HOLOMONITOR® APP SUITE PROTOCOL

SINGLE CELL TRACKING

This protocol helps to set up a Single Cell Tracking using HoloMonitor® M4 and the HoloMonitor® App Suite software. The HoloMonitor® Single Cell Tracking facilitates label-free characterization of heterogeneous live cell behavior over time on an individual cell level. The HoloMonitor® Single Cell Tracking can also be used to analyse fluorescence data obtained with HoloMonitor® M4FL, for more information see HoloMonitor® Fluorescence Capture protocol.

REQUIREMENTS:

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

OUTPUT:

- Plots of cell movement and morphological features over time
- Interactive cell family trees
- Calculation spread sheets of all data including graphs and population averages

REANALYSIS:

- Guided assays

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

- In-depth assays

Single Cell Tracking | Cell Morphology

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®

EXPERIMENT SETUP

1. Choose Application
2. Define experiment
3. Set duration and capture intervals
4. Check image quality
5. Start capture

RESULTS & ANALYSIS

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

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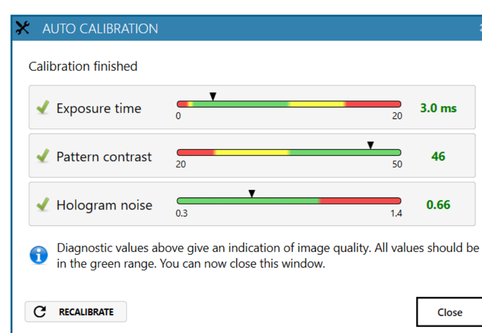
PREPARATIONS

Materials

- ✓ **HoloMonitor® M4**, placed inside the incubator
- ✓ **HoloMonitor® App Suite** software
- ✓ **Cell culture vessel.** Please check our list with recommended vessels.
- ✓ **HoloLid™** for the selected vessel
- ✓ **Vessel holder** for the selected vessel
- ✓ **Cells**
- ✓ **Setup and Operation Manual** for HoloMonitor ®M4

Steps

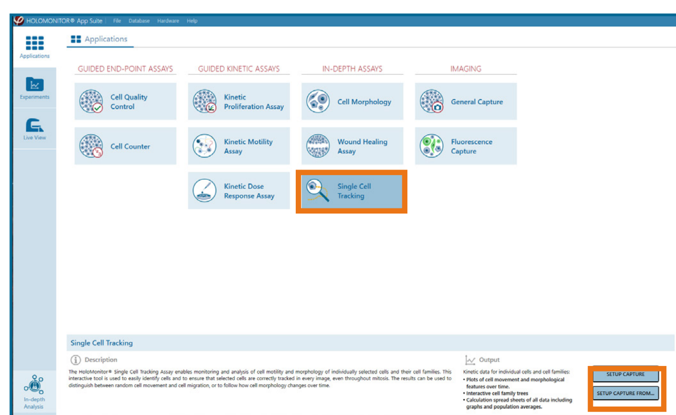
1. Seed the cells with about **5 % confluence** (ca. 6000 – 11000 cells/cm²).
   ▶Please note that too few cells may lead to inadequate results due to auto-focus failure.
2. Place the vessel in the incubator and let **cells attach** for 2-24 hours.
3. **Start** the **software** and wait for complete instrument **initialization**.
4. Run an **auto-calibration**. With successful calibration, the instrument is ready to use.
5. **Sterilize** the **HoloLids™** according to the **HoloLid™** sterilization and use 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	80241	71131	2.5 mL/well	1.90	NA
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 according to the table above.
8. **Replace** the standard lids with the **HoloLid™**.
9. **Put** the vessel **holder** with the sample on the **HoloMonitor® M4 stage**.
10. Select the **Single Cell Tracking** application and proceed by clicking the **Setup Capture** button.

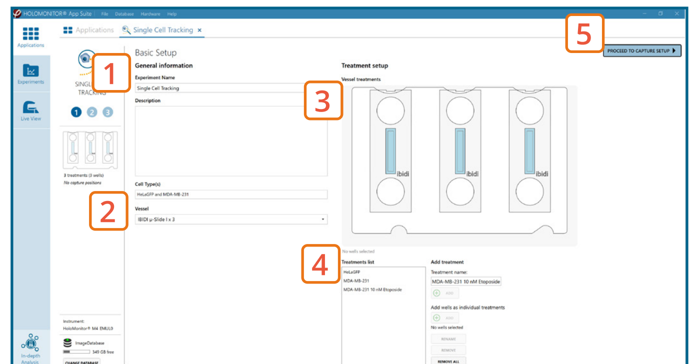


AppSuite main window with selected Single Cell Tracking application

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: Select the experiment time settings and choose capture positions

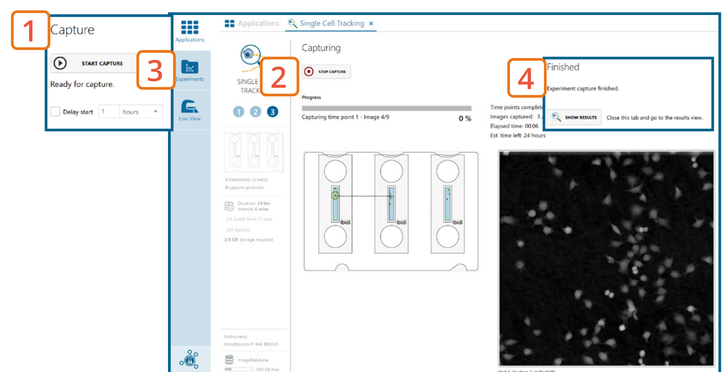
1. Adjust the default settings for **duration** and **interval**.
2. Add **capture positions**: The position list is open by default. Click positions on the vessel map and add them to the position list with the **Add current location button**. In case the image quality is poor, a warning sign ⚠ appears. **Adjust focus** or **position location** if necessary.
3. Ensure that the **storage requirement** for the experiment does not exceed the computer capacity.
   ► When running an experiment, **data needs to be stored on the computer** connected to the instrument. Storing data on an external drive (e.g. connected via USB or internet server) may cause data loss due to erratic USB connections or poor internet connection.
4. Run a full or quick **validation** of the selected positions to ensure **good image quality**.
5. When satisfied with the experiment setup, click **Proceed to Capture**.

Capture Setup window

MANUAL FOCUS ADJUSTMENT: The focusing tool is located in the **Controls tab**. Move the black square or click on the Arrow buttons to move the stage up and down. Save an adjusted focus setting for the selected position by using the **Apply Current Focus** button. For details, consult the Setup and Operation Manual.

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 button.
   ► It is NOT possible to restart the experiment once it has been stopped.
3. Go to the **Experiments tab** and open your ongoing experiment to preview the captured images during the run.
   ► Wait for the experiment to finish before starting In-depth Analysis.
4. When the Experiment capture finishes, click the **Show Result** button to get directly to the **Results page**.

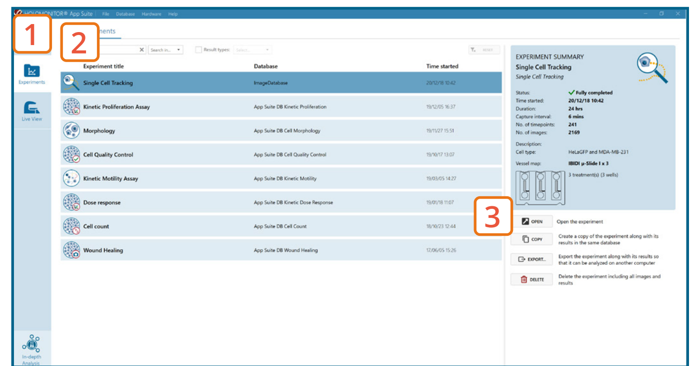


Capture window

RESULTS & ANALYSIS

Experiments tab

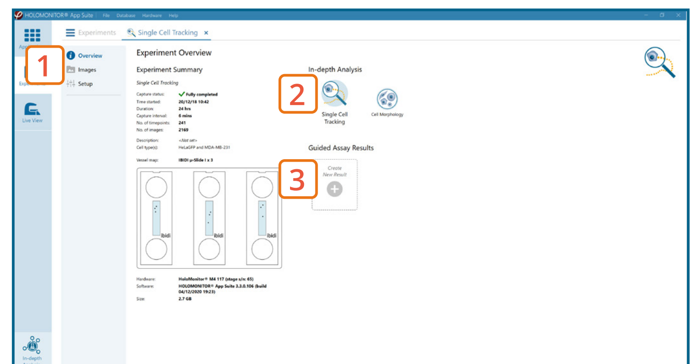
1. Click Experiments to see a **list of the experiments**.
2. Click on the experiment title to open an **experiment summary**.
3. Click **Open** to open the results page.



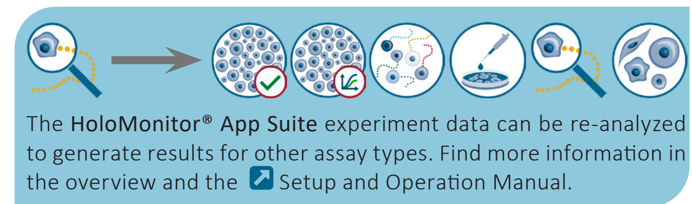
Experiments tab

Experiment overview tab

1. See the experiment **summary**, view all **images** and go to the experiment **setup** by choosing the respective tab.
2. Generate **in-depth analysis** data from the captured images by clicking on the **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 respective button.



Experiment overview tab



The **HoloMonitor® App Suite** experiment data can be re-analyzed to generate results for other assay types. Find more information in the overview and the Setup and Operation Manual.

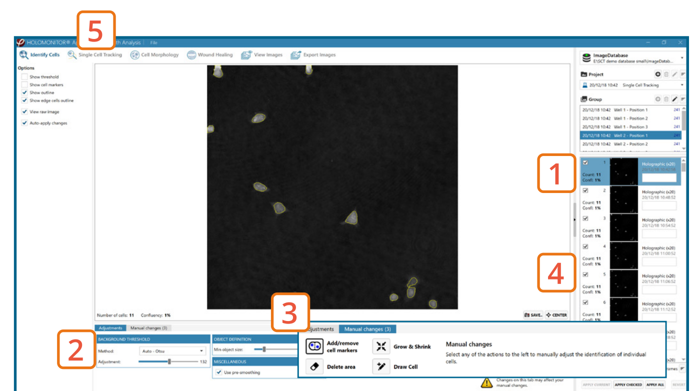
In-depth analysis — identify cells tab

✓ First step for single cell tracking analysis is to identify the cells.

✓ The software automatically calculates cell count and confluence for each frame.

✓ Review the image quality and adjust or exlude frames from results analysis. See the **Image quality guide** for more information.

1. Start by selecting a **frame of interest**, so the image is displayed. You can alter the viewing options.
2. **Adjust** the background **threshold** and **object size** so the mask best fits the cell segmentation and click **Apply** (Current/ Checked/ All) to use the settings for the frames and to display the **automatic calculation** of **cell count (#/frame)** and **confluence (%)**.
3. **Manual changes** are possible. Add/remove cell markers or grow/ shrink cell, delete cell area or draw cell area. These changes will **only** be applied to the **currently selected frame**.
4. **Check** cell segmentation in frames throught the experiment to make sure the applied threshold fits. Scrolling through the frame list and checking for abnormal jumps in cell count or confluence values may help see if any adjustments are necessary.
5. Once satisfied with the cell identification, **click** on the **Single Cell Tracking** tab to begin analysis.



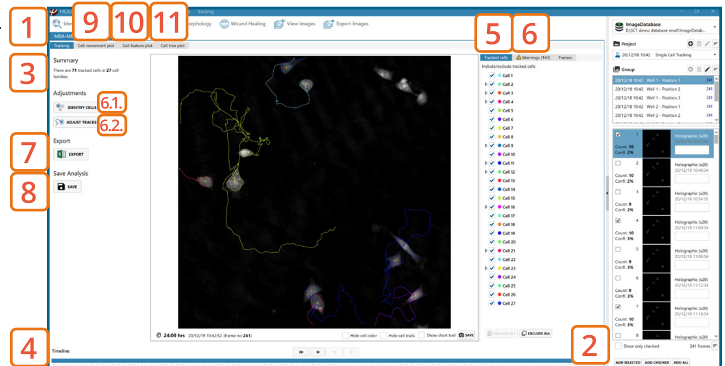
Identify cells tab

It is **important for in-depth analysis that cell identification is done correctly**. Thus, the more thoroughly the segmentation is performed, the better analysis results are in the following steps.

It is the **users responsibility to verify** that the software algorithm was correctly applied during analysis and that results are accurate.

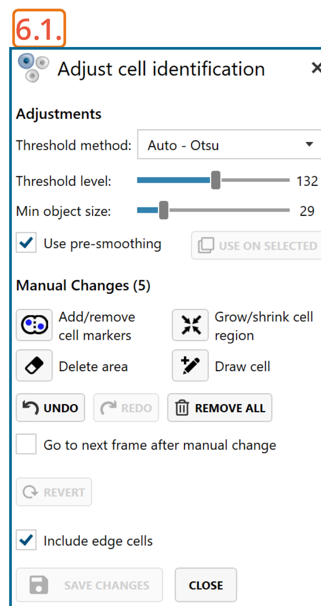
In-depth analysis — single cell tracking tab

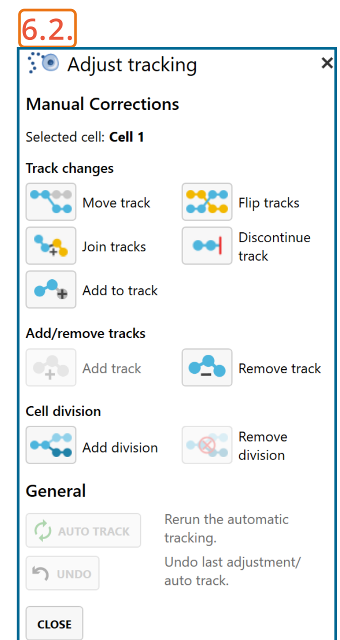
- ✓ Review the image quality and include/ exclude frames from analysis. See the **Image quality guide** for additional information.
- ✓ For further analysis all results can be exported.
- ✓ For more details, see the **Setup and Operation manual.**



Spatial Cell Tracking tab

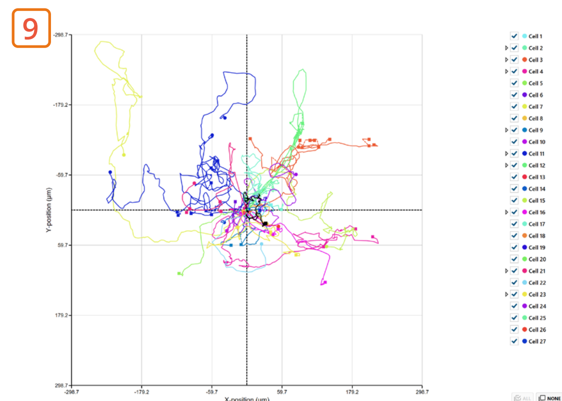
1. Click **New Analysis** to start a new tracking analysis or load a previous one by clicking the Open button.
2. **Add** at least **two frames** to the analysis using the Add Selected/ Add Checked/ Add All button.
3. **Software** automatically tracks all the cells in the included frames from a time-lapse.
   ▶ At the top left corner there is a **summary** of the number of cell and cell families tracked.
4. Use the **timeline slider** to view cell tracks over time in the time-lapse and evaluate cell tracking status.
5. **Include** and **exclude** cells and/or cell families in the tracking by checking/unchecking tick boxes in the **tracked cells** list.
   ▶ **Tracking information** and **cell features** for cells and/or cell families included in tracked cells list will be added to the **Excel export** file.
6. A list of **possible warnings** resulting from tracking issues such as cells leaving the field of view, overlapping or dividing. Resolve the warnings by:
   6.1. first **adjusting** specific cell and or full frame **segmentation** using **Identify cells** funtions.
   ▶ Segmentation changes created here are applied globally and can influence the results in other In-depth assays.
   6.2. then **adjusting tracks** manually. For more details on each button function, see the **Setup and Operation manual.**
   6.3. or **choosing** to **ignore** the warning.
   ▶ **Repeat** steps 6.1. to 6.3. until all warnings are resolved and cell tracks are correct.
7. **Export** the tracking data to **Excel. Cell features** for cells and cell families included in step 5 are exported. The exported file can **include**: cell feature averages, cell feature data for all individually tracked cells and cell families, as well as cell movement and cell tree plots.
8. **Save** tracking analysis to return to it later.
9. In the **Cell movement plot tab**, the tracking data for the selected cells and/or cell families is displayed





Analysis tips:

- Always start resolving warnings by adjusting cell identification. If this step fails, only then adjust tracking manually.
- Use Auto Track funtion after manual track adjustments.

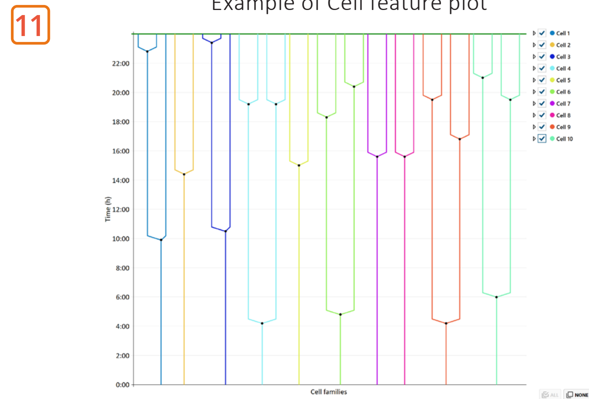


Example of Cell movement plot

in a plot showing the **cell track traces.** Plots can be saved and scale/display options can be changed.

10. In the **Cell feature plot tab**, cell feature changes of selected cells and/or cell families can be viewed over time. Select the feature of interest from the drop-down list. Plots can be saved and scale/display options can be changed.
11. In the **Cell tree plot tab**, cell family trees for selected cell families feature changes of selected cell families are shown. Plots can be saved and scale/display options can be changed.

Example of Cell feature plot



Example of Cell tree plot

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 ↗.

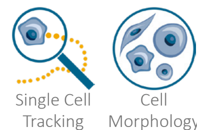
Generating Guided Assay results

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. 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.

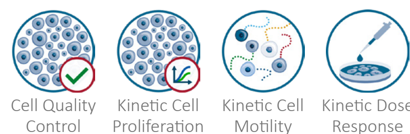
   ► For further data analysis steps, please see the respective assay protocol ↗ .
4. **Press create.**

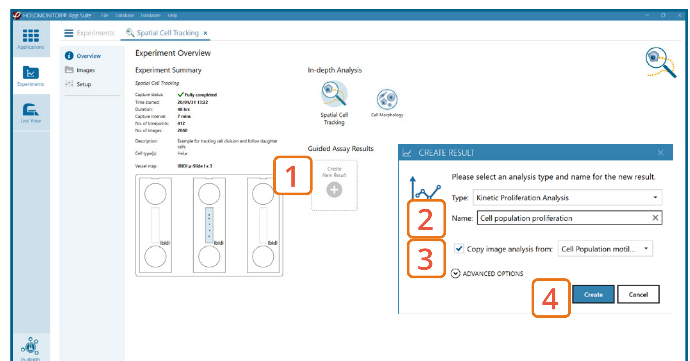
Obtain these results from the Single Cell Tracking data:

In-depth assays



Guided assays





Experiment overview tab

When **creating a New Guided Assay** result from **Single Cell Tracking** experiment - the **first** analysis will take some time, as the software needs to evaluate image quality and perform image analysis.