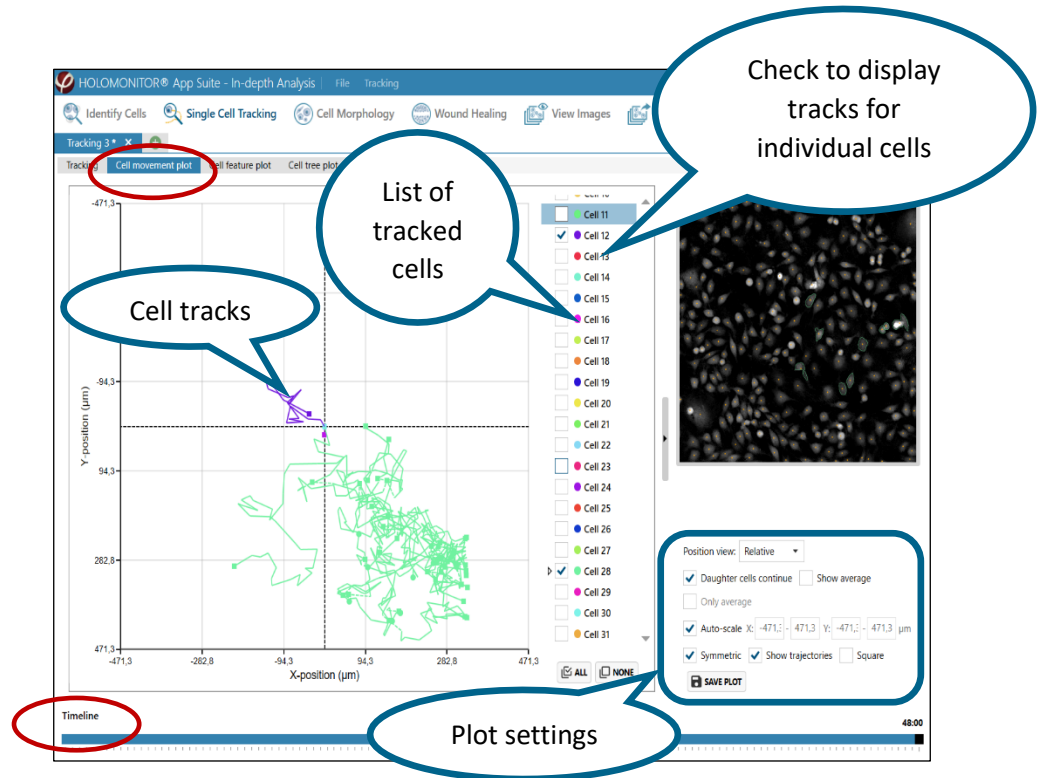


Cell numbers might change in the re-opened tracking file.

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.



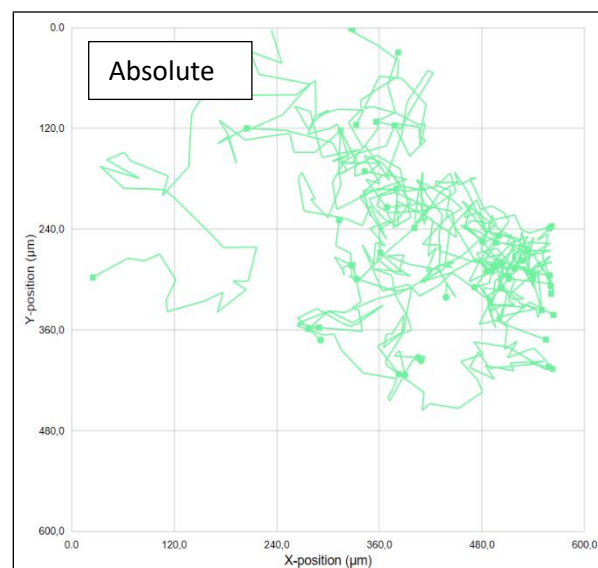
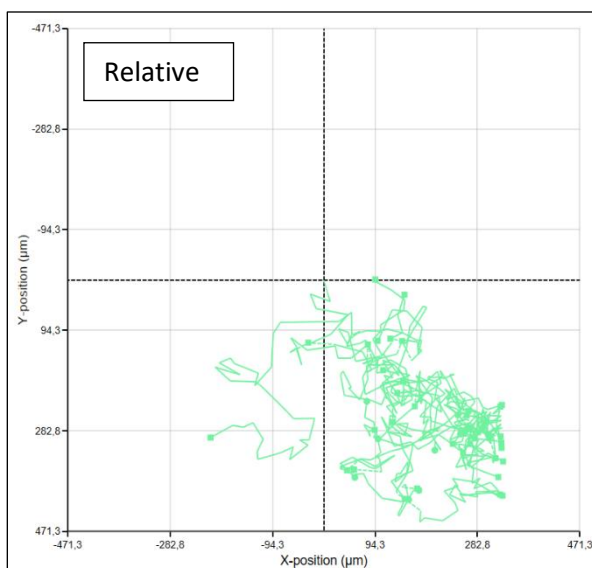
Plot settings

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 (X=0, Y=0) for cell movement. With **Absolute** view, the cell movements are shown with the same coordinates as in the image frame.

The screenshot shows the Plot settings panel. It includes a 'Position view' dropdown set to 'Relative'. There are several checkboxes: 'Daughter cells continue' (checked), 'Show average' (unchecked), 'Only average' (unchecked), 'Auto-scale X' (checked), 'Symmetric' (checked), 'Show trajectories' (checked), and 'Square' (unchecked). There is also a 'SAVE PLOT' button.



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

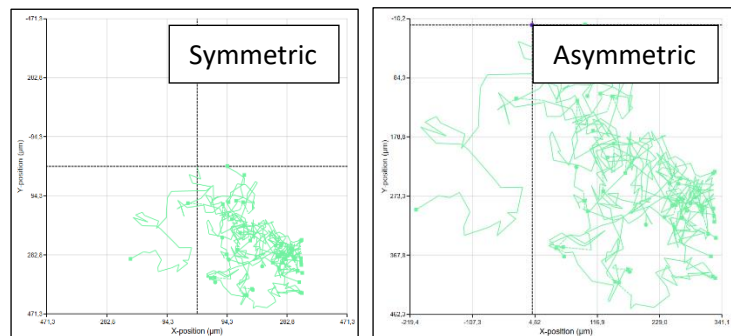
With only average checked, all other tracks will be hidden.

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.

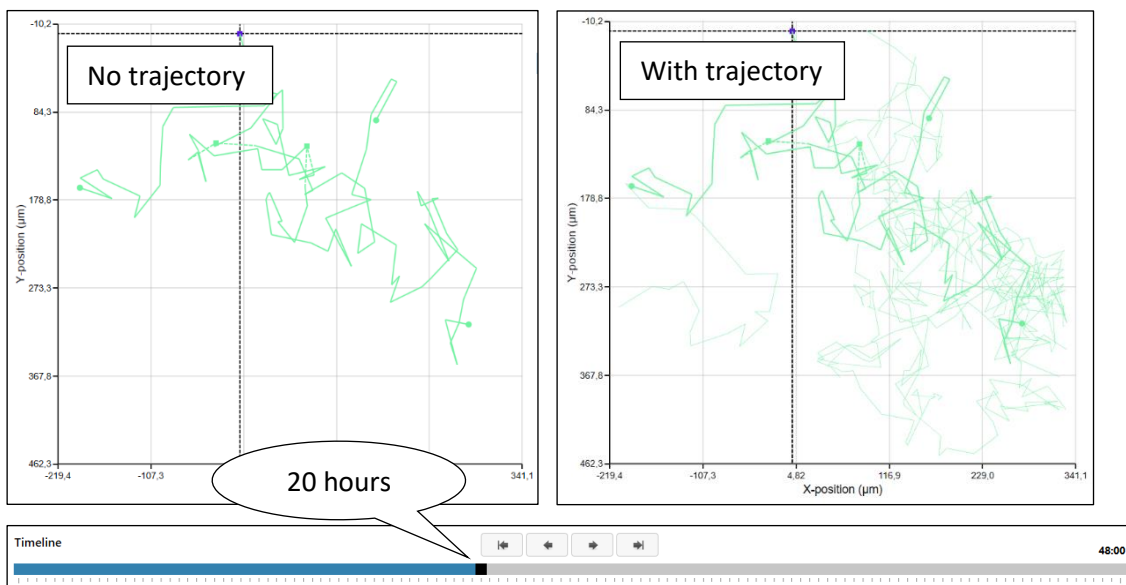
Symmetric

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

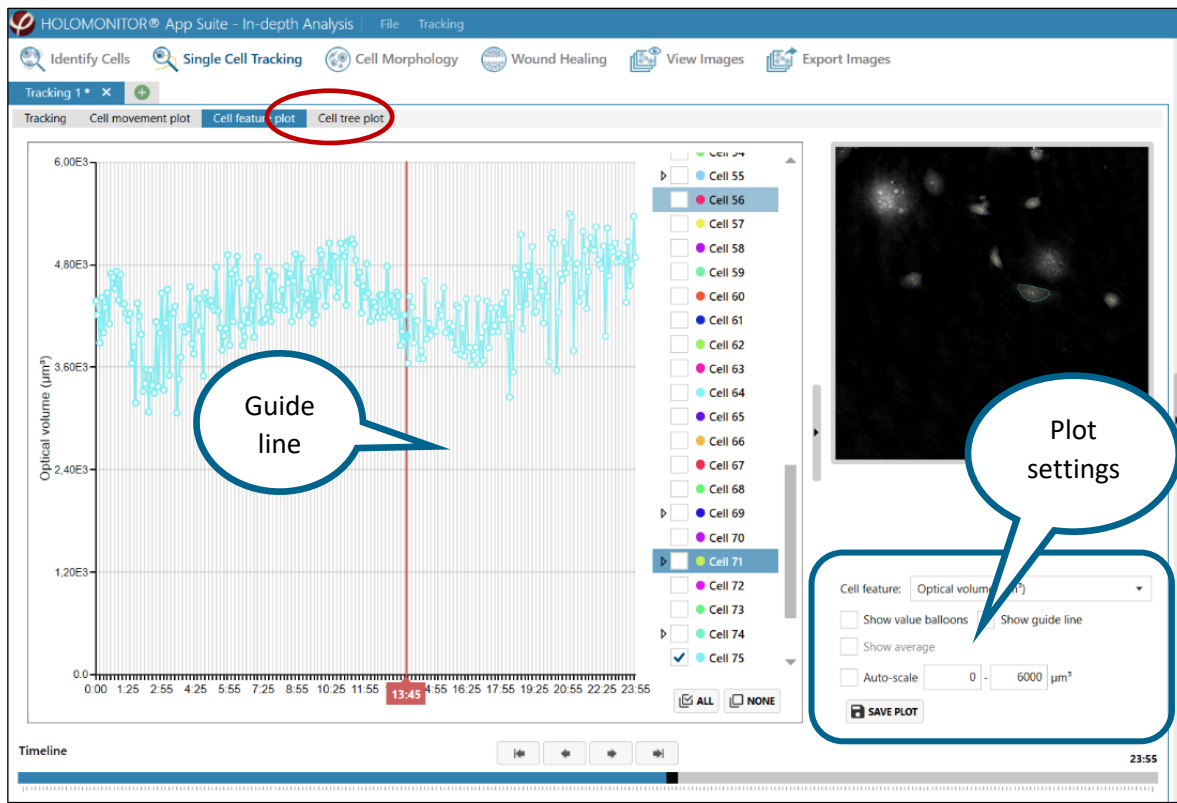
Checking **Square** shows a plot that is square, meaning X and Y axis min-max values are the same.

Save plot

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

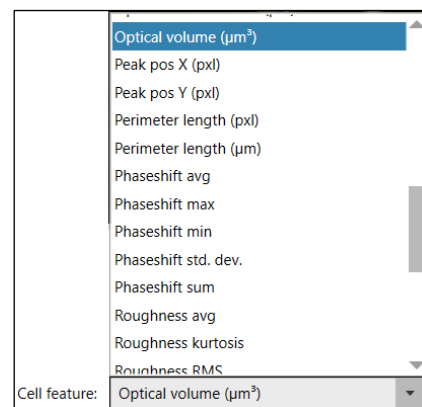
Show value balloons displays the value at the current time-point.

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, bitmap, or .gif format.



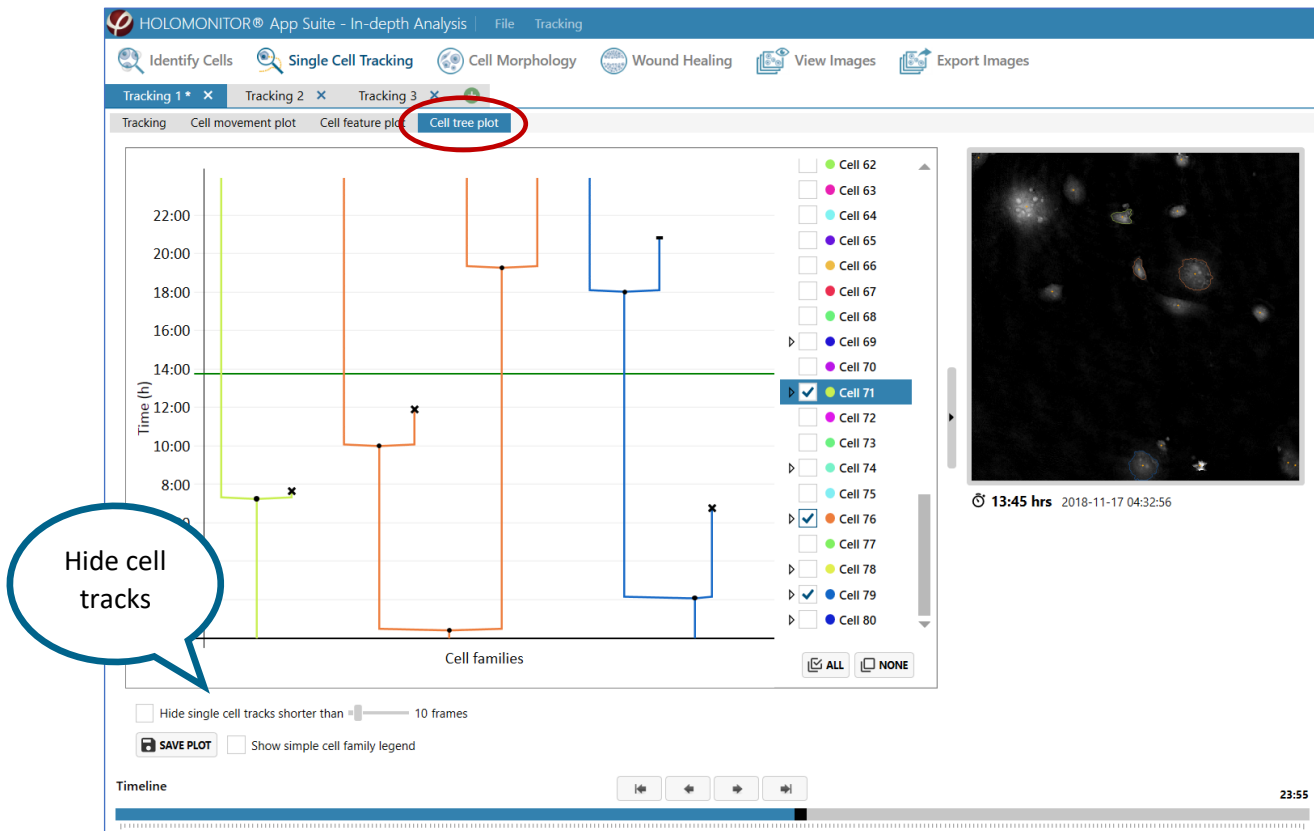
Cell tree analysis

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

- + symbolizes that the tracking of the cell ends. May be caused by, e.g., cell death or cell engulfment.
- symbolizes that the cell is traveling out of the frame, and track is therefore discontinued
- symbolizes cell division

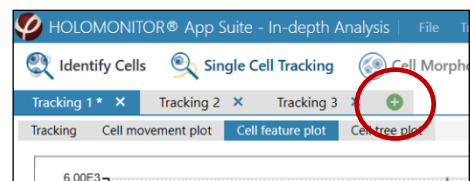
In the tracked cell list, cell families are numbered and color-coded for easy identification. When Cell-4 divided, the daughter cells were named Cell 4-1 and Cell 4-2. When Cell 4-1 divided, the daughter cells were named Cell 4-1-1 and Cell 4-1-2 and so on. All the cells in a family have the same color.

There is a possibility to hide cell tracks which are present during less than 2-100 frames.



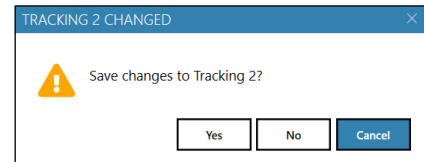
Multiple analyses

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

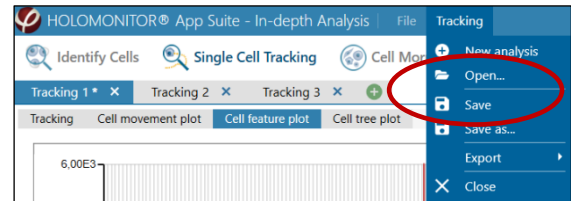


Save or open tracking analyses

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



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



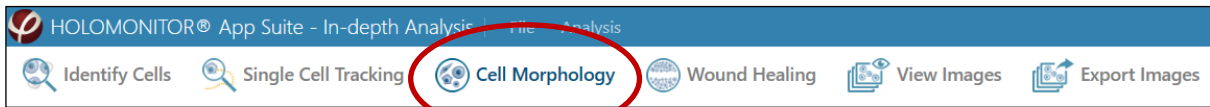
The analyses are saved as separate files to the computer. They are not integral parts of the software and need to be backed up separately.



In the saved analysis cell family selections are not saved – all analyses for all tracks/cell families will be saved.

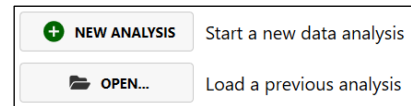
Cell Morphology

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



Start a new analysis

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



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, and then it is time saving to analyze only the frames that are needed. The frames can be chosen by selecting, sorting or checking options.

Image selection

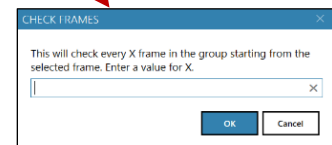
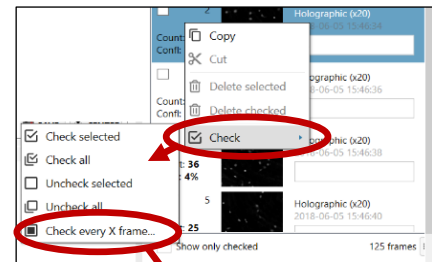
Several images can be added simultaneously if they are all selected. The SHIFT key can be used to select several consecutive images and the CTRL key to select non-consecutive image frames.

Image sorting

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

Check options

The **Check options** can be used to analyze subgroups of images. 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 other frames is displayed when the frame is selected in the Source frame list.

Add data from different experiments or treatments

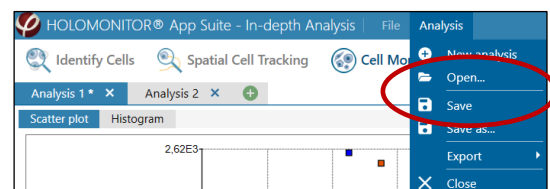
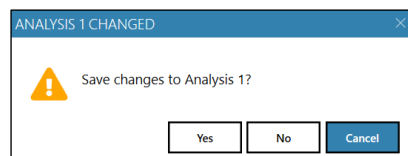
Data from different experiments and treatments can be added to the same analysis if they are stored in the same database.

Multiple analyses in parallel

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

Save or open morphology analyses

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



There is a **Save** option in the top menu as well, and there it is possible to open saved analyses.

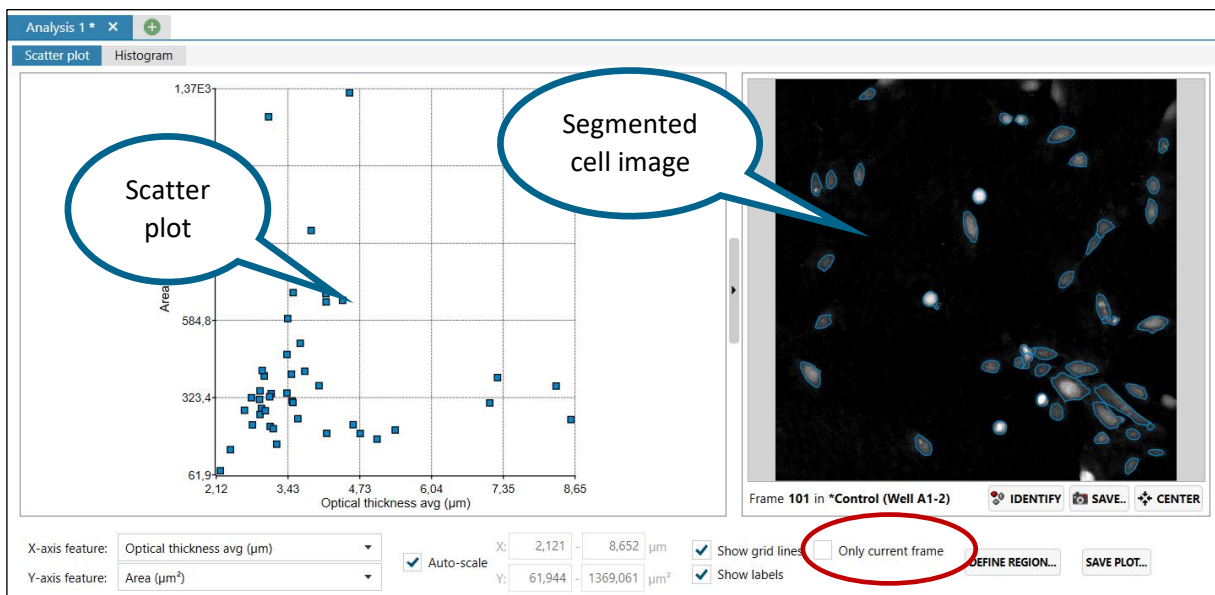


The analyses are saved as files to the computer, and are not integral parts of the software and need to be backed up separately.

Display results as scatter plot

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

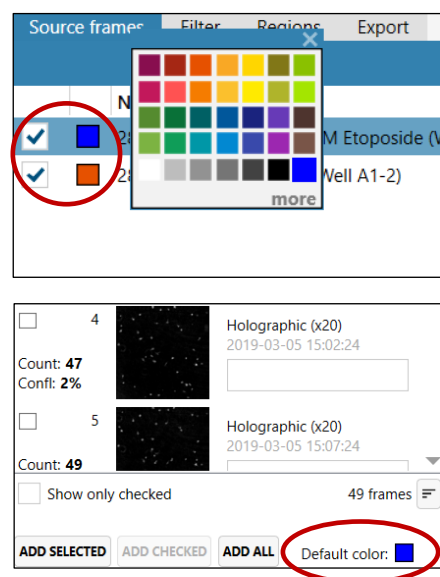
If several frames are represented in the plot, the data points from all images will be displayed in the plot simultaneously, while the frame that is selected in the **Source frames** table is displayed in the viewer.



Data point coloring

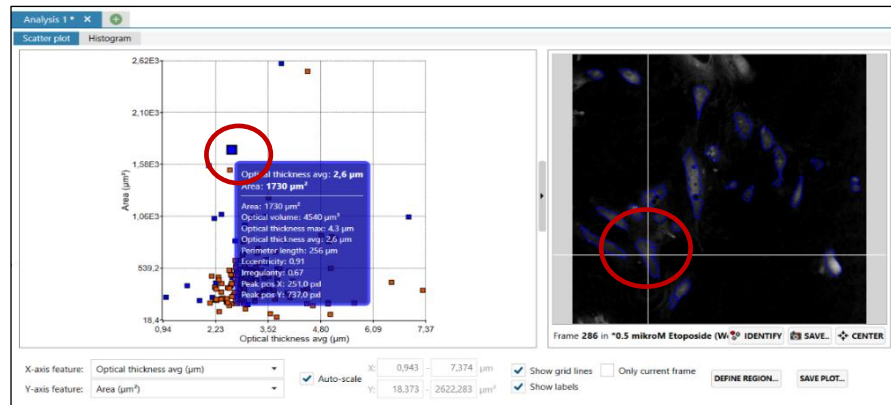
The data points will be displayed in the plot with the color shown in the color box in the **Source frames** table, found below the **Scatter plot** tab. By default, all data points from all frames are set to be shown in blue. Thus, to separate between frames and/or time points and/or treatments/conditions, 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.

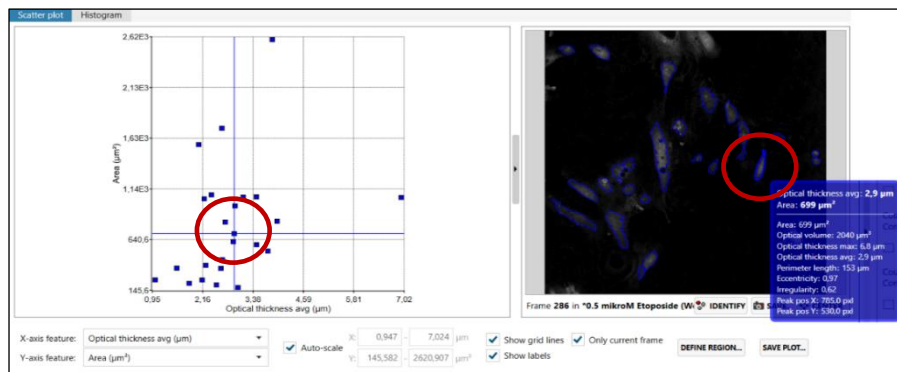


Identify which data point corresponds to which cell

Hover with 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.

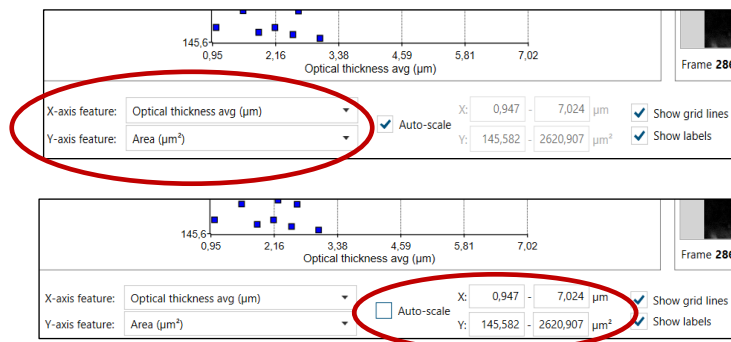


It works the other way around as well. Hover over a certain cell in an added image frame to identify the corresponding data point by a crosshair. Cell feature information for that cell will be displayed in a box.



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.

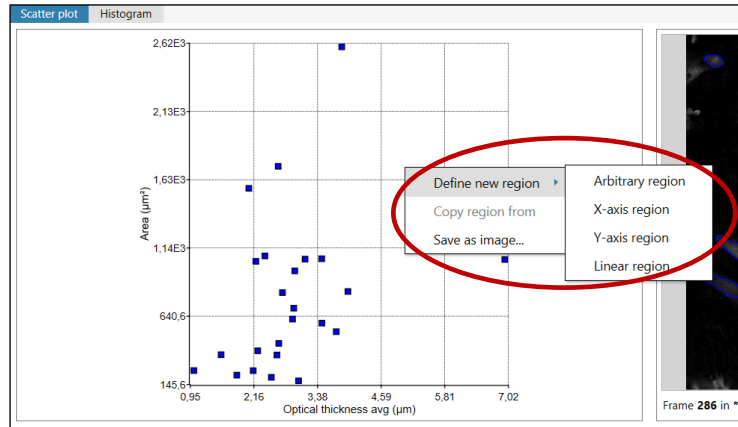
Alternatively, the scatter plot area can be changed with the mouse scroll button. Left click in the plot and scroll using the mouse scroll button to zoom in or out.

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 using flow cytometry. To open a **region menu**, right-click while hovering over the plot. When **Define new region** is selected, a region menu will appear.

Alternatively, there is a **Define Region** button below the **Scatter plot** tab, to the right of **Auto-scale**.



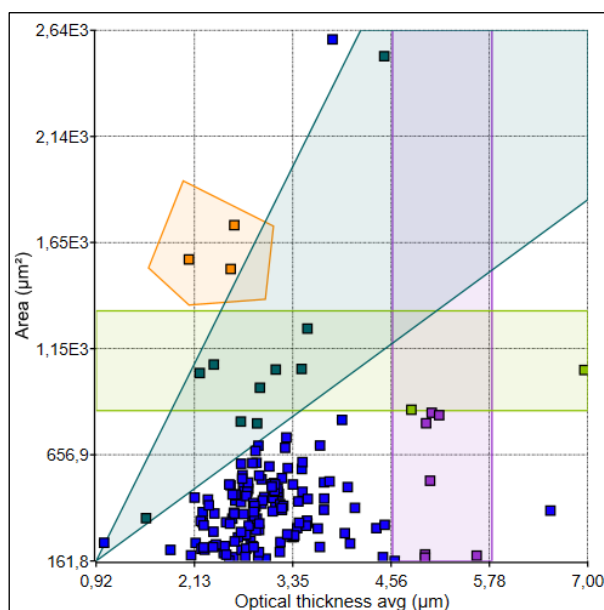
Select a region type by clicking it. There are four region types:

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

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

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

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



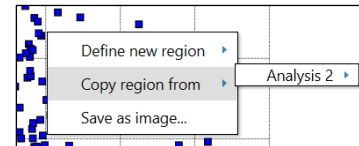
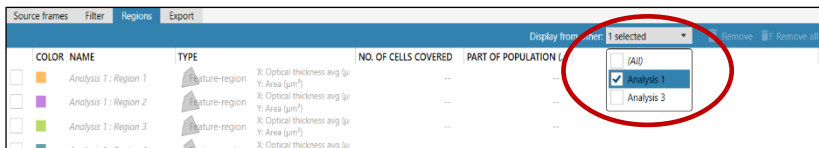
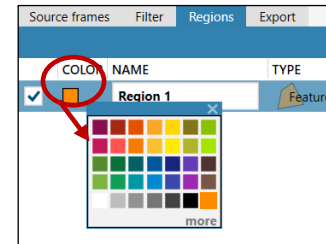
The Regions tab

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

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

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

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

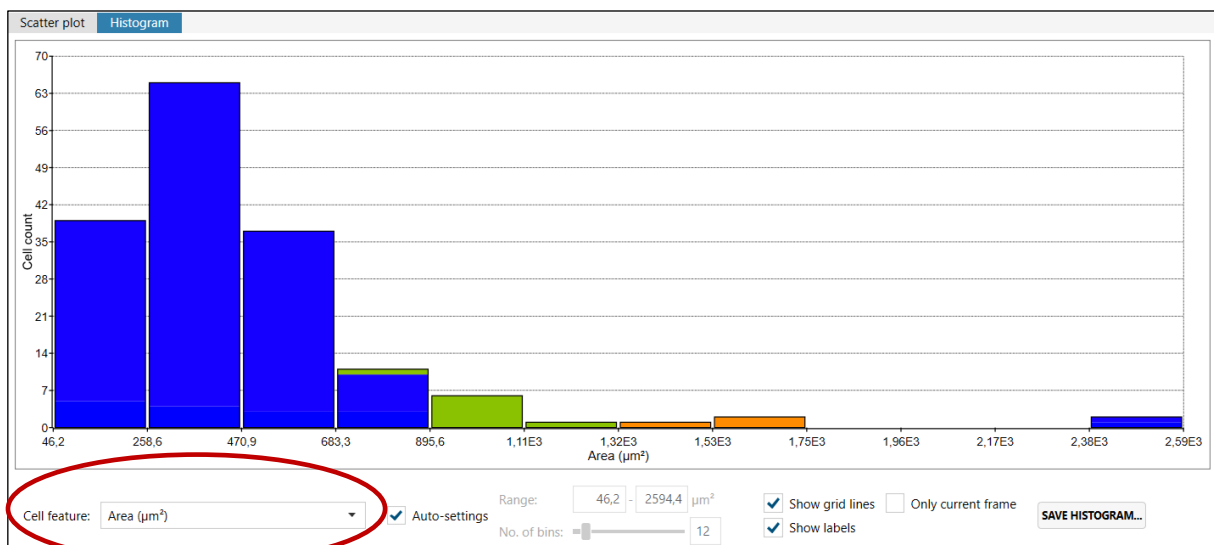


Display results as histograms

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.

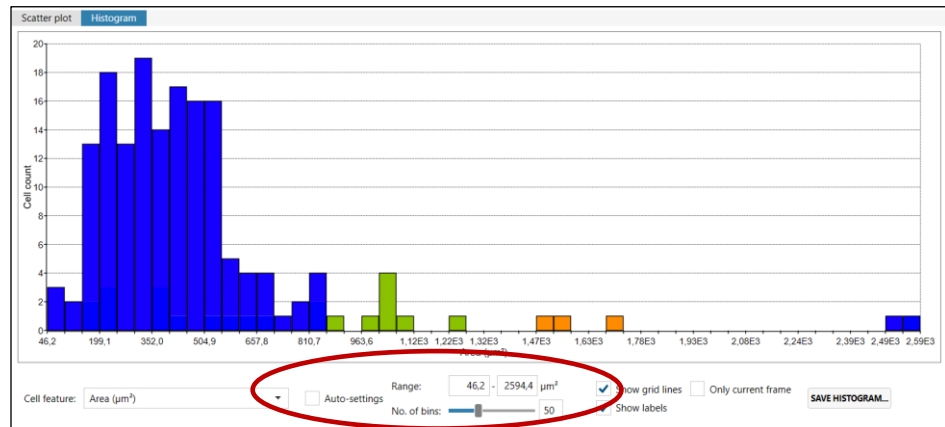
Change the histogram x-axis units

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

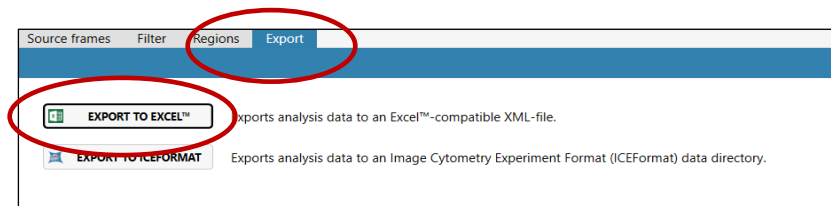
The histogram x-axis interval is set automatically to fit in all values from all added frames. To make these changes, **Auto-settings** must be unchecked and enter the minimum, maximum values. The number of bins can be changed by using the slider of typing a number in a text box.



Export cell data

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

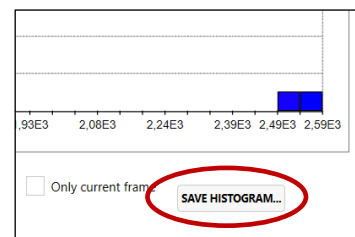
- Click one of the **Export** buttons to export all cell data. A file browser window will open. The file will automatically be named with date but it is possible to rename the file.
- Save the file to an appropriate place. All data relating to the image frames added to the current plot are exported.



Save images, plots and histograms

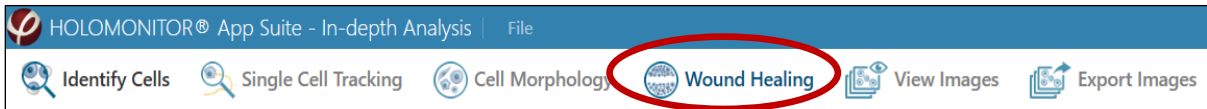
Images of the current cell image, plot and histogram can be saved as bitmap, GIF, JPEG, PNG or TIFF by using the **Save**, **Save Plot** and **Save Histogram** buttons.

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

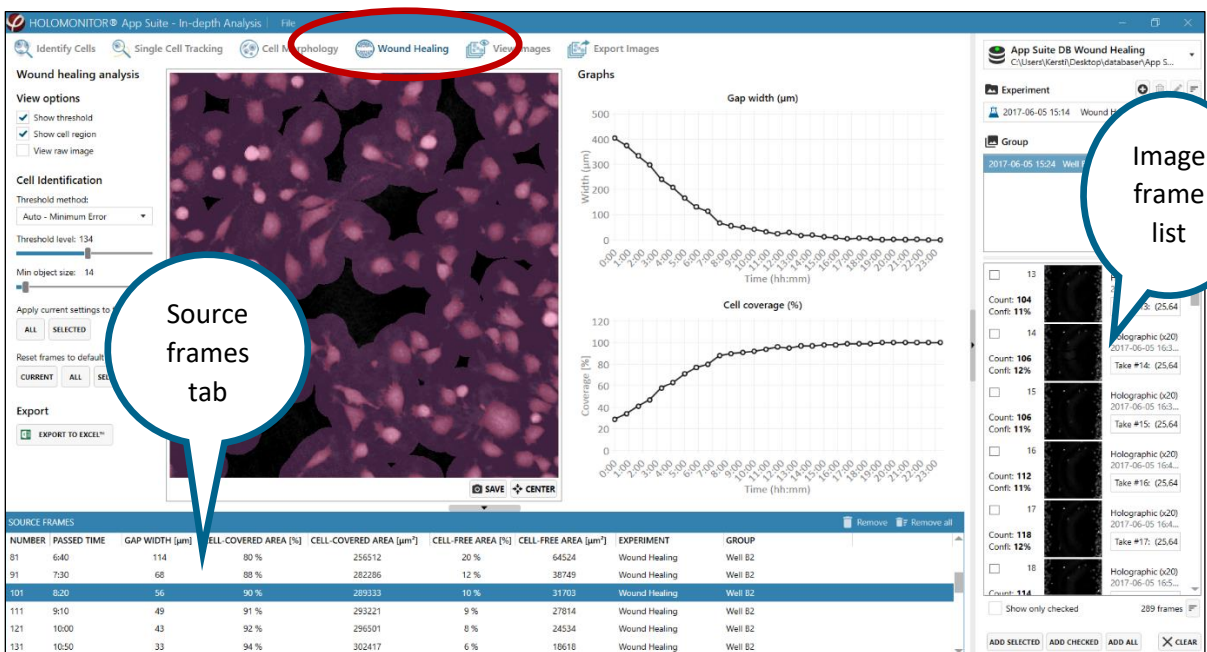


Wound Healing

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



There is no need to identify the cells in the frames before a **Wound Healing** analysis.



Start a new analysis

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

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

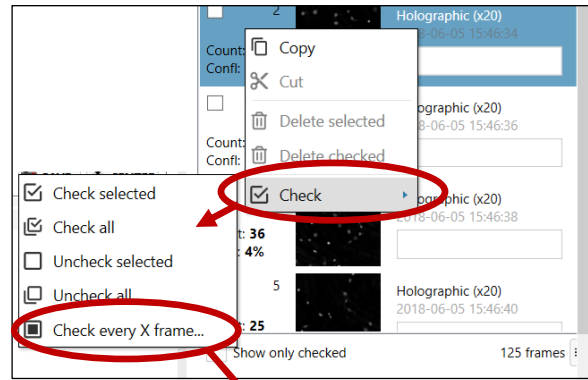
Add images

Select 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.

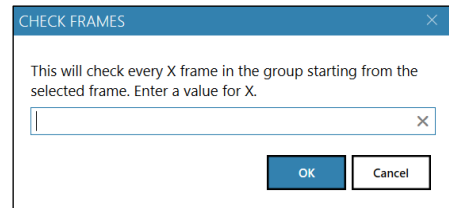
Several images can be added simultaneously if they are all selected. The SHIFT key can be used to select several consecutive images and the CTRL key to select non-consecutive image frames.

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 (=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**.



SOURCE FRAMES									
NUMBER	PASSED TIME	GAP WIDTH [µm]	CELL-COVERED AREA [%]	CELL-COVERED AREA [µm ²]	CELL-FREE AREA [%]	CELL-FREE AREA [µm ²]	EXPERIMENT	GROUP	
61	6:40	114	80 %	256512	20 %	64524	Wound Healing	Well B2	
91	7:30	68	88 %	282286	12 %	38749	Wound Healing	Well B2	
101	8:20	56	90 %	289333	10 %	31703	Wound Healing	Well B2	
111	9:10	49	91 %	293221	9 %	27814	Wound Healing	Well B2	
121	10:00	43	92 %	296501	8 %	24534	Wound Healing	Well B2	
131	10:50	33	94 %	302417	6 %	18618	Wound Healing	Well B2	

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.

Display options

Adjust cell identification

Graphs

Gap width (µm)

Cell coverage (%)

NUMBER	PASSED TIME	GAP WIDTH [µm]	CELL-COVERED AREA [%]	CELL-COVERED AREA [µm ²]	CELL-FREE AREA [%]	CELL-FREE AREA [µm ²]	EXPERIMENT	GROUP
1	0:00	404	29 %	92317	71 %	228718	Wound Healing	Well B2
11	0:50	375	34 %	108426	66 %	212609	Wound Healing	Well B2
21	1:40	334	41 %	131280	59 %	109455	Wound Healing	Well B2
31	2:30	298	47 %	152374	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

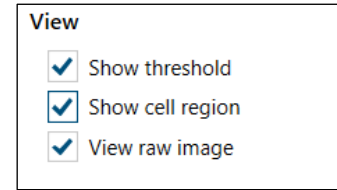
Adjust the display options

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

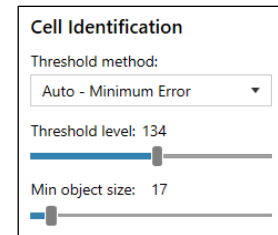
View raw image removes all colorsets and other image adjustments.



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.

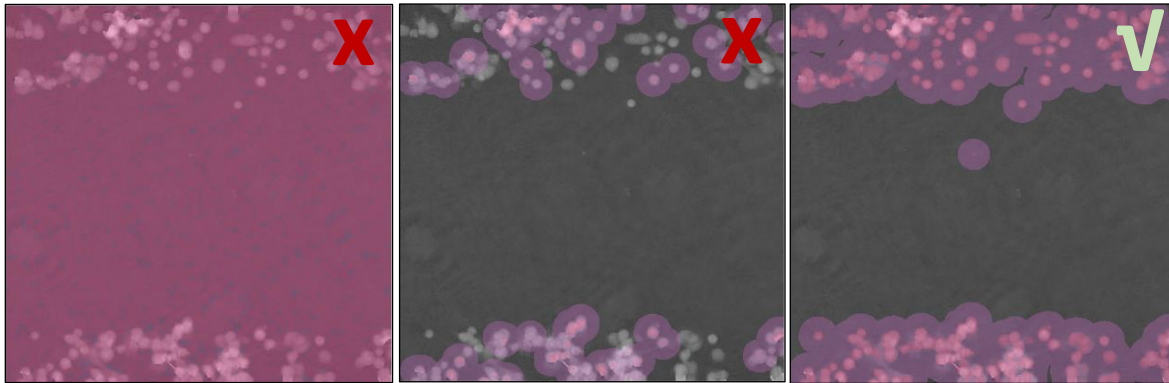
Double adaptive mean is the same as Double Otsu, but with two adaptive mean threshold masks.

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.

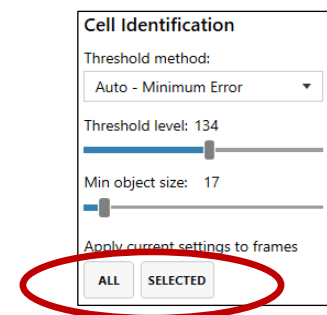
When the sliders are used, the threshold actively shows the boundaries of the cells. The images below show a too low threshold which includes too much background (left), a too high threshold which excludes too many cells (middle) and a correct threshold setting (right).



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 not show a big deviation, but instead it should increase steadily (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 [μm^2]	CELL-FREE AREA [%]	CELL-FREE AREA [μm^2]	EXPERIMENT	GROUP	
1	0:00	404	29 %	92317	71 %	228718	Wound Healing	Well B2	
11	0:50	375	34 %	101426	66 %	212609	Wound Healing	Well B2	
21	1:40	334	41 %	131180	59 %	189455	Wound Healing	Well B2	
31	2:30	298	47 %	151374	53 %	168661	Wound Healing	Well B2	
41	3:20	241	58 %	134758	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.

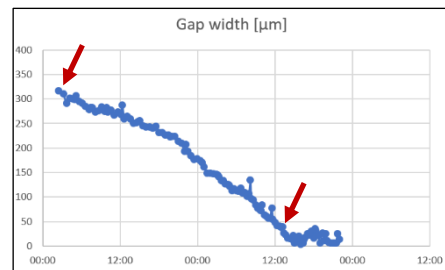


Cell Front Velocity

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 and divide with the number of hours between the values.

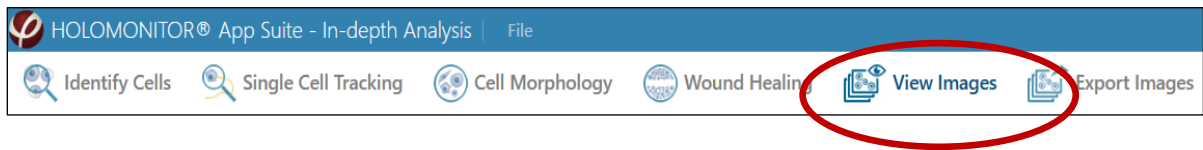
In this example: $(321-25) \mu\text{m}/36 \text{ h} = 8 \mu\text{m}/\text{h}$

This can also be performed in the Excel data sheet. Note that it is important to use only data from the linear phase of the slope.



View Images

In **View Images**, all captured images can be viewed, colored, and the software focus can be adjusted.

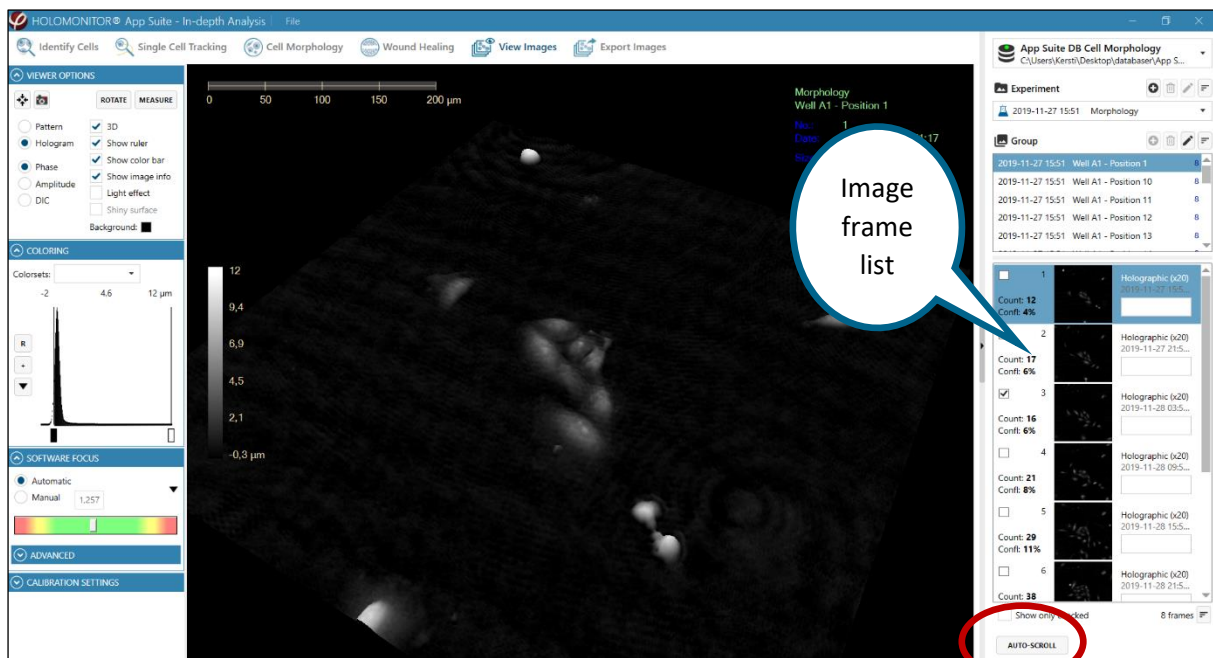


View an image

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, 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.

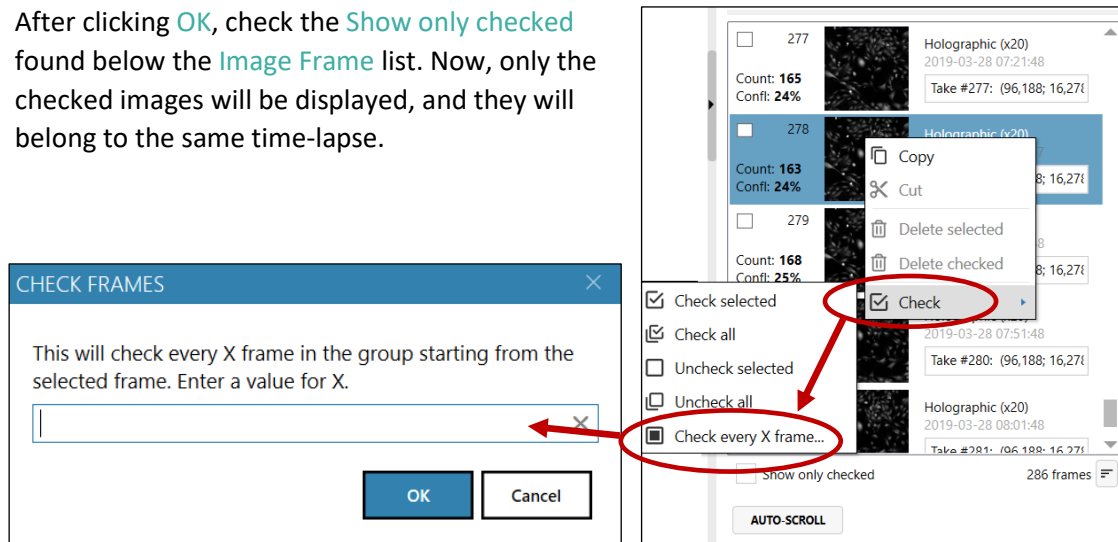
To flip and move a holographic 3D image, click, hold and drag the image using the right mouse button on the image.

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 thereafter **Check every X frame**. In the example above, 7 would then be entered in the **Check frames window**.
- After clicking **OK**, check the **Show only checked** found below the **Image Frame** list. Now, only the checked images will be displayed, and they will belong to the same time-lapse.




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


Use the **sorting** button, which is found below the **Image frame** list, to sort the images after position.

The images will then be displayed as consecutive time-lapses when **Auto-scroll** is active.

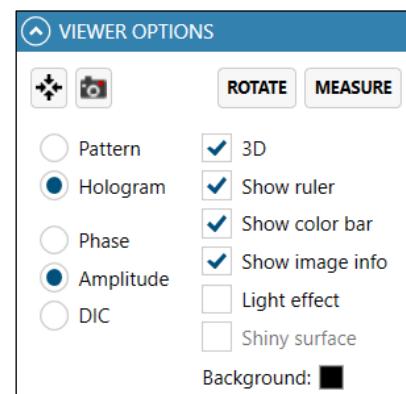
Use Viewer Options to change image display

The holographic image display can be 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

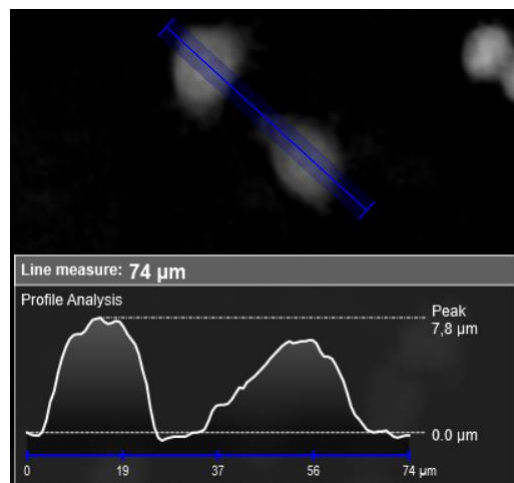
The **Measure** button activates the line measuring function, a simple tool for interactively measuring distances and cells.

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

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

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

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



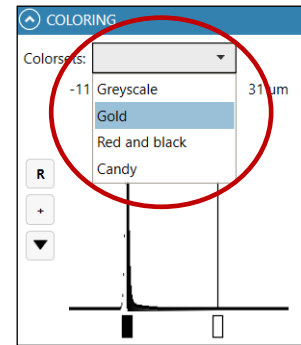
Change image coloring

All images in **In-Depth** originally appear in gray scale. If colors are needed or wanted, use the **Coloring** panel to the left of the **View area**.

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

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

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



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

The **+ button** adds new color to a Colorset. A colored triangle representing the new color which appear beneath the histogram. Alternatively, right-click on the x-axis and select **Add color**.

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

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

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

Save the current color settings to a previously saved Colorset by left clicking the button and choose **Save**. 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**.



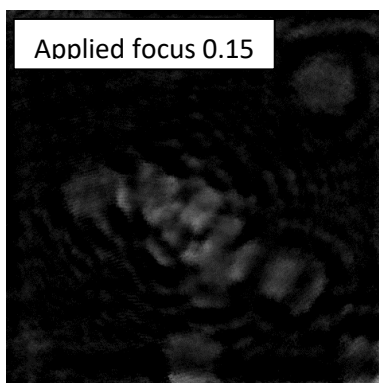
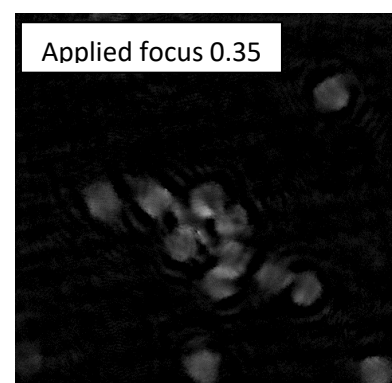
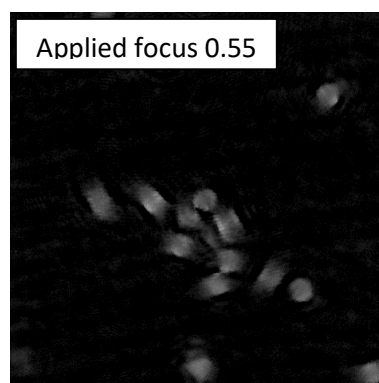
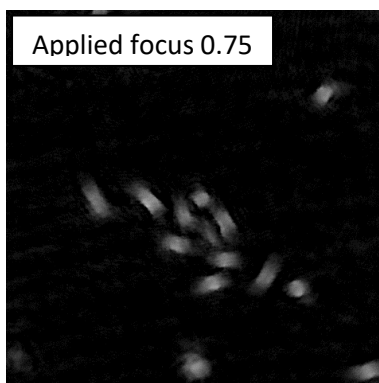
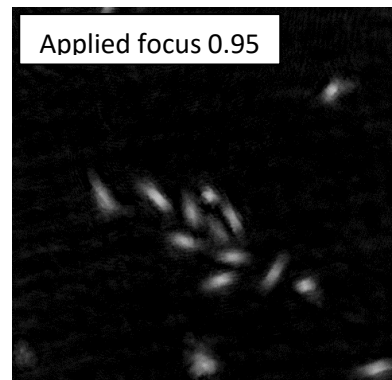
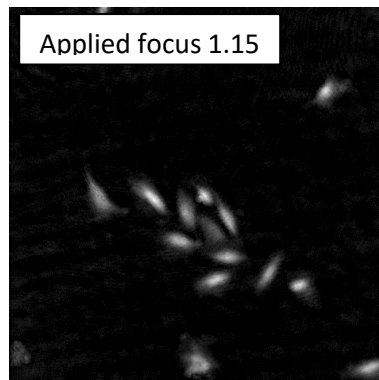
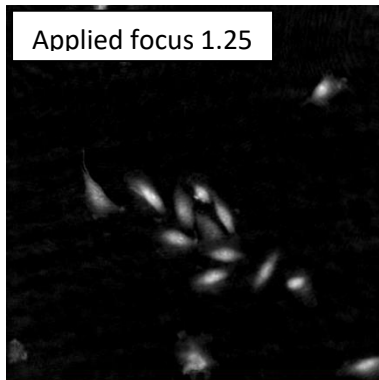
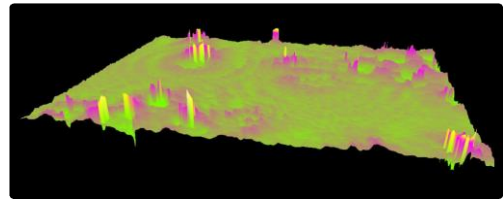
Colorsets are stored with the databases. Thus, if a colorset is created in one database, it is not found in a different database, even on the same computer/harddrive.



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.

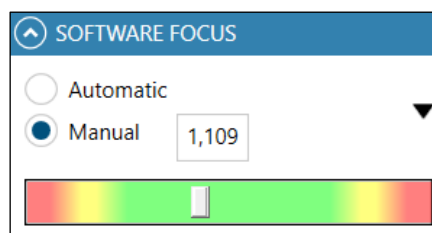
The **software focal distance** in mm can then be changed either by entering a value manually or by using the slider beneath the text box.

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

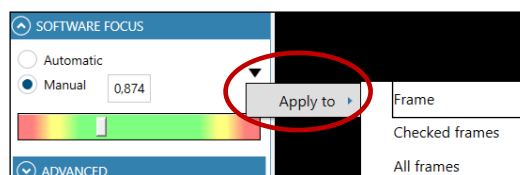
To replace the manual changes with the automatic focus, activate **Automatic** in the **Software focus** panel and then use the  button to apply the update to either the **current frame**, **all frames** or **all checked frames**.



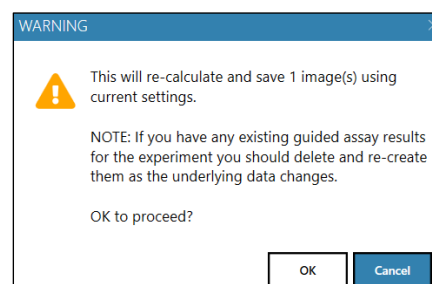
Activating Automatic focus will reset the software focus to the original values. These are the values that were applied when the image was captured. They may not be the optimal focus settings.



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.

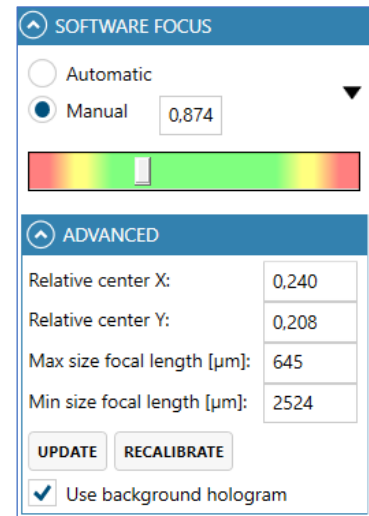


Advanced

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.

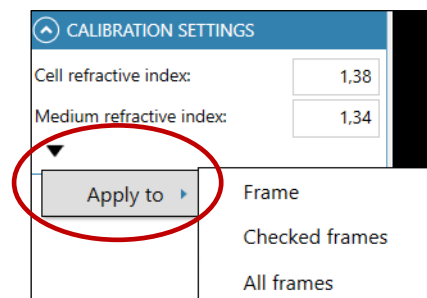


Change default cell and medium refractive index

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 .

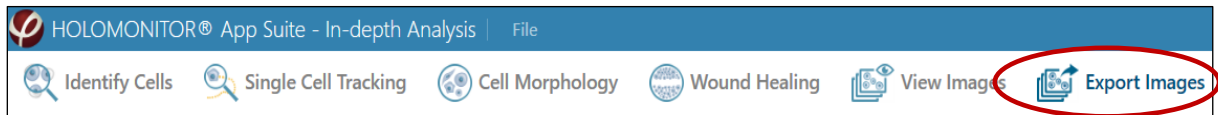
The default refractive index settings can be changed in the **Calibration side** panel.

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.



Export Images

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



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

Add and remove image frames

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

Add selected

Click the **Add selected** button, found below the **Image Frame** list, to add a single selected image frame or several selected frames.

Use the shift-key to select several consecutive images and the Ctrl-key to select non-consecutive images.

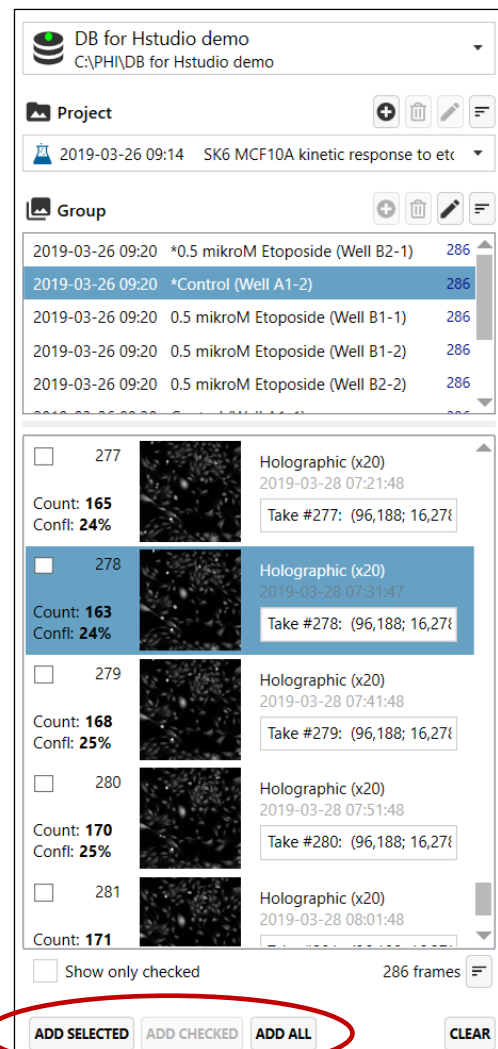
Add checked

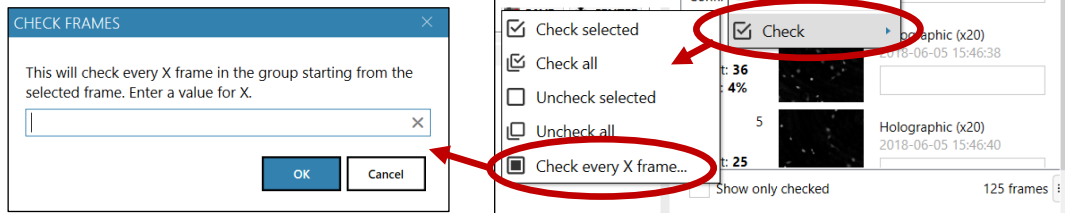
Images can also be selected and added by checking the box to the left of each image, and then clicking the **Add checked** button.

Add every X frame

Often not every image is required. Use the check-functions to select fewer images if for example images have been captured every five minutes, but only every 30 minutes are needed.

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





Add all

Add all image frames by clicking **Add All**.

Drag and drop

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

Source frames

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

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

View area

The **View Area** contains a view of the currently active added image as well as thumbnails of all the added images. Click an image thumbnail, to display the image in the **View area**.



Adjust image display

Viewer options

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

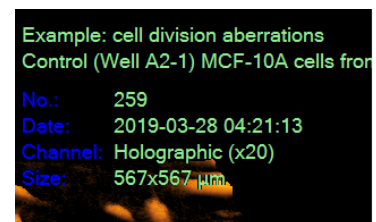
3D displays the image as a 3D representation.

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

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

Show Image Info displays additional information associated with the image:

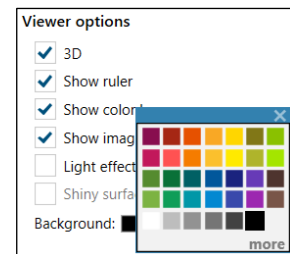
- First row specifies in which **Experiment** the image is located.
- Second row specifies in which **Group** the image is located.
- No. specifies which number the image has in the group.
- Date specifies the capture date and time of the image.
- Channel specifies the image type (holographic) and objective magnification.
- Size specifies image width and height in μm .



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

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

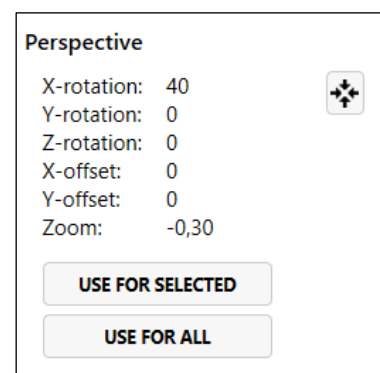
Background color is by default set to black. Left-click the Background color box to select a new color.



Zoom, move or flip the image

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

- To zoom, left click the cell image in the **View area** and then use the mouse scroll button.
- To move the image to a desired location in the **View area**, click, hold and drag using the left mouse button.
- To flip and move the 3D image, click, hold and drag using the right mouse button.
- Click one of the **Use for** buttons to apply the changes to either the added images that are selected or to all added images.

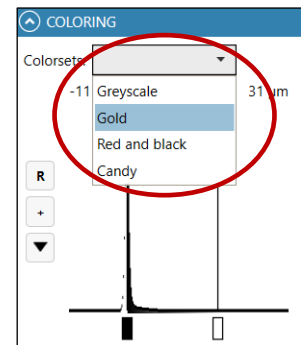


Change image coloring

All images in **In Depth** originally appear in gray scale. Use the **Coloring** panel, which is found to the left of the **View area**, to add or change the pseudo coloring to the images. The colors that are applied will color the image relatively to the thickness of the cells.

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

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



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

- **The R button** rescales the coloring to better utilize the dynamic range of the image. This button needs to be operated every time the image coloring is mis-adjusted.
- **The + button** adds new color to a Colorset. A colored triangle representing the new color will appear beneath the histogram. Alternatively, right-click on the x-axis and select Add color.
- **The arrow button** has several functions.
 - **Change the color** by right-clicking on a colored triangle beneath the histogram and select a new color, alternatively left-click the arrow 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 arrow button and choose **Save as**.
 - **Save the current color settings to a previously saved Colorset** by left-clicking the button and choose **Save**. Note that this will overwrite the settings previously saved.
 - **Delete a Colorset** by selecting it in the drop-down **Colorset list**. Then left click the button and select **Delete colorset**.



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



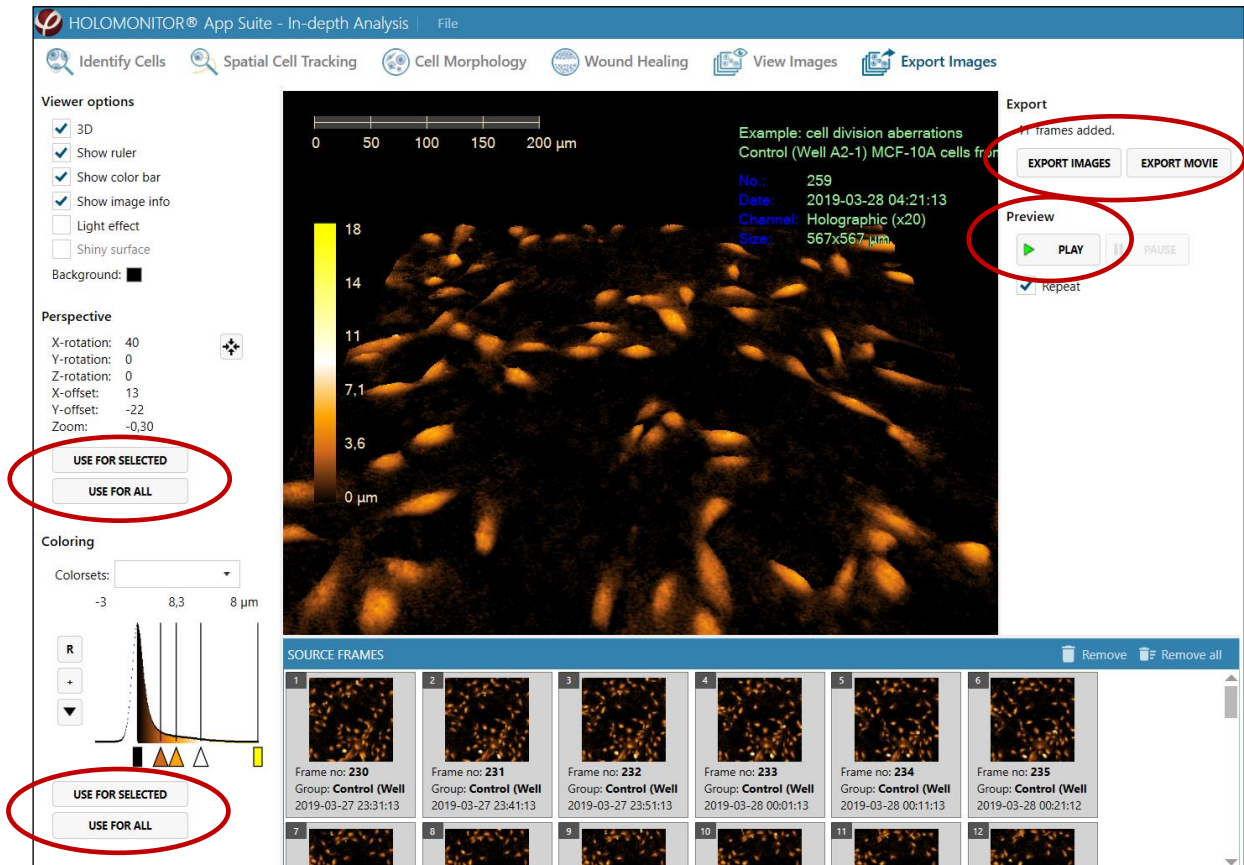
Note that no colorsets carry over from the App Suite assays to In Depth and vice versa.

Export a movie

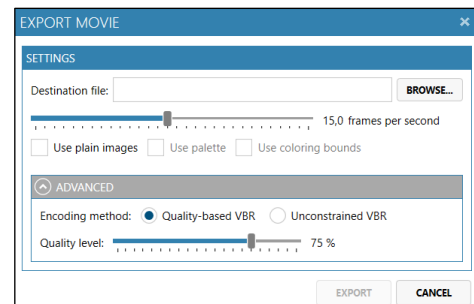
When image display, perspective and color look good, click the **Use for all** buttons in both the **Perspective** panel and the **Coloring** panel.

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

If the preview is satisfactory, click the **Export Movie** button to open the **Export Movie window** (see image below).



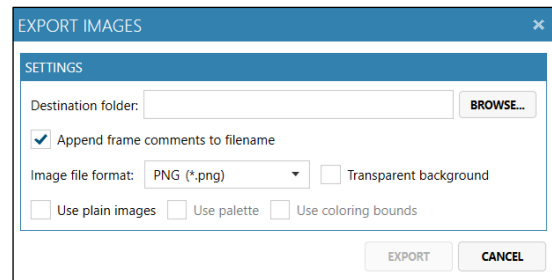
- Select the destination folder by browsing.
- Select video format. Standard setting is mp4.
- Move the slide bar to set the number of frames per second that will be shown in the movie.
- If desired, use **Advanced settings** to adjust the image quality specifications.
 - **Quality based VBR**: The quality and size of the exported movie file depends on a user-set target quality level for the movie,
 - **Unconstrained VBR**: The quality and size of the exported movie file depends on a target bitrate (amount of data per second) set by the user.
- When ready, click **Export**.



Export images

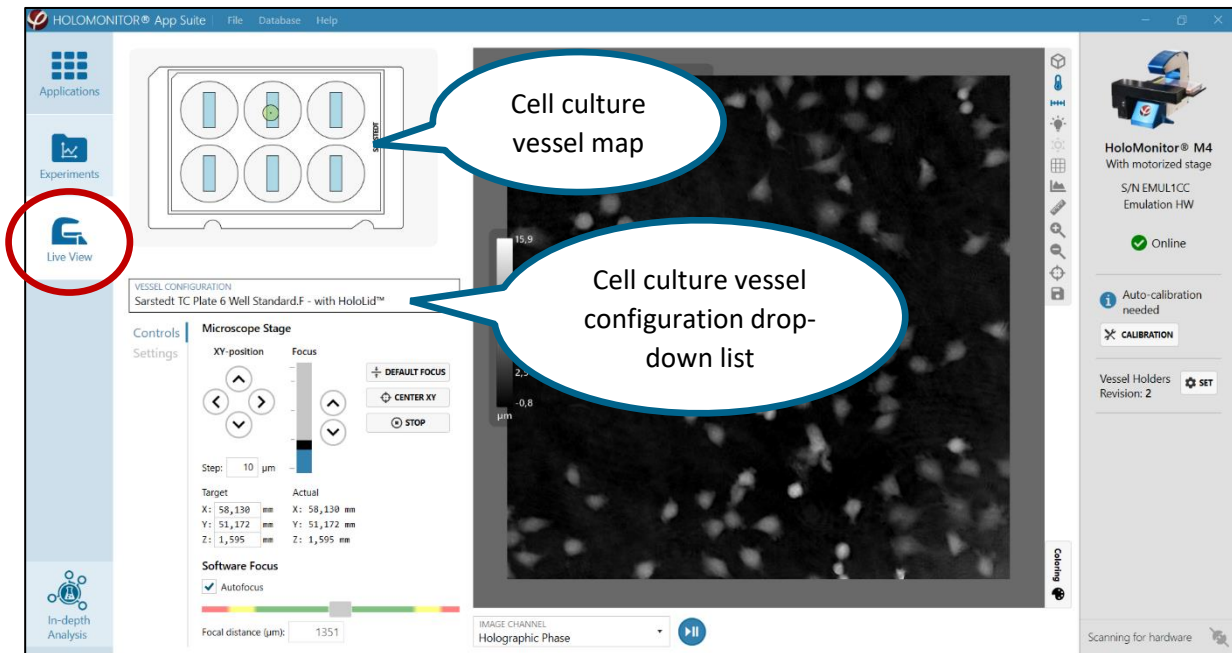
When image display, perspective and color look good, click the **Use for all buttons** in both the **Perspective** panel and the **Coloring** panel. Then click the **Export Images** button to open the **Export Images window**.

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



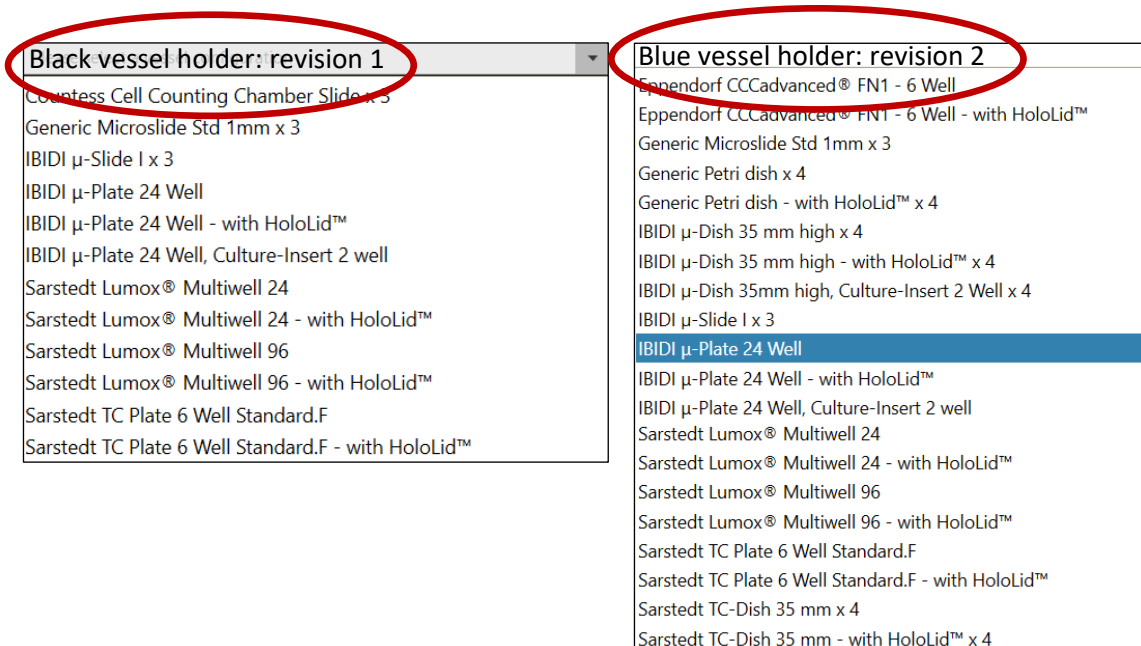
Live View

Live View allows the user to study cell samples without setting up an experiment, thus allowing HoloMonitor M4 to function as a microscope. For this function to be active, an instrument needs to be connected and an active AppSuite license is needed.



Cell culture vessels

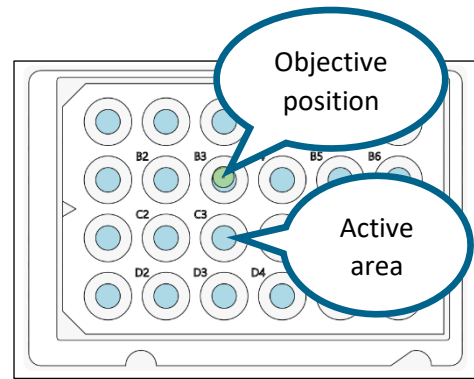
In the upper left corner of the **Live View Window**, a cell culture vessel map is displayed. Select the correct map using the **Vessel Configuration** dropdown list. The following vessels, depending on the vessel holder, are supported:



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. Too big steps can crash the objective into the sample.

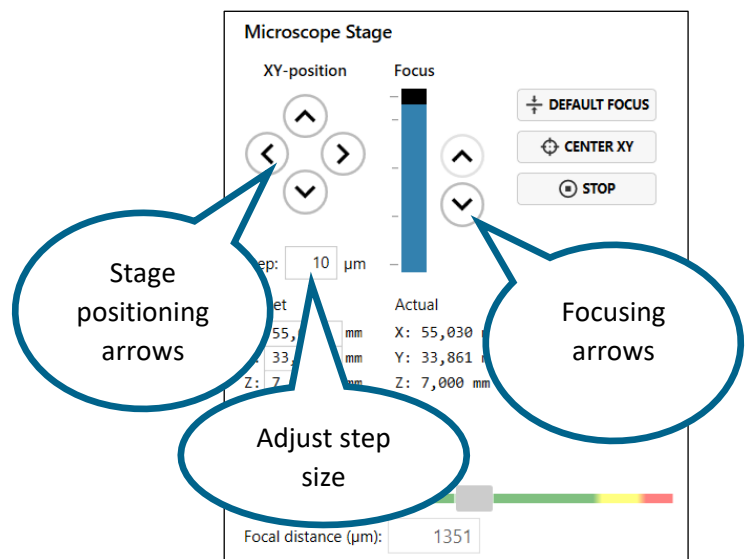
Coordinates of the current position as well as target coordinates are given below the arrow buttons. The target coordinates can be entered manually.

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

Clicking the [Center XY](#) button will move the stage to the center position of the vessel.

Clicking the [Stop](#) button will immediately stop all stage movement.

Click the [Default Focus](#) button to replace the current focus with the pre-set focus settings



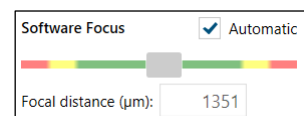
Focusing

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 M4 was installed, but sometimes focus needs to be adjusted by the user, e.g. if the cell culture vessel has a coating that makes autofocusing difficult.

HoloMonitor M4 has both a coarse mechanical and a fine software focus. They need to cooperate for the image to be in focus. Use the mechanical focus, *i.e.* move the motorized stage, to move the cells to the approximately correct distance from the objective. The software focus

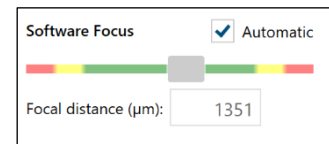
then finds and calculates the cell images. The [Software Focus](#) indicator shows how well the software can focus an image at the current mechanical focus setting. The software can only find focus when the cell culture vessel is at the approximately correct distance from the objective.



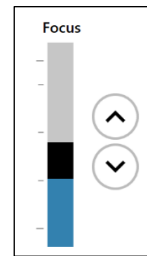
Focusing in practice - semi automatic focusing

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.

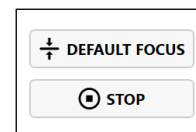


The black box in the **Focus** bar represents the motorized stage. Move the black box up or down to move the motorized stage up or down. Click the two arrows for smaller stage movements up or down.



Note that the step length applies to all directions of stage movement: X, Y and Z. 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.

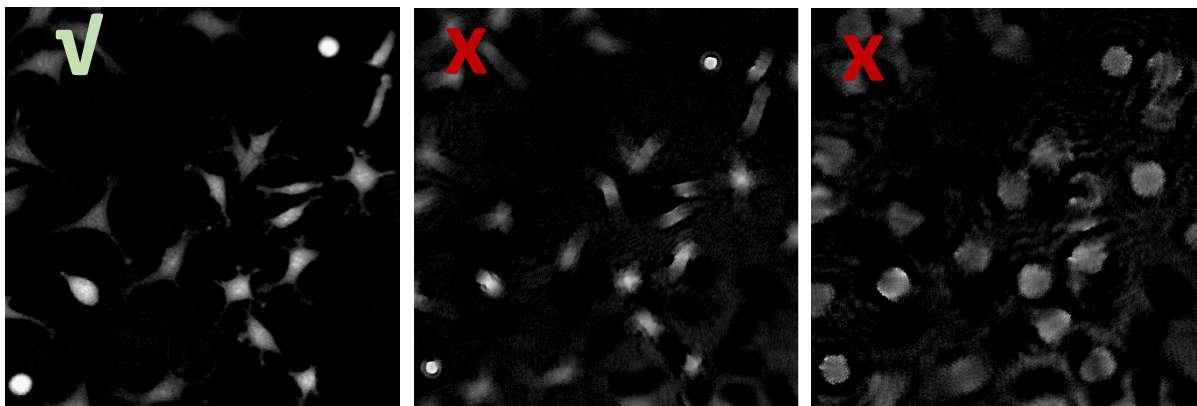
Manual focusing

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 (left), slightly out of focus (center) and totally out of focus (right).

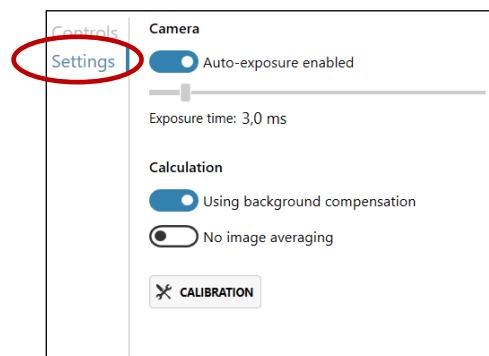


Settings

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.

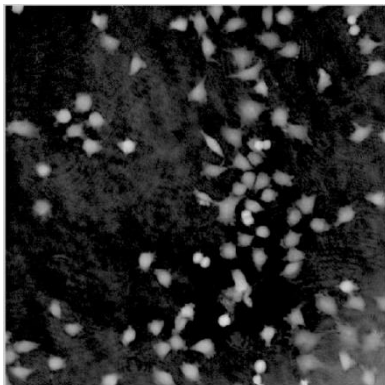
Image channels

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

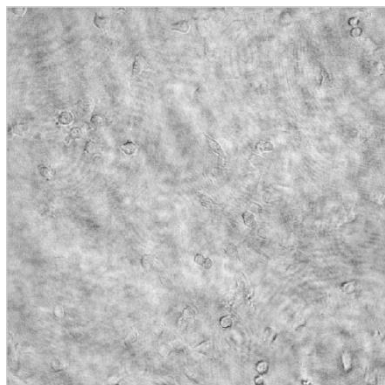
The **Holographic Phase Image** is a reconstructed image based on the light phase information in the hologram.

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

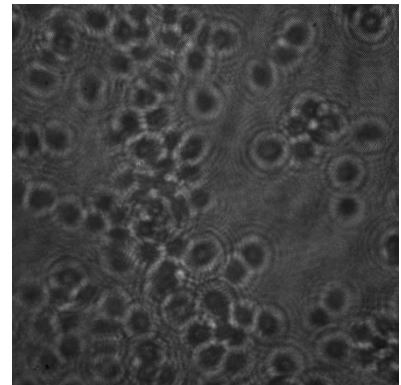
The **Hologram image** is captured by the camera and is the result of interfering light waves. The hologram is the basis for the reconstructed holographic cell images.



Holographic phase image



Holographic amplitude image



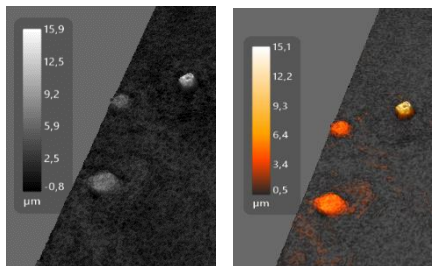
Hologram image

Adjusting image presentation

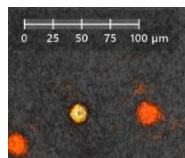
To the right of the **Live image** view window there are multiple functions that adjust the presentation of the live image (see image to the right). Blue functions are active while gray functions are inactive.

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).

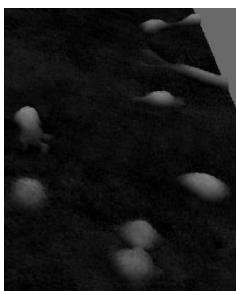


Show XY scale displays a horizontal scale bar representative of the distance in X and Y (See image to the right).

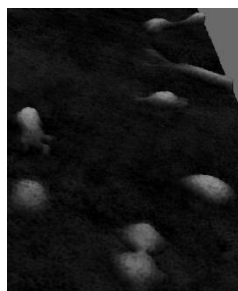


Enable Lighting applies an artificial light source to the image which may sometimes render an improved image (See image below).

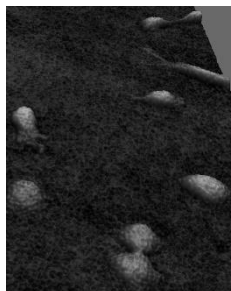
Enable Shiny surface shows the rendered surface with a reflective visual effect. Shiny surface is only active when Lighting is active.


















Standard image



Enabled Lighting

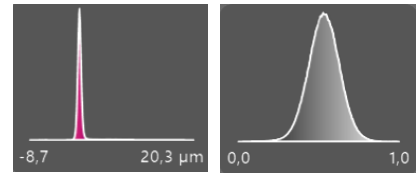


Enabled Lighting and Shiny surface


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

[Show background grid](#) adds a grid in the background.

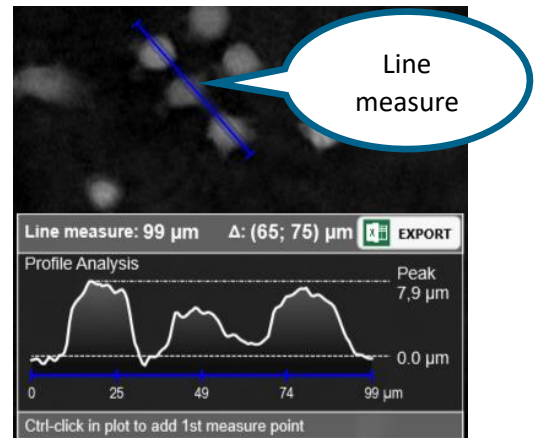
[Show image histogram](#) shows a histogram overlay of the pixel values in the image. For a phase image this equals a histogram of the optical thickness values (left image) and for other image channels such as camera it shows a histogram of the pixel intensity values (right image).



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

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.

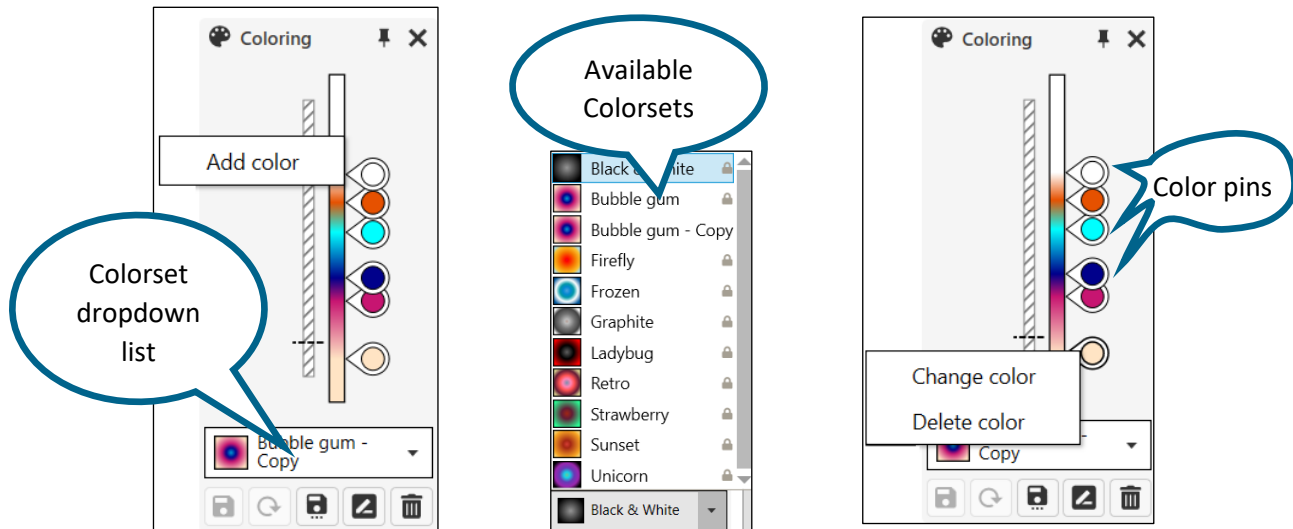
[Save view as image file](#) saves the image in the [Live window](#) exactly as it is shown as bmp, gif, jpg or png files.

[Show color controls](#) opens [Coloring](#) (see image below) which is used to adjust the color balance of the image and to color the image 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 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** are used to

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

Connected instruments

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

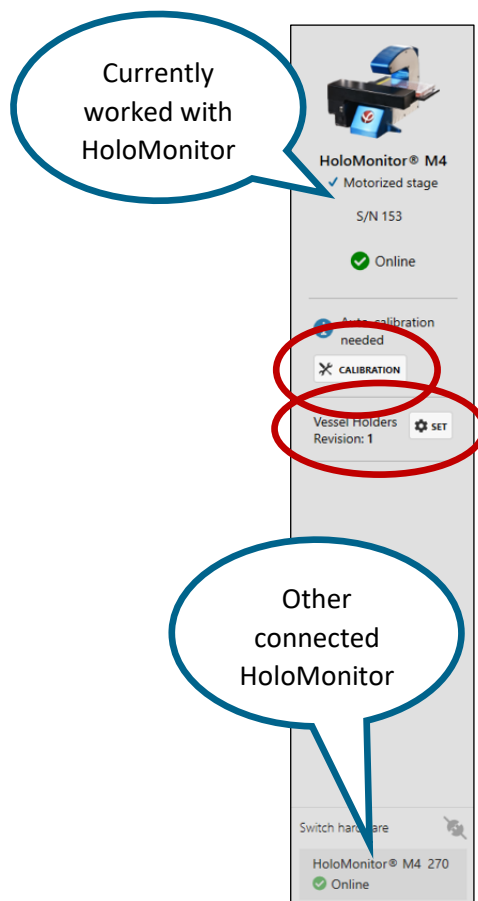
Working with two instruments

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

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



The same vessel holder revision must be used with the two HoloMonitor M4s, and both experiments must be saved to the same database.



Auto-calibration needed

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

Vessel Holders revision

There are two lines of vessel holders for the HoloMonitor M4. They are black (Revision 1) or blue (Revision 2). It is necessary to have the correct revision selected, as the [Vessel Maps](#) are different for the different revisions. Use the [Set button](#) to set the correct revision of vessel holders. All new HoloMonitor M4s are delivered with the blue holders.

Database Handling

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



Access the Database Handler

Access the database handler through [Database](#) in the top menu 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 M4.

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

In the [Database Handler](#), there are buttons 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.

The screenshot shows the HOLOMONITOR App Suite interface. At the top, there are two buttons: "CREATE NEW" and "OPEN", both circled in red. Below them is a list of databases. A blue arrow points to a database icon with the text "Display arrow". Another blue arrow points to a database with a red status icon and the text "Inaccessible database". A third blue arrow points to a database name in the list with the text "Opens when right clicking a database name". A context menu is open over one of the databases, showing options like "Paste", "Move...", "Delete...", "Export...", "Import...", "Import from", "Find Hstudio™ experiments...", "Sort experiments by", and "Remove from list". A fourth blue arrow points to the "Location" field in the database details panel with the text "Click to go to database location in explorer". The database details panel shows "Name: Holoscope Data", "Location: C:\Users\PHI\Desktop\Holoscope Data", "Drive usage: 189 GB free", "Storage size: Not calculated", and "Contents: 0 experiments.". At the bottom of the details panel, a row of buttons (EXPORT..., IMPORT..., MOVE..., DELETE..., REMOVE FROM LIST) is circled in red.

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.

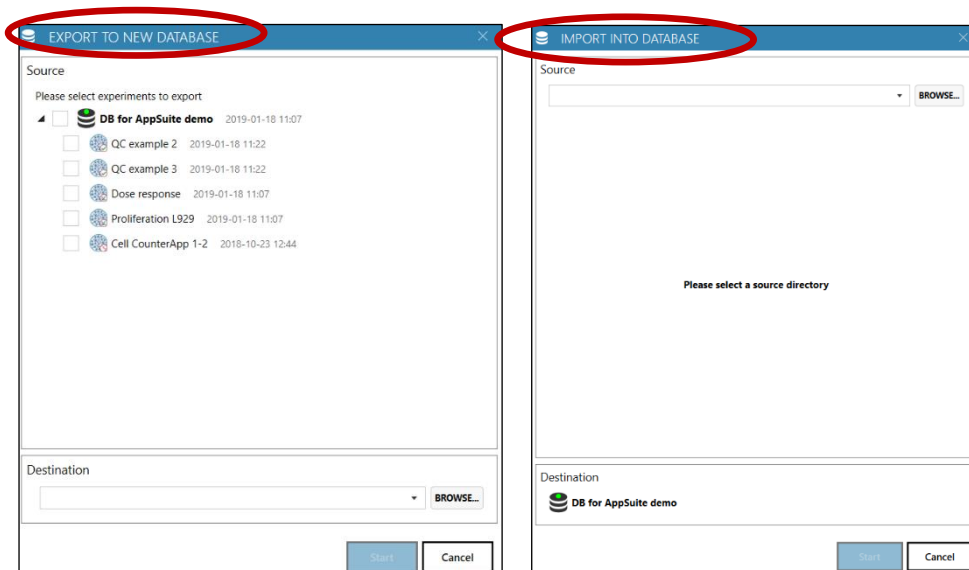
Export and import databases

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 **Database Handler** list of databases. Right click the selected database and select **Find Hstudio experiments**. Any available Hstudio experiments will be shown in a list. Select an experiment and click **Add**. This will convert the Hstudio data to App Suite data.

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



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



Not all older Hstudio experiments are possible to convert to AppSuite experiments. If that is the case, experiments will still be available for analysis in AppSuite 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 little arrow beside a database in the **Database Handler** to show the **Experiments** included in the database. Right-click the **Experiment** and select **Copy**. Right click the desired database and paste the copied **Experiment** into the database.

	Cut	Ctrl+X
	Copy	Ctrl+C
	Delete	Del
	Rename	F2

Backup/restore HoloMonitor data

Create a database backup

Using the **Database Handler**, 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 the **Database Handler** 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.

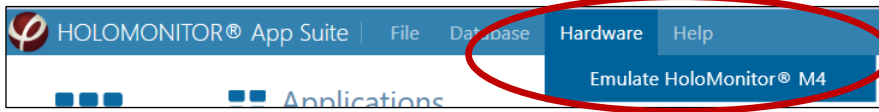


Databases can always be copied and moved using windows explorer, just like any folder or map in the computer. Ensure that all files in the database folder are copied, otherwise the data cannot be accessed.

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.

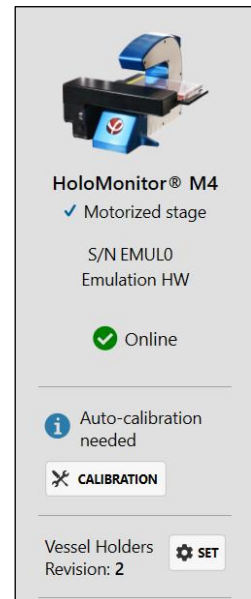
HOLOMONITOR EMULATOR



The **HoloMonitor M4 Emulator** is a virtual HoloMonitor M4. When activated it gives access to all live features of the software as if a HoloMonitor M4 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 M4.



HoloMonitor® M4

- ✓ Motorized stage

S/N EMUL0
Emulation HW

✓ Online

i Auto-calibration needed

✕ CALIBRATION

Vessel Holders
Revision: 2 **SET**

TROUBLESHOOTING

Startup

The software cannot find the HoloMonitor M4

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.

If the software still is unable to find the HoloMonitor M4, contact support@phiab.se or your local representative.

The Auto-calibration does not pass

All calibrations values are in the red

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

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

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

Check the laser

- Is the red light shining on the objective?
- If 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 M4 positioned correctly and tightened?
- Inspect the Laser tip. Unscrew the Laser connection from the back of HoloMonitor and see if the tip is scratched or dirty.
- When the laser connection is detached from the HoloMonitor M4, check if the laser light shines in a strong and perfect red circle when shining on a white paper. If not, it is either dirty, or the tip could be damaged or cracked.

If the laser tip needs cleaning

- Wipe the tip with a dry cotton swab in one direction.
- Take a new cotton tip and clean the fiber connector.

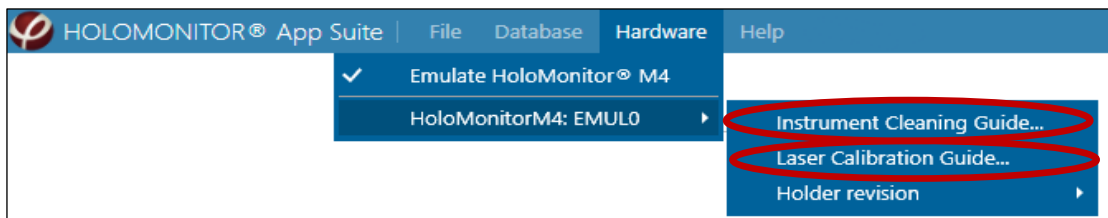
- Carefully reconnect the laser, paying attention that it is aligned properly. The guiding metal ridge on the laser connection must slip into the guiding notch in the connector socket at the back of the HoloMonitor M4 (see the section for Hardware installation for pictures). Connections must be finger tight.

After checking the laser, recalibrate

After testing all above, re-run the **Calibration** wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range 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 could indicate that the laser needs periodic recalibration. Go to the top menu bar, select **Hardware** and then the current HoloMonitor M4. Activate the **Laser Calibration Guide** and follow the instructions. Afterwards, re-run the **Auto-calibration** wizard. After successful auto-calibration you should see all values (black arrow heads) in the green range 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 M4. Go to the top menu bar, select **Hardware** and then the current HoloMonitor M4. Activate the **Instrument Cleaning Guide** and follow the instructions. You may need to repeat the cleaning 2-4 times depending on how dirty the optics are. Afterwards, re-run the **Auto-calibration** wizard. After successful auto-calibration all values (black arrow heads) should be in the green range 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.se or your local distributor for further assistance.

Experiment setup

There is no vessel map for petri dishes

The HoloMonitor M4 has black cell culture vessel holders

HoloMonitor M4 with motorized stage is delivered with a set of cell culture vessel holders. At present there are two revisions of the holders which can be distinguished by their color.

Revision 1 is black, and **Revision 2** is blue. In App Suite, there are **Vessel Maps** to match the holders. All new HoloMonitor M4 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 \varnothing 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 service@phiab.se.

The cell culture vessels used do not follow the recommended specifications
Switch to the recommended cell culture vessels.

It is impossible to focus the live image

The cell culture vessel is scratched or smudged

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

There is condensation on the inside of the cell culture vessel

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

There is condensation on the outside of the cell culture vessel

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

The objective and the laser window need cleaning

Clean the instrument according to the **Cleaning instructions** in 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 M4 are not loose and that they do not touch anything that vibrates. Ensure that no vibrations come from sources like centrifuges or hard-working air vents. Make sure that the incubator is leveled and stands without disturbances.

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

The cell culture vessels are not the recommended versions.

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

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

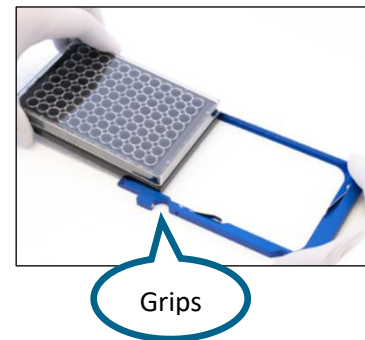
There are two revisions of cell culture vessel holders, the **Black (Revision 1)** and the **Blue (Revision 2)** holders. Their versions are slightly different, and unless the correct version is set, the **Vessel Maps** will not correlate with the actual wells.

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

Ensure that well A1 (Sarstedt, 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.se 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 looks garbled

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

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

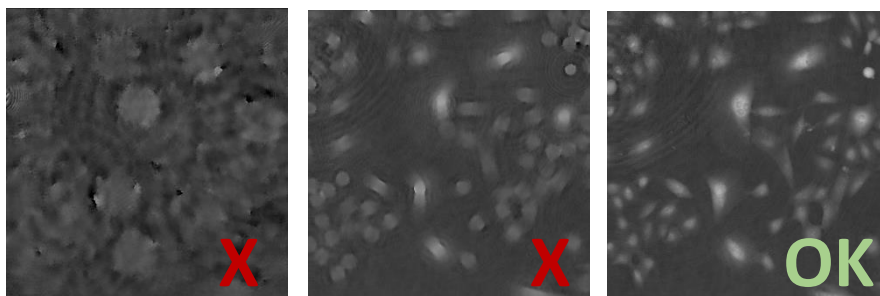
Clean the instrument as described in the [Cleaning](#) instructions in the software.

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™.

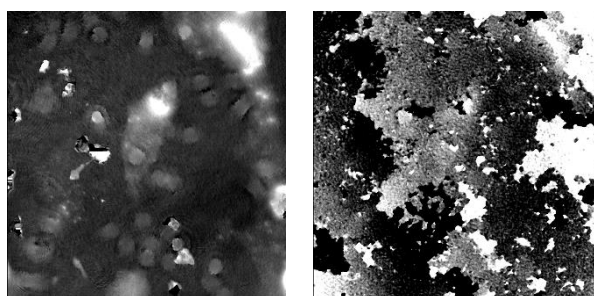
Re-calibrate according to the [Calibration](#) procedure.

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

Wrong focus



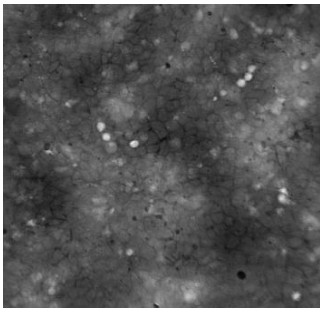
Condensation



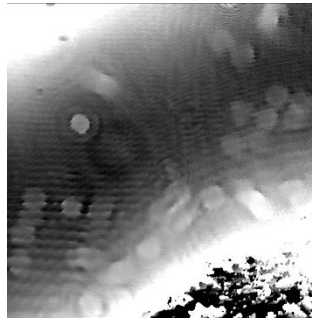
Some condensation

Full condensation

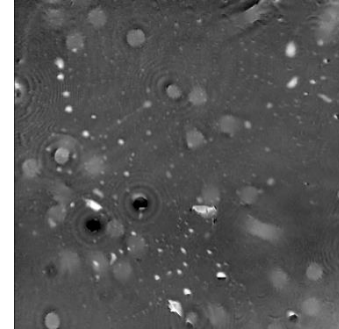
Cells in many layers



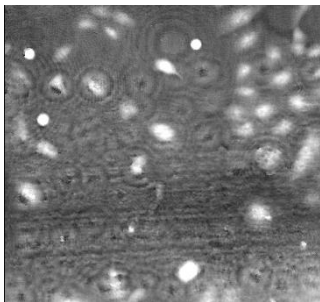
Air bubble



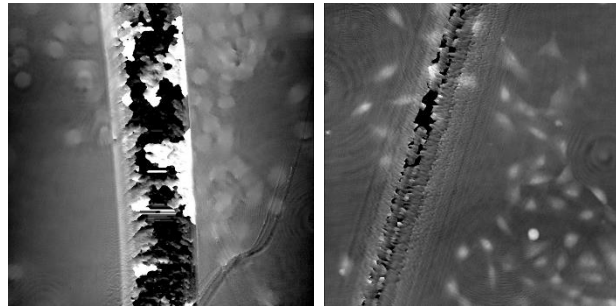
Dirt & Particles



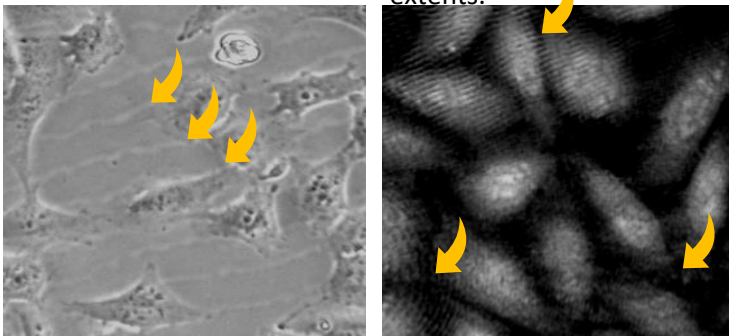
Smear



Small and big scratches



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.se for advice.

Condensation has formed inside HoloMonitor M4

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

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

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

The live cell image is black

The laser unit may not be connected properly.


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

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.

Database Handler

There are no databases in the Database Handler

All databases have been removed from the Database Handler.

Browse to locate and open databases.

There are no databases on the computer.

Create a new database for the experiment.

The databases in the Database Handler cannot be accessed.

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



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

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.se for further assistance.

The motorized stage needs to be serviced

Contact your local distributor or support@phiab.se for further assistance.

The capture was interrupted

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

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

There was a power failure

The instrument will not automatically restart an interrupted experiment.

The data was stored on an external hard drive

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

The computer updated software automatically and then restarted

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

HoloMonitor losing 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 Assays

There are no databases in the Database Handler

All databases have been removed from the Database Handler

Browse to locate and open databases.

The databases in the Database Handler cannot be accessed

If 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 Database Handler and 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

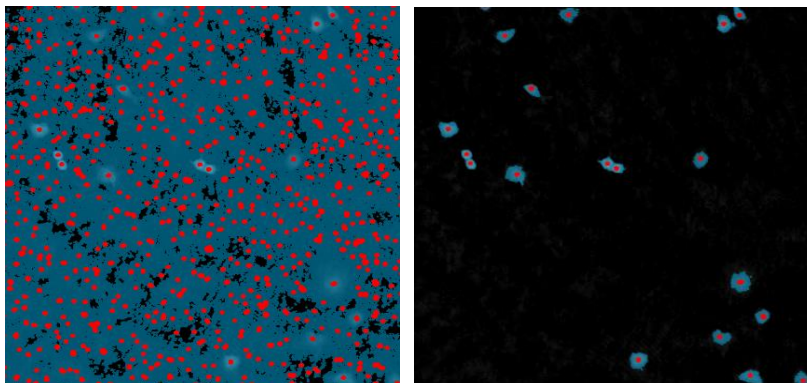


External drive USB connection or internet connection were interrupted.

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.

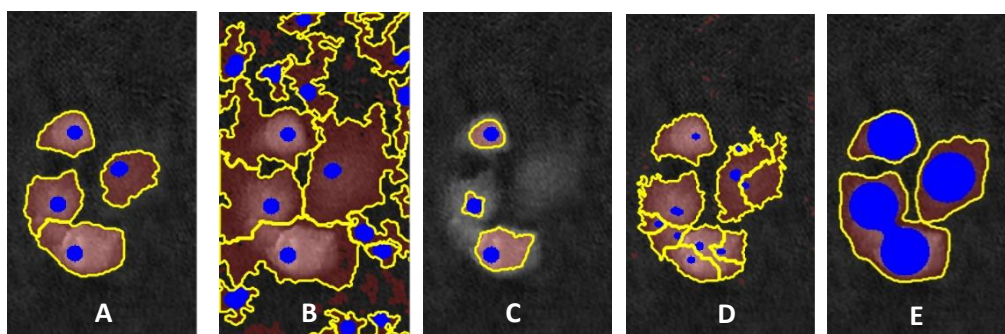
The threshold setting methods cannot identify individual cells if the cells grow very close to or on top of each other. Adjust the **Background threshold** in **Adjustment tab**. Make sure that only the background and not any cells are defined as background. Then use the confluence to determine the amount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

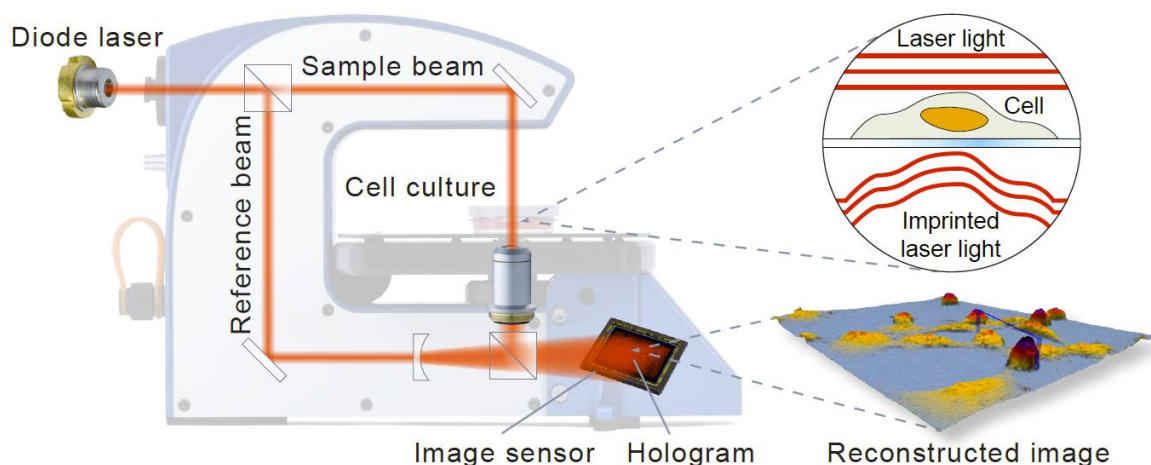
TECHNOLOGY

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, 2013; Kim 2010.

In short

HoloMonitor M4 creates label-free holographic cell images. Laser light is divided into a reference and an object beam. As the object beam passes through the sample, the light is slowed down and a phase delay is imprinted on the light beam. By subsequently merging the sample and the reference beam, an interference pattern is created. This interference pattern is the hologram. It is recorded by an image sensor and then used to numerically reconstruct a so-called phase image, which is displayed and analyzed (Mölder et al. 2008). In other words, all calculations are based on how much the light slows down when it passes through the cells.



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

Calculations

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

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

ANALYSIS FEATURES

Output Variables for Guided Assays

Output variable statistics

In App Suite, output variables are typically presented as an average value, sometimes including a variation measure, such as Coefficient of Variation (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 (μm^2)

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

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

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

Cell Count (cells/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 ($\mu\text{m}/\text{h}$)

The motility speed is based on the mean cell distance from one frame to the next, divided by the time between frames. The Mean Cell Speed is based on all cells in the frame.

Mean Cell Diameter (μm)

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

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

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

Mean Cell Volume (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

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

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

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

Optical Volume

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

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

Optical thickness

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

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

Optical thickness max

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

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

Optical path length

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

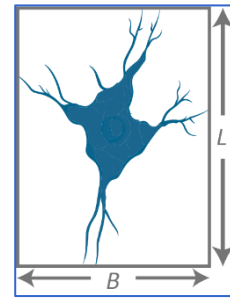
$$OPD_{avg} = T_{avg} \cdot (n_c - n_m) = \lambda \cdot \frac{\varphi_{sum}}{N}$$

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

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

Boxed length and breadth

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



Boxed center position x and y

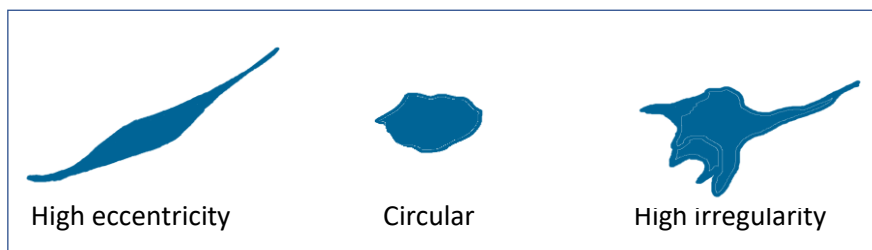
The geometric center position of an area-minimized (aligned) bounding box that encloses the cell.

Centroid position x and y

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

Eccentricity

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



Hull convexity

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

Irregularity

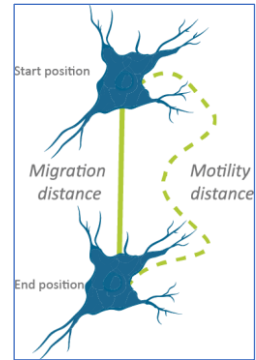
A measure of how much the circumference of the cell deviates from the circumference of a perfect circle. A value of 0 means the cell is circular and higher values mean a longer, more irregular outline. The calculations are based on a threshold setting that distinguishes background from cell.

Migration distance

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

Motility distance

The motility distance is the actual distance traveled by the cell between the starting point and the end point of the cell path.



Motility speed

The motility speed is the motility distance divided by the time it took for the cell to travel from the starting point to the end point.

Migration directness

How direct the cell movement towards its endpoint is ($\text{migration distance} / \text{motility distance}$). A number close to one represents a direct movement. A number below 0.5 represents a non-direct random movement.

Peak position

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

Perimeter length

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

Phase shift

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

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

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

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

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

Phase shift sum

A different way of calculating the texture correlation. The phase shift sum of a cell (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. φ_i is the average phase shift in radians created by the area imaged by object pixel i divided by 2π .

Roughness

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

The different ways to look at the roughness may reveal different effects of cell treatments.

Roughness avg.

The average of the roughness distribution.

Roughness kurtosis

How peaked the value distribution is. A positive kurtosis distribution has a sharper peak and longer, fatter tails (e.g. a Student's t distribution) while a negative kurtosis distribution has a more rounded peak and shorter, thinner tails (e.g. a Bernoulli distribution). A neutral kurtosis distribution (=0) corresponds to a normal distribution.

Roughness RMS

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

Roughness skewness

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

Shape convexity

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

Texture

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

<i>Texture cluster shade</i>	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.
<i>Texture cluster tendency</i>	Indicates into how many clusters the gray levels present in the image can be classified.
<i>Texture contrast</i>	Measures the thickness contrast between a pixel and its neighbor.
<i>Texture correlation</i>	Measures the joint probability occurrence of specified pixel pairs, i.e. how likely it is that pixels with certain phase shifts occur together.
<i>Texture correlation info1</i>	A different way of calculating the texture correlation.
<i>Texture correlation info2</i>	A different way of calculating the texture correlation.
<i>Texture energy</i>	Provides the sum of squared elements in the gray-level co-occurrence matrix. Also known as uniformity or the angular second moment.
<i>Texture entropy</i>	Entropy is a statistical measure of randomness that can be used to characterize the texture of the cell image.
<i>Texture homogeneity</i>	Measures how many different gray levels the image holds.
<i>Texture maxprob</i>	High values occur if one combination of pixels dominates the pixel pairs in the window.

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