HOLOMONITOR® APP SUITE PROTOCOL

SPATIAL CELL TRACKING ASSAY

This protocol helps to set up a Spatial Cell Tracking Assay using HoloMonitor® M4 and the HoloMonitor® App Suite software. The HoloMonitor® Spatial Cell Tracking Assay facilitates label-free characterization of heterogeneous live cell behavior over time on a individual-cell level.

REQUIREMENTS:

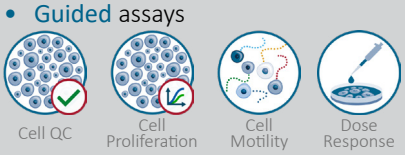
- HoloMonitor® M4, placed in incubator
- HoloMonitor® M4 App Suite
- Culture vessel of choice with cells
- HoloLid™ for selected vessel
- Vessel holder for selected vessel

OUTPUT:

- Movement diagram
- Diagram of features over time
- 30+ invidual cell parameters
- Calculation spread sheets of all parameters, including population averages

REANALYSIS:

- Guided assays



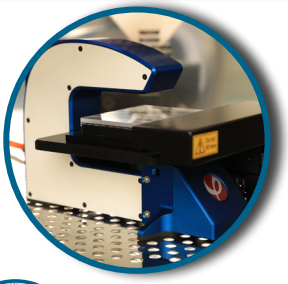
- In-depth assays

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®

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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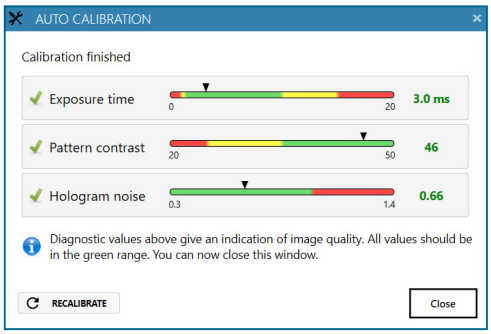
PREPARATIONS

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 Operational Manual** for HoloMonitor ®M4

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.
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	HoloLid™	Final volume	Growth area, cm²/well
Sarstedt TC-dish 35 (cat. 83.3900)	71110	3.0 mL/well	8.0
Sarstedt TC 6-well plate (cat. 83.3920.005)	71120	3.0 mL/well	8.8
Sarstedt lumox® 24-multiwell plate (cat. 94.6000.014)	71130	1.8 mL/well	1.9
Sarstedt lumox® 96-multiwell plate (cat. 94.6000.024)	71140	170 µL/well	0.2
ibidi® µ-dish 35 mm, high (cat. 81156)	71111	2.5 mL/well	3.5
ibidi® µ-plate 24 Well Black (cat. 81156)	71131	2.5 mL/well	1.9

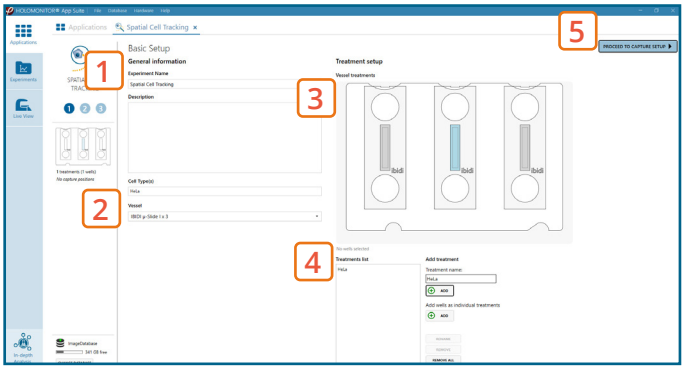
7. **Slide** the cell culture **vessel** onto the Vessel **holder**, its grips facing towards you. Ensure that the vessel is parallel to the holder. There is a spring that holds the vessel in place.
   ►When using multi-well plates, place them with the cut-off corner to the left.
8. **Replace** the standard lids with the **HoloLid™.**
9. **Put** the vessel **holder** with the sample on the **HoloMonitor® M4 stage** and click it to secure.
10. Select the **Spatial Cell Tracking Assay** and proceed by clicking the **Setup Assay** button.

AppSuite main window with selected Spatial Cell Tracking **Assay**

EXPERIMENT SETUP

1 Basic setup: describe the experiment and assign treatments/conditions 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 and conditions** 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**.

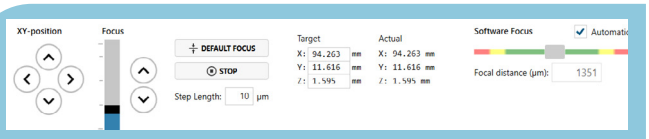


Basic Setup window

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.
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**.

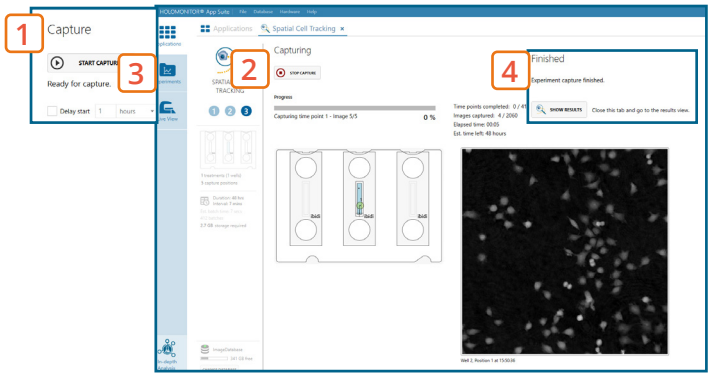
Capture Setup window



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 Operational 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. Note that 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**.

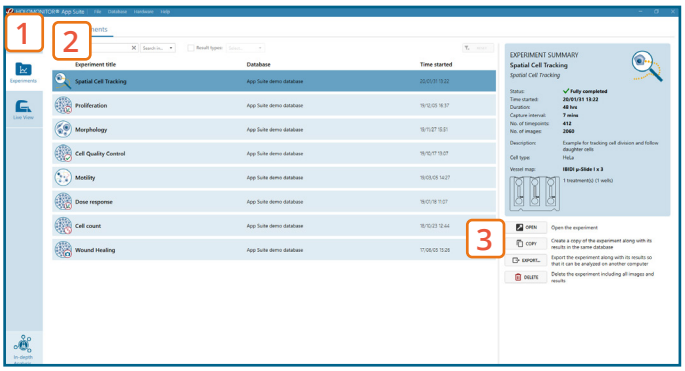


Capture window

RESULTS & ANALYSIS

Experiments tab

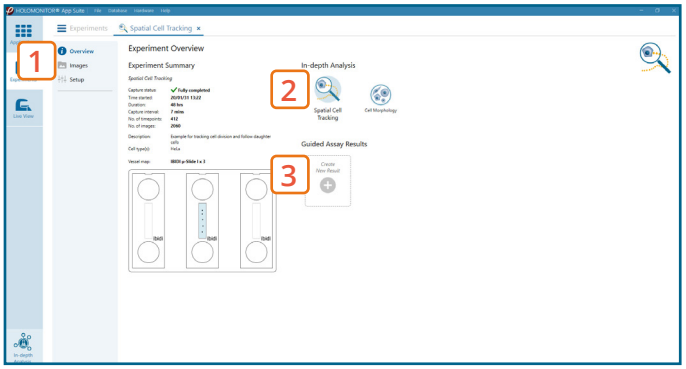
1. Click Experiments to see a **list of the experiments**.
2. Click on the experiment title to open an **experiment summary**.
3. Click **Open** to open the results page.



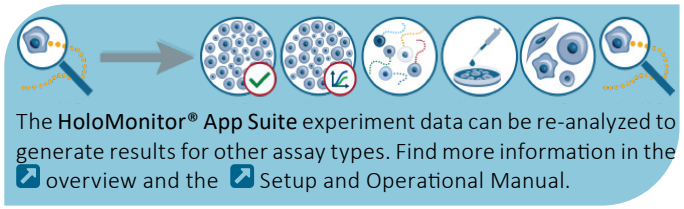
Experiments tab

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 **Spatial 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



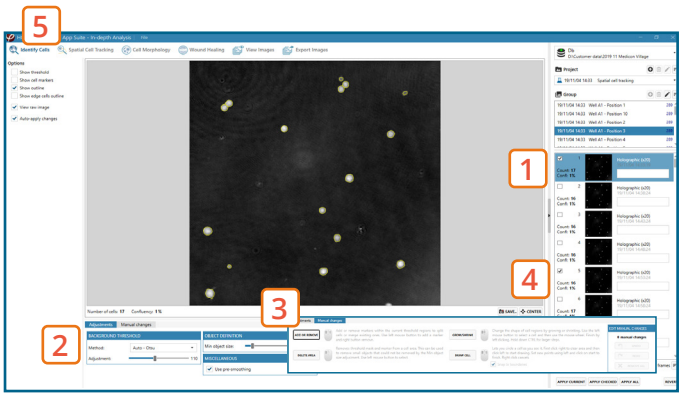
In-depth analysis — identify cells tab

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

✓ The software automatically calculates cell count and confluence of each image.

✓ Review the image quality and include/ exclude images from results analysis. See the **Image 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 fix the settings to the frames and to display the **automatic calculation** of **cell count (#/frame)** and **confluence (%)**.
3. **Manual changes** are possible. Add/remove or grow/ shrink a cell, delete a cell area or draw a cell area yourself. These changes will **only** be saved to the **currently selected image**.
4. **Check** cell segmentation for some images in the beginning, middle and end of the experiment to make sure the applied threshold fits. Scrolling through the image list and checking for abnormal jumps in cell count or confluence values may help as well to see if adjustments are necessary.
5. Once satisfied with the cell identification, **click** on the **Cell Spatial Cell Tracking** tab to start morphology analysis.



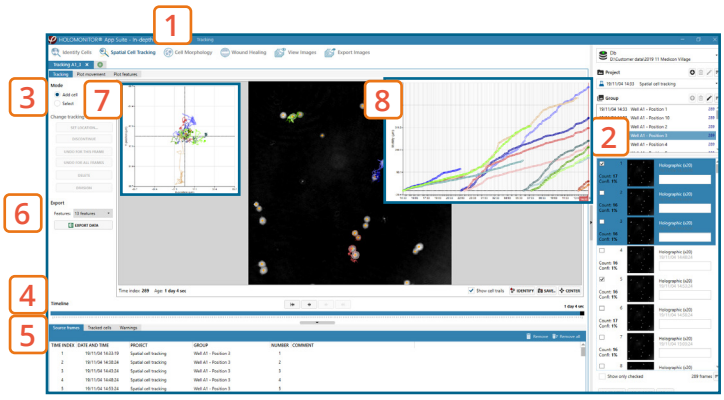
Identify cells tab

In-depth analysis — spatial cell tracking tab

✓ Review the image quality and include/ exclude images from results analysis. See the image guide for more information.

✓ For further analysis all results can be exported.

✓ For more details, see the **Setup and Operational 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 either by drag-and-drop or using the Add Selected/ Checked/ All button.
3. **Click** on a **cell** in the image to **start tracking** it. You can start/ stop tracking cells at any time point by using the **Change tracking** functions.
4. Use the **timeline slider** to view the colorful cell tracks over time in the image.
5. The **table** contains:
   —a list of **source frames** for this tracking analysis
   —a list of the **tracked cells** with color coding and displaying information on cell heritage, tracking status, age, motility (mm), motility speed (mm/ h), migration (mm) and directness (migration/ motility)
   ►More information on these parameters can be found in the **Setup and Operational manual**.
   — a list of **possible warnings** resulting from tracking issues such as cells leaving the field of view, overlapping or dividing. Resolve the warnings by clicking on the ⚠ icon, using the **Change Tracking functions** or choose to select the **Ignore** checkbox.
6. You can **export** the tracking data to **Excel** and choose the included **cell features** from the drop-down list. The exported data include information on the frames used, average cell feature values and selected cell feature data for all individually tracked cells.
   ►It is possible to **save** your tracking analysis to return to it later.
7. In the **plot movement tab**, the tracking data of the selected cells is displayed in a diagram showing the **cell track traces.** You can save the plot and change its scale / display options.
8. In the **plot features tab**, cell attribute changes of the chosen cells can be viewed over time. Select the feature of interest from the drop-down list. You can save the plot and change its scale / display options.