HOLOMONITOR® APP SUITE PROTOCOL

FLUORESCENCE CAPTURE

This protocol helps to set up a Fluorescence Capture using HoloMonitor® M4FL and the HoloMonitor® App Suite software. The HoloMonitor® Fluorescence Capture provides holography time-lapse images for further analysis by any other HoloMonitor® App Suite application and accompanying fluorescence images for Single Cell Tracking and Cell Morphology analysis.

REQUIREMENTS:

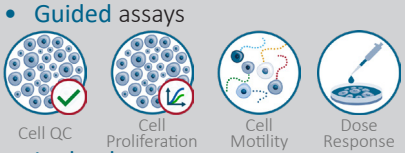
- HoloMonitor® M4 with M4FL, placed in incubator
- HoloMonitor® M4 App Suite
- Culture vessel of choice with cells, including fluorescent cells for positive control
- HoloLid™ for selected vessel
- Vessel holder for selected vessel

OUTPUT:

- Time-lapse images
- Time-lapse videos
- Fluorescence intensity values
- Selected application data analysis

REANALYSIS:

- Guided assays



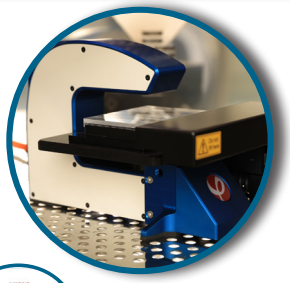
- In-depth assays

WORK FLOW:

PREPARATION

1. Seed cells and let adhere
2. Perform Auto-calibration
3. Add treatment
4. Put vessel(s) on vessel holder and place HoloLid™
5. Put vessel holder with cells on HoloMonitor®
6. Calibrate Fluorescence focus and overlay

EXPERIMENT SETUP



1. Choose Application
2. Define experiment
3. Set duration and capture interval
4. Check holographic image quality and focus
5. Check Fluorescence image focus
6. Start capture

RESULTS & ANALYSIS

1. Open Results Page
2. Perform In-Depth analysis
3. Export results
4. Create new results

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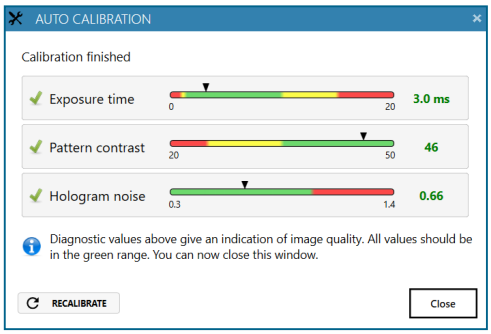
PREPARATIONS

Materials

- ✓ **HoloMonitor® M4FL**, placed inside the incubator
- ✓ **HoloMonitor® App Suite** software
- ✓ **Cell culture vessel.** Please check our ↗ list with recommended vessels.
- ✓ **Cells,** including a **positive fluorescence control**
- ✓ **HoloLid™** for the selected vessel
- ✓ **Vessel holder** for the selected vessel
- ✓ ↗ **Setup and Operational Manual** for HoloMonitor ®

Steps

1. Seed the cells with about **5 % confluence** (ca. 6000 – 11000 cells/cm^2).
   ►Please note that too few cells may lead to inadequate results due to auto-focus failure.
   ►Please note that a positive fluorescent control that is representative of your exmeriment set up is essential.
2. Place the vessel in the incubator and let **cells attach** for 2-24 hours.
3. **Start** the **software** and wait for complete instrument **initialization**.
4. Run an **auto-calibration**. With successful calibration, the instrument is ready to use.
5. **Sterilize** the **HoloLids™** according to the specific ↗ **HoloLid™** protocol.
6. Add the **treatment** to your cells. The final working volumes per well, essential for using **HoloLids™**, are shown in the table:



Successful auto-calibration window

Vessel	Vendor cat. number	HoloLid™	Final working volume	Growth area, cm²/well	Vessel cut in a holder
Sarstedt TC-dish 35	83.3900	71110	3.0 mL/well	8.00	NA
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
ibidi® µ-dish 35 mm, high	81156	71111	2.5 mL/well	3.50	NA
ibidi® µ-plate 24 Well Black ID 14 mm	82426	71132	1.5 mL/well	1.54	left
Eppendorf CCCadvanced® FN1 - 6 well	0038110010	71150	3.0 mL/well	9.40	bottom right

7. **Slide** the cell culture **vessel** onto the Vessel **holder**, its grips facing towards you. Ensure that the vessel is parallel to the holder. There is a spring that holds the vessel in place.
   ►When using multi-well plates, place them with the cut-off corner oriented according to the table.
8. **Replace** the standard lids with the **HoloLid™.**
9. **Put** the vessel **holder** with the sample on the **HoloMonitor® M4 stage**.

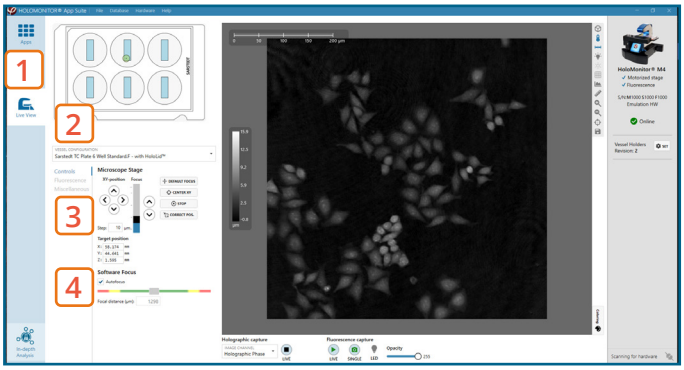
CALIBRATING FLUORESCENCE SIGNAL IN LIVE VIEW

1 Focus Holography image

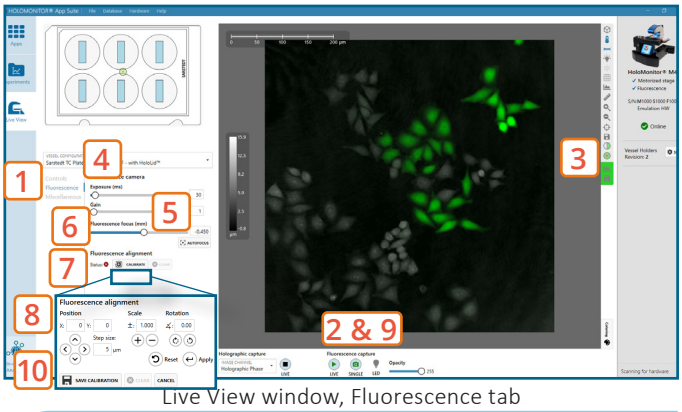
1. Go to **Live View Tab** on the left side panel.
2. Select the correct **vessel map** from the drop-down list.
3. Find a position with cells. Preferably, the position should be as centered in the vessel as possible.
4. In **Controls**, make sure that the holography image of the cells is in **focus by** adjusting the **Z-position** of the stage using the up/down arrows. Ensure that **Autofocus** is ticked and then adjust the **Z-position** until the Software focus bar shows **1300±5**.
   ► The **Z-position** will be saved with the calibration and used as the default **Z-position** during **Capture Setup**.

2 Calibrate Fluorescence Focus Plane

1. Go to the **Fluorescence** tab.
2. Turn on **Fluorescence live capture** by pressing the **Play** button (or take a **Single** image repeatedly) while manually adjusting the **Fluorescence focus** until you get a fluorescence signal.
   ► You can use the arrow keys on the keyboard.
3. Check that the view is set to mixed channel ( ).
4. Set **Exposure time** and **Gain**. For more information see HoloMonitor® M4FL Setup and Operation Manual.
   ► Increasing **gain** value might be beneficial when the fluorescence signal is weak, and App Suite struggles to find focus automatically.
5. Press **Autofocus** to automatically set best focus offset.
6. If the software fails to find optimal focus, adjust it manually by adjusting the **fluorescence focus** slider.
7. Press **Calibrate**.
8. Use the **Position**, **Scale** and **Rotation** controls to adjust and align the fluorescence overlay to fit the holographic image.
   ► This is an iterative step. Thus, position, scale, and rotation adjustments do not need to be performed sequentially.
   ► If needed, you can decrease the Step size to make finer adjustments. You can also change the opacity of the fluorescence overlay to compare it with the holographic image better.
9. Turn Off ( ) the Fluorescence **Live** Capture.
10. Press **Save calibration** when the fluorescence focus and alignment are as good as possible.
11. Go to the **Apps** tab and select the **Fluorescence Capture Application** and proceed by clicking the **Setup** button.



Live View window, Controls tab



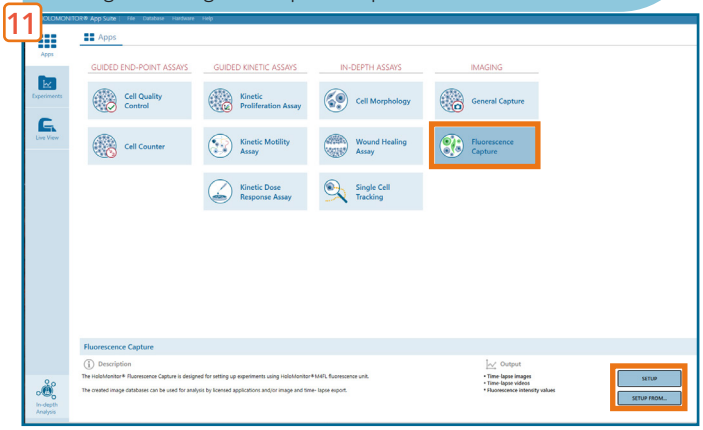
Live View window, Fluorescence tab

Fluorescence signal intensity can be evaluated using the measure tool (or histogram), which are found to the right of the Live View image.

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

If the **arrows** in **Controls** for **Microscope Stage** were used in step 1.3 to navigate to the location used for calibration, the holographic position might need to be corrected:

i. Go to **Controls**.
ii. Turn OFF the **Holographic Phase channel** by pressing the **Stop** button ( ).
iii. Press **Correct Pos**.
iv. Turn ON the **Holographic Phase channel** by pressing the **Play** button ( ).
v. Turn OFF the **Holographic Phase channel** by pressing the **Stop** button ( ).
vi. Take **Single** Fluorescence Capture.
vii. Check if Holographic Channel and Fluorescence Channel images are aligned. Repeat steps if needed.

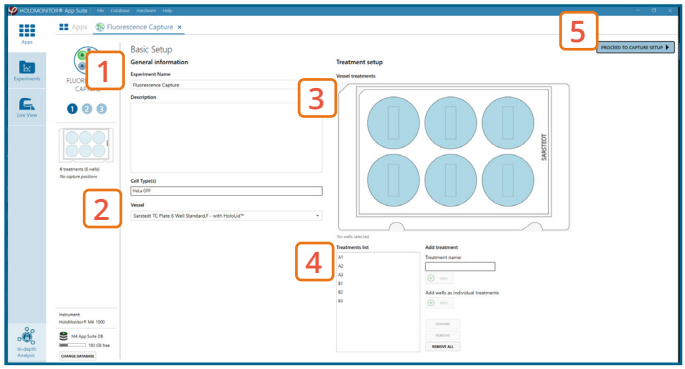


App Suite main window with selected Fluorescence Capture

EXPERIMENT SETUP

1 Basic setup: describe the experiment and assign treatments to the wells

1. Enter the experiment **name**, optional experiment **description** and cell types.
2. Select the correct **vessel map** from the drop-down list.
3. Map **treatments** on the vessel map. **Select wells** by marking them with the left mouse button while moving the cursor over the relevant well/s.
4. Add the **treatment name/s** in the text box below the vessel map and click **Add** /press Enter. It is possible to add wells as **individual treatments**. Marked well/s are light blue, selected wells will appear dark blue.
5. Proceed to **Capture setup**.



Basic Setup window

2 Capture setup: Set experiment time setting, choose and validate capture positions

1. Adjust the default settings for **duration** and **interval**.
2. **Set** Fluorescence capture **interval**.
   ► Captures set too often will induce phototoxicity.
3. Setup capture positions.

3.1. Setting capture locations:

3.1.1. Use the software **automatically** pre-set positions, change the number of positions if needed by pressing the **Regenerate pattern** button.

3.1.2. If automatically generated positions are not suitable, find and set the positions **manually** (see *Capture Setup: Manually choose and validate capture positions* section).

3.2. Setting Holographic focus:

► This step can be skipped if the Holographic **Software focus** was set manually to **1300±5**.

3.2.1. Go to the **Holography** tab.

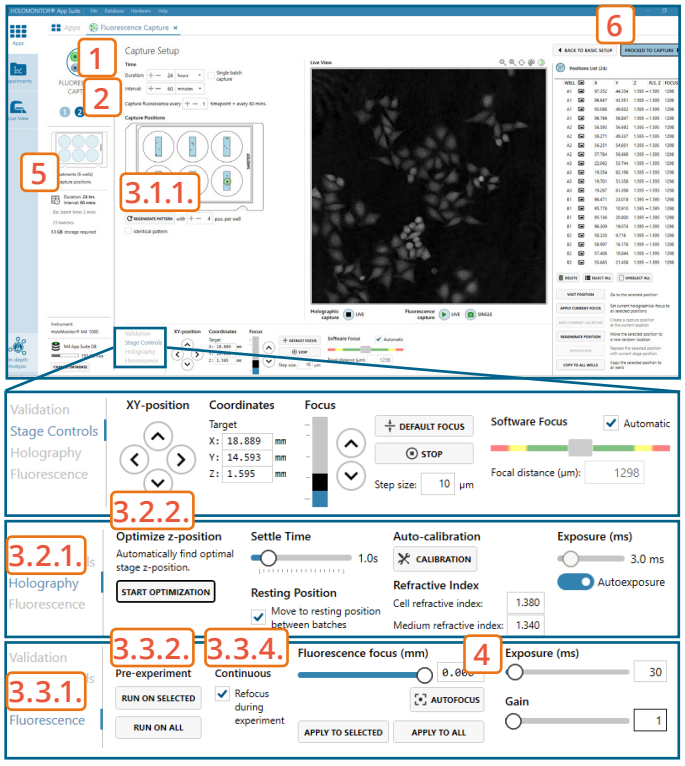
3.2.2. **Automatically** optimize the Z-position by pressing the **Start Optimization button**. The software automatically tries to set a Z-value, where the **Software focus** would be as close as possible to **1300±5**. Values for the inspected positions are displayed in the **Position list**.

► This function also validates the image quality. For more information about image quality, consult *App Suite Setup and Operation Manual.*

3.2.3. If the Automatically set **Z-value** does not lead to a **Software focus** of **1300±5**, adjust the **Z-value manually** (see *Capture Setup: Manually choose and validate capture positions* section).

3.3. Setting Fluorescence focus:

3.3.1. Go to the Fluorescence tab.



Capture Setup window with opened setup tabs

For the **best Fluorescence imaging result**, the Holographic Image Software focus value should be as close as possible to **1300±5**. Adjusting the Actual Z-value for each position might be necessary.

3.3.2. To automatically adjust the Fluorescence focus value for selected positions that have fluorescence signal at the beginning of the capture or all positions, press **Run on Selected** or **Run on All** under **Autofocus Pre-experiment**.

► This step can be skipped for positions/wells where the fluorescence signal appears later during the capture.

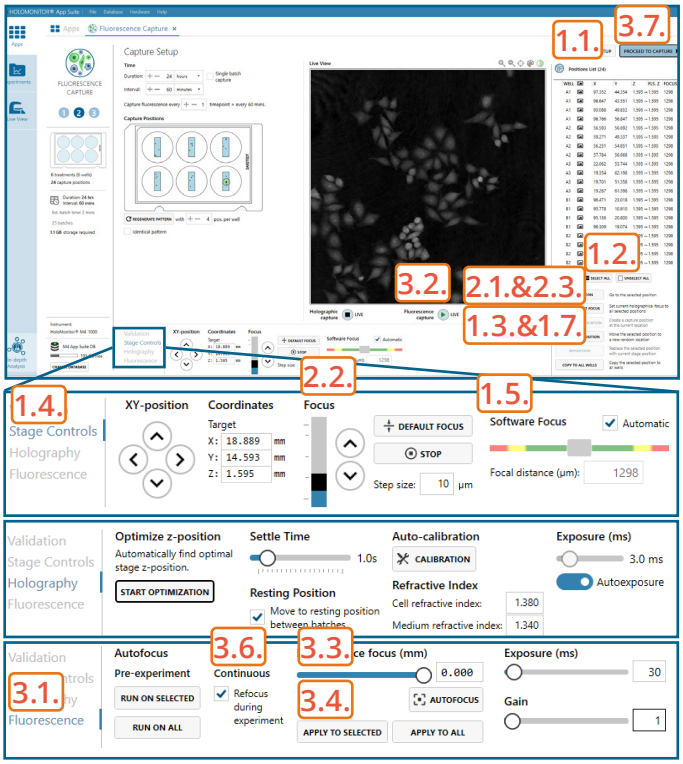
3.3.3. To **manually** adjust the Fluorescence focus value for the selected position or all positions, see *Capture Setup: Manually choose and validate capture positions* section.

3.3.4. Enable **Continuous Refocus during experiment** for the software to adjust Fluorescence focus during the capture.

4. Adjust the fluorescence **exposure time** and **gain** if needed in the **Fluorescence tab**.
5. Ensure that the **storage requirement** for the experiment does not exceed the computer capacity.
6. When satisfied with the experiment setup, click **Proceed to Capture**.

Capture setup: Manually choose and validate capture positions

1. Setting capture locations:
   1.1. Click the **positions list** button to view the list.
   1.2. Press the **Select all** button and **Delete** to remove automatically generated positions.
   1.3. Press **Add Current Location** to add Fluorescence calibration position if wanted.
   1.4. Find a position with cells using arrows in **Stage Controls** or by clicking on the vessel map.
   1.5. Adjust the **Z-value** to the position where the **Software focus** is **1300±5**.
   1.6. If satisfied, press **Add current location**.
   1.7. **Repeat** until satisfied.
2. Setting Holographic focus:
   2.1. **Visit** the position.
   2.2. Change position **Z-value** using Focus arrows in **Stage Controls**. Once the **Software focus** is **1300±5**, press **Apply Current Focus**.
   2.3. If satisfied, proceed to the next position. Otherwise, find a new location.
3. Setting Fluorescence focus:
   3.1. Go to the **Fluorescence** tab.
   3.2. Start **Fluorescence live** feed.
   3.3. Use **Fluorescence focus** slider to achieve the sharpest fluorescence image.
   3.4. Once satisfied, press **Apply to Selected** or **Apply to All**.



Capture Setup window with opened setup tabs

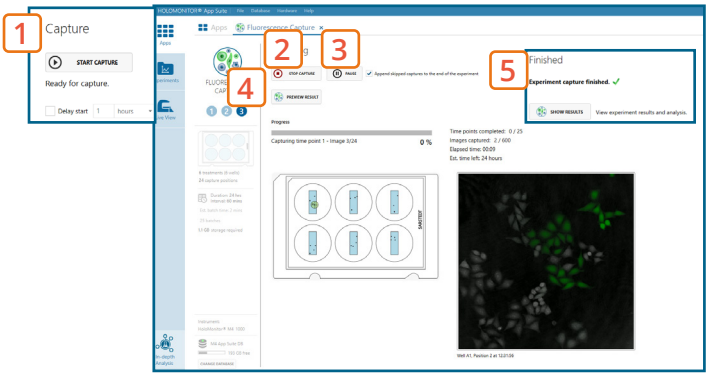
3.5. Repeat for other positions if needed until satisfied.

3.6. Enable **Continuous Refocus during experiment** for the software to adjust Fluorescence focus during the capture.

3.7. Click Proceed to capture.

3 Capture: Review the experiment in real-time during the time-lapse

1. Click **Start Capture**.
2. To stop the experiment ahead of time, click the **Stop Capture** button.
   ► It is **not** possible to restart the experiment once it has been stopped.
3. To pause the experiment, click the **Pause** button. Press **Resume** button to continue the experiment.
   ► Missed frames can be appended to the end of the experiment or skipped.
4. Click on **Preview Results** to preview the captured images during the run.
   ►Wait for the experiment to fully finish before starting data analysis.
5. When the Experiment capture finishes, click the **Show Result** button to go directly to the **Experiment Overview** tab.

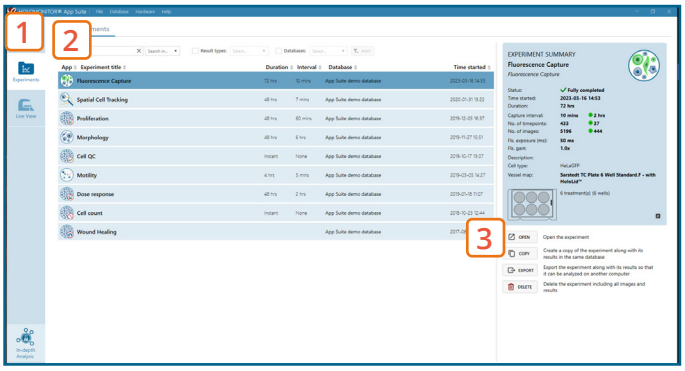


Capture window

RESULTS & ANALYSIS

Experiments tab

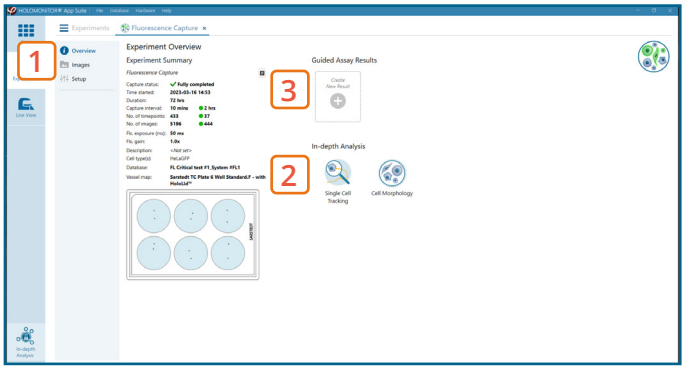
1. Click Experiments to see a **list of the experiments**.
2. Click on the experiment title to open an **experiment summary**.
3. Click **Open** or double-click left mouse button to open the results page.



Experiments tab

Experiment overview tab

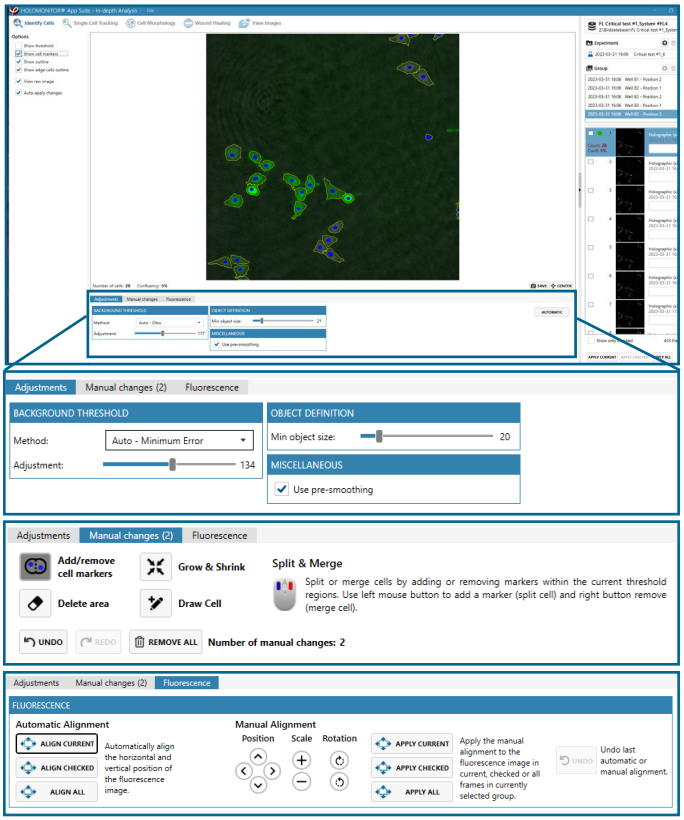
1. See the experiment **summary**, view all **images** and go to the experiment **setup**.
2. Generate **In-depth analysis** data (fluorescence green area (µm²), fluorescence green average, fluorescence green max, fluorescence green min, fluorescence green std, and fluorescence green sum) from the captured images by clicking on the **Cell Morphology** or **Single Cell Tracking** icon. A **new window** for the In-depth analysis will open.
3. **Create New Guided Assay Results** from this experiment by clicking the button.
   ► Note that only the DHM images will be analysed using the guided assay analysis.



Experiment overview tab

In-depth analysis

- ✓ The first step for **Cell Morphology** analysis and **Single Cell Tracking** is to identify the cells.
- ✓ The software automatically calculates cell count and confluence for each image.
- ✓ Review the image quality and include/ exclude images from results analysis. See the **Image guide** for more information.
- ✓ In the **Identify cells** under **Fluorescence tab**, adjust **Holographic** and **Fluorescence Image overlay** automatically or manually for individual frames, selected frames, or all frames.
- ✓ Follow corresponding **Cell Morphology** or **Single Cell tracking** application protocols for data analysis.



Identify cells window

One experiment — multiple results

- ✓ This section helps to reanalyse data between different applications using HoloMonitor® App Suite software.

Generating In-depth Assay results

1. In the **Experiment overview page** select the In-depth application icon for wanted result.
2. **Follow** the respective assay protocol .

Generating Guided Assay results

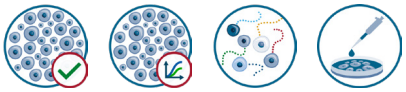
1. In the **Experiment overview page** under Guided Assay Results select **Create New Result**.
2. **Choose type** of analysis in the pop up window and **name** the new result.
3. If there are previous analyses it is possible to tick **copy image analysis from** and **select** the experiment to copy from. This will copy the image analysis settings from the selected result including all changes.
   ► For further data analysis steps, please see the respective assay protocol .
4. **Press create**.

Obtain these results from the Fluorescence Capture data:

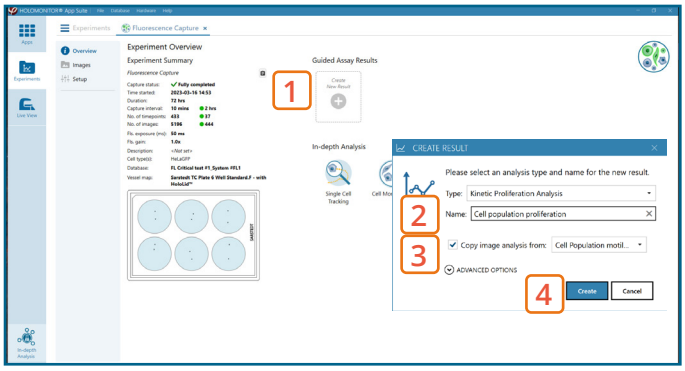
In-depth assays

Single Cell Tracking | Cell Morphology

Guided assays



Cell Quality Control | Kinetic Cell Proliferation | Kinetic Cell Motility | Kinetic Dose Response



Experiment overview tab