## MATERIAL

- **HoloMonitor® M4** placed inside a cell incubator.

- **Hstudio software,** version 2.7.3 or later.

- **Culture vessel**: ibidi® Culture-Insert 2 Well in ibidi µ-Dish 35 mm, high (cat. # 81176) or ibidi Culture-Insert 2 Well in ibidi µ-Plate 24 Well (cat. # 80241) and protocols for their use, provided by your local ibidi distributor.

- PHI **HoloLid™** for selected ibidi vessel and protocol for its use; HoloLid 71111 for ibidi µ-Dish 35 mm and HoloLid 71131 for ibidi µ-Plate 24 Well. HoloLid product information and protocol is available here.

- PHI **vessel holder** for selected ibidi vessel. For information regarding vessel holders contact PHI at support@phiab.se.

- **Cells** suspended according to the ibidi protocol.

- Setup and Operation Manual for using HoloMonitor M4, if the user is unfamiliar with the imaging procedures.

  We recommend that each experiment is performed at least three times to acquire sound statistics.
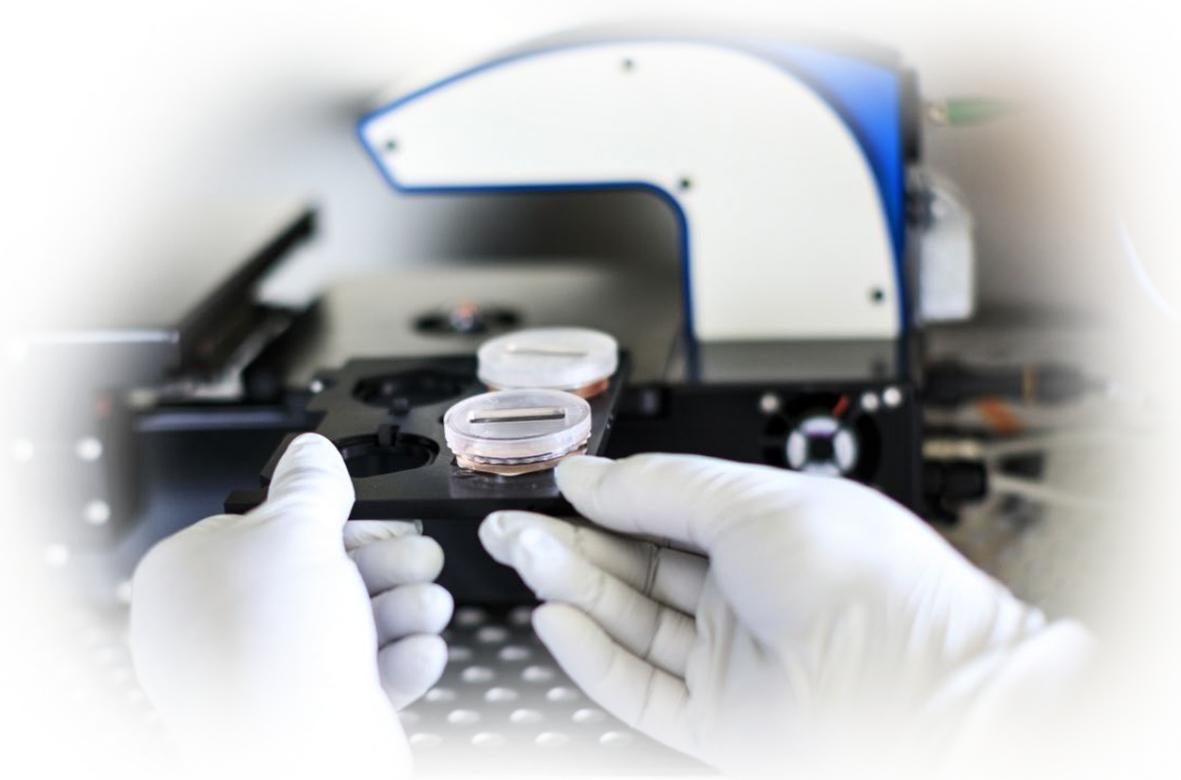
# PREPARATION

1. Seed the cells. When starting the wound healing experiment, the cell density in the wells should be approximately 90 %. Seed the cells according to the ibidi protocol. Note that the surface areas in the inserts are 0.22 cm$^2$ each. To avoid bubbles forming when seeding the cells, reverse pipetting is recommended.

2. Add the treatment according to experimental setup, either at seeding or when the inserts are removed and the wound healing experiment begins.

3. Put the cell culture vessel with the standard lid into the cell incubator and allow the cells to attach.

4. Sterilize the *HoloLids* according to the *HoloLid protocol*.

5. Start-up *HoloMonitor* and calibrate the system as instructed by the Hstudio software. The values achieved should lie within the green area of the calibration results bar.

6. Put on the standard lid.

7. Look at the cells in the inserts to ensure 90 % confluence.

8. Remove the inserts according to the ibidi protocol; there will now be a gap in the cell layer. Remove the cell culture medium. If there are many floating cells in the cell cultures, rinse the cultures once with medium, and then add new medium. The final working volumes, essential for using *HoloLid*, are 2.5 ml for both types of cell culture vessels, ibidi Culture-Insert 2 Well in ibidi µ-Dish 35 mm, high (cat. # 81176) and ibidi Culture-Insert 2 Well in ibidi µ-Plate 24 Well (cat. # 80241). Remember to take into account that the volume of the treatment adds to the final working volume.

> ### Tip
> Use pre-warmed cell culture medium. Otherwise there is a high probability of condensation forming on the outside of the vessel, causing image disturbances.

9. Put the standard ibidi lid onto the cell culture vessel.

# IMAGING

## For imaging with a motorized stage

1. Wipe off the **Vessel holder** with alcohol and put it in to the LAF-bench, the grips facing down.

2. Place the cell samples on to the **Vessel holder**. Place the sample as shown below and make sure the cell gaps are aligned as indicated by the green arrows, i.e. the cell front is parallel with the green arrow:
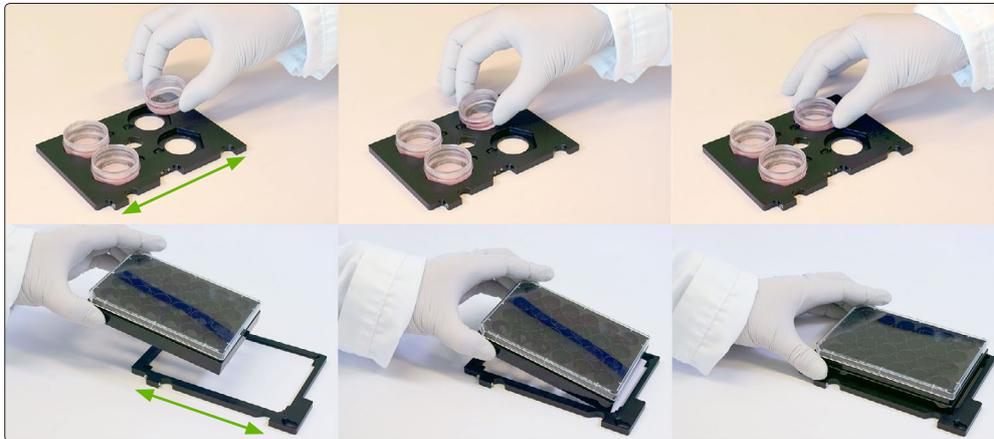


3. Replace the standard lids with the appropriate **HoloLid**, following the HoloLid protocol.

4. Thereafter place the **Vessel holder** with the samples on the HoloMonitor stage (right image).

5. Go to the **Live capture tab** in the Hstudio software and select the appropriate vessel template in the **Stage position side panel**:



| Vessel | Template |
|--------|----------|
| ibidi Culture-Insert 2 Well in ibidi μ-Dish 35 mm | ibidi Culture-Insert 2 Well in μ-Dish 35 mm |
| ibidi Culture-Insert 2 Well in ibidi μ-Plate 24 Well | ibidi μ-plate 24 well with Culture Inserts |

6. Create a **Project** for image storage.

7. Check **Timelapse** and type the total time and interval of the time-lapse capture. For good statistics, the speed of the cells should be considered when setting the duration of the time-lapse, as well as the interval between image captures. The faster the cells move, the shorter the time-lapse and the closer between captures. Typically, a 12 hour time-lapse with an interval of one hour between captures is adequate. At least 10 captures are recommended to acquire enough data. If the user later wants to track individual cells in addition to the gap closure analysis described in this protocol, by using the Hstudio tracking module, it is recommended to set the interval to 5 minutes between captures.

8.  Find each gap by left-clicking the center of the light blue squares in the *Stage position side panel* (right image). The stage can be moved in *Step size* increments by using the arrow buttons or the arrow keys on the numerical pad.

9.  When a gap has been found, click the *Remember button* to save the current position and focus. A saved capture position is indicated by a small red dot.

10. Click *Advanced Setup* and check *Multiple destination groups*.

11. Make sure *One group per well* is unchecked.

12. Click *Save and close*.

13. Click the *Capture button* in the *Capture side panel*.

14. Go to the *View image tab* and review the first set of images for quality.

15. Await the multiple time-lapse capturing to finish.
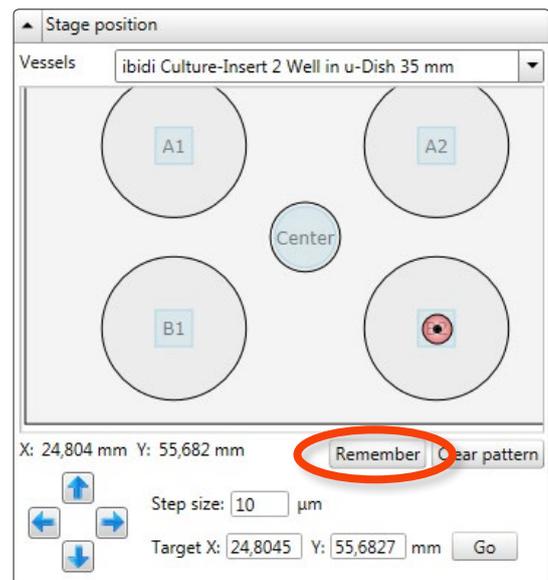
## For imaging with a fixed stage

1.  Replace the standard lid with the appropriate *HoloLid*, following the HoloLid protocol.

2.  Place the sample on the *HoloMonitor stage* using the # 3 distance plate.

3.  Go to the *Live capture tab* and ensure that the images are well focused. Adjust the software focus, if required.

4.  Create a *Project* and one *Group* per sample, i.e. "Control1 Day 1" as one sample.

5.  Check *Timelapse* and type the total time and interval of the time-lapse capture. For good statistics, the speed of the cells should be considered when setting the duration of the time-lapse, as well as the interval between image captures. The faster the cells move, the shorter the time-lapse and the closer between captures. Typically, a 12 hour time-lapse with an interval of one hour between captures is adequate. At least 10 captures are recommended to acquire enough data. If the user later wants to track individual cells in addition to the gap closure analysis described in this protocol, by using the Hstudio tracking module, it is recommended to set the interval to 5 minutes between captures.

6.  Click *Capture*.

7.  Go to *View image tab* and review the images for quality.

8.  Await the time-lapse capturing to finish.

# ANALYSIS

The gap closure of a wound healing experiment can be analyzed without prior cell identification.

1. Go to the **View Images tab** and remove bad images.

2. Select the **Wound healing tab**:
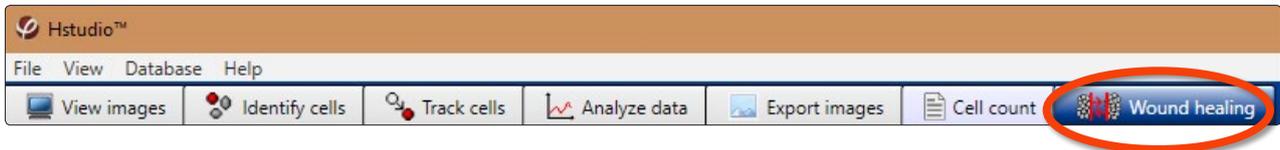


## Start the analysis

Add images from a wound healing time-lapse image series to the analysis by using the **Add selected** or the **Add all button**, which are found below the **Image frame list**. Several images can be added simultaneously if they are all selected or checked. The shift key can be used to select several consecutive images and the ctrl-key to select non consecutive image frames. If e.g. every fourth image should be added, check the images by right clicking the mouse, selecting **Check every X frame** and type "4" select **Add checked**.
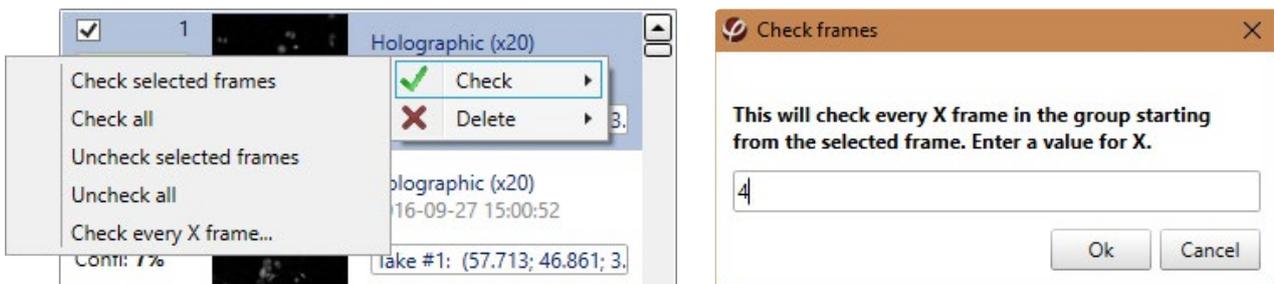


## Setting the threshold

1. Once the images are added, the threshold should be set. Use the **Threshold level** slider to set a threshold for an image at the beginning of the sequence. The slider button is moved by clicking and holding the left mouse button onto it.
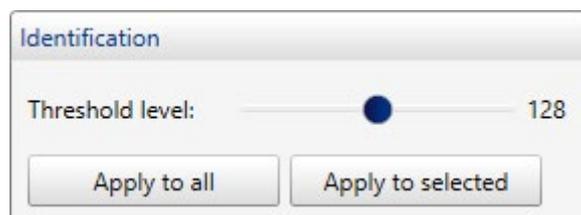


2. Then select an image in the middle and at the end and make sure that the threshold suits both of them. The threshold should cover as many cells as possible in each image, but no background.



| Number | Group | Project | Comment | Gap Width [µm] | Cell-covered area [%] | Cell-covered area [µm²] | Cell-free area |
|---|---|---|---|---|---|---|---|
| 10 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #10: (82,847; 6,042; 4,103) in A2 | 427 | 25 % | 79196 | 75 % |
| 14 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #14: (82,847; 6,042; 4,103) in A2 | 406 | 28 % | 90743 | 72 % |
| 18 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #18: (82,847; 6,042; 4,103) in A2 | 395 | 30 % | 97412 | 70 % |
| 22 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #22: (82,847; 6,042; 4,103) in A2 | 385 | 32 % | 103153 | 68 % |
| 26 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #26: (82,847; 6,042; 4,103) in A2 | 377 | 33 % | 107199 | 67 % |

3. While moving the slider, the threshold actively shows the boundaries of the cells. The images below show a too low threshold (left), a too high threshold (middle) and a correct threshold setting (right).
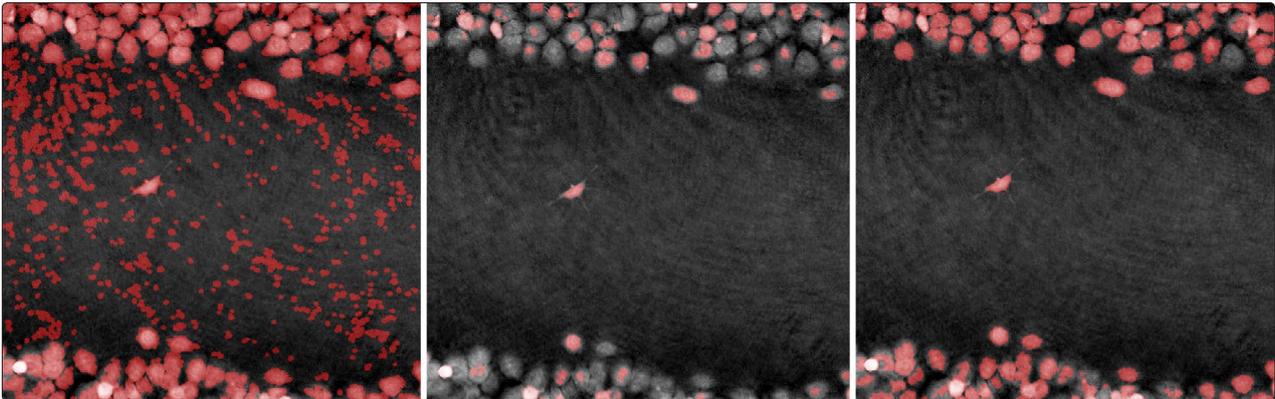


**Tip**

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. Below is an example example showing a steady increase in cell covered area with one exception (Number 14, highlighted). To solve this, select the deviating image, and then correct the threshold setting for that image.

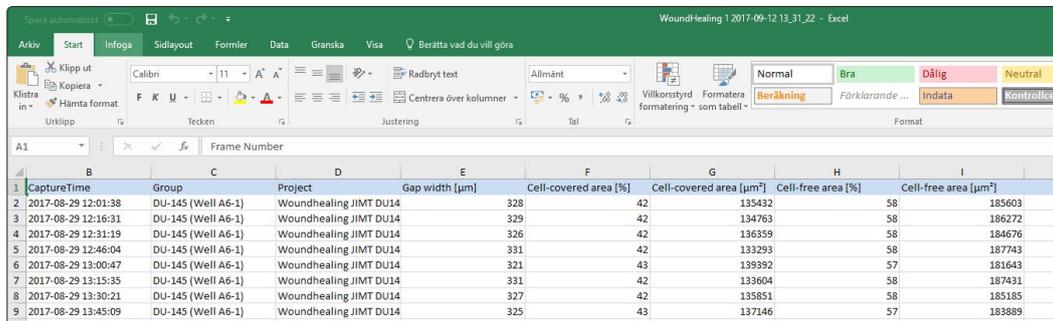| Number | Group | Project | Comment | Gap Width [µm] | Cell-covered area [%] | Cell-covered area [µm²] | Cell-free area |
|--------|-------|---------|---------|----------------|-----------------------|-------------------------|----------------|
| 10 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #10: (82,847; 6,042; 4,103) in A2 | 427 | 25 % | 79196 | 75 % |
| 14 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #14: (82,847; 6,042; 4,103) in A2 | 128 | 77 % | 248505 | 23 % |
| 18 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #18: (82,847; 6,042; 4,103) in A2 | 395 | 30 % | 97412 | 70 % |
| 22 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #22: (82,847; 6,042; 4,103) in A2 | 385 | 32 % | 103153 | 68 % |
| 26 | Capture#1 juni 16 (Well A2) kontroll | woundhealing 24 well | Take #26: (82,847; 6,042; 4,103) in A2 | 377 | 33 % | 107199 | 67 % |

4. After releasing the mouse button, the resulting mask is applied:
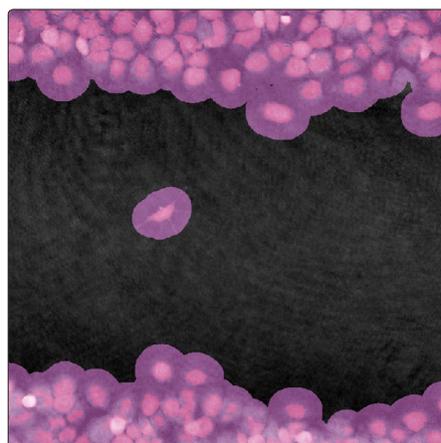


5. When a suitable setting has been found, apply the setting to all images by using the **Apply to all frames button**.

## Export results

1. When the threshold setting has been applied to all frames in the analysis, export the results to an XML file by clicking the *Excel Export button*. In the resulting xml file, gap width in μm, as well as cell covered area and cell free area, in % and $\mu m^2$, are given.
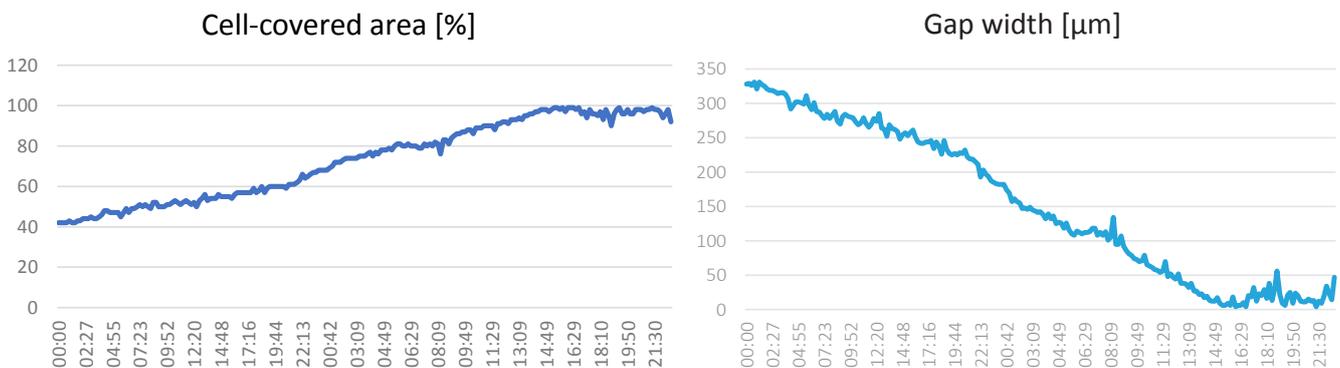


2. By using Excel formulas, a column with time points from start can be created. Add a column to the right of *Capture time* and type the formula as shown in the figure below. The column needs to be formatted as time.



3. Create diagrams to visualize the gap width, cell covered area or cell free area over time:



4. Calculate the speed of the cell front (*Cell front velocity*) by using the first value for gap width within the linear phase of the slope and divide with the time value at the end of the linear phase. In this example: 321 μm/16 h = 20 μm/h. This can also be performed in the *Excel data sheet*. However, it is important to use only data that are lying within the linear phase of the slope.

# TRACKING OF MOTILITY AND MIGRATION OF CELLS AT THE GAP FRONT

Use the tracking capabilities of the HoloMonitor software. Start the tracking approximately one hour into the time-lapse. This will give the cells time to start moving out from the denser parts of the cell layer, making it possible to track individual cells. Even if individual cells cannot be tracked, cell movement can be followed by tracking 2-3 cells that move together in a clump.

Ensure that the cells that move into the gap are well identified/segmented in the *Identify cells tab*. Please note that the segmentation performed previously in the *Wound Healing tab* does not apply to the tracking analysis. The other cells may not be possible to segment properly. The image frames in a wound healing time-lapse change very much from the start of the time-lapse until the end. Therefore, the same segmentation settings probably cannot be used all through the time-lapse.

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Tip

- To make the cell tracking easier, the cell identification can be set with a cell area that is slightly smaller than perceived in the image.

- Due to technical reasons, the software may identify cells in the middle of the gap even though it is empty. As long as those artifacts do not disturb the tracking analysis, they can be safely ignored.

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1. Go to the *Track cells tab:*
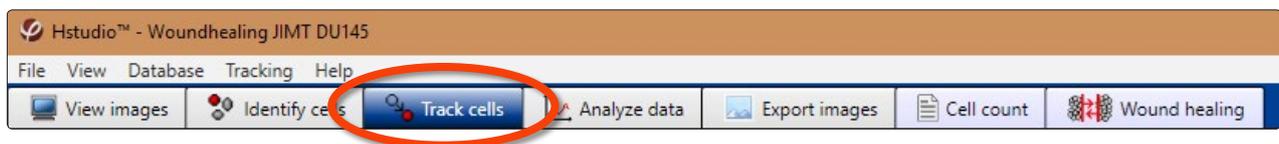


1. Choose *New Analysis*. Add frames from one position to the tracking analysis. Repeat the procedure to create two identical analyses.

2. Every item in the frame with an orange **+** can be tracked. Add front cells to be tracked by clicking on them. In the first of the two identical analyses add cells migrating from the lower cell front upwards (right image), and in the second analysis add cells migrating from the upper front and down.

3. Move the *Timeline slider* to the right to see the tracks of the selected cells. Adjust any tracking errors.



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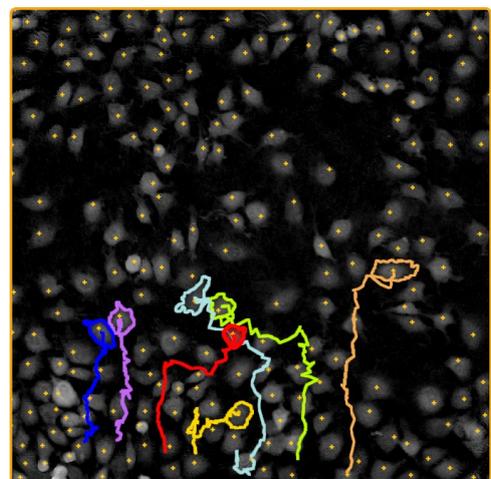
4. Go to *Plot movement* and check all cells to be included in the plot. The colored tracks show the movements of the selected cells, with the origin as the starting point for each cell. The plots can be saved as an image in several formats. If different samples (treatments) are to be compared, it is recommended to adjust the X- and Y-scales to be identical for all samples before image export.
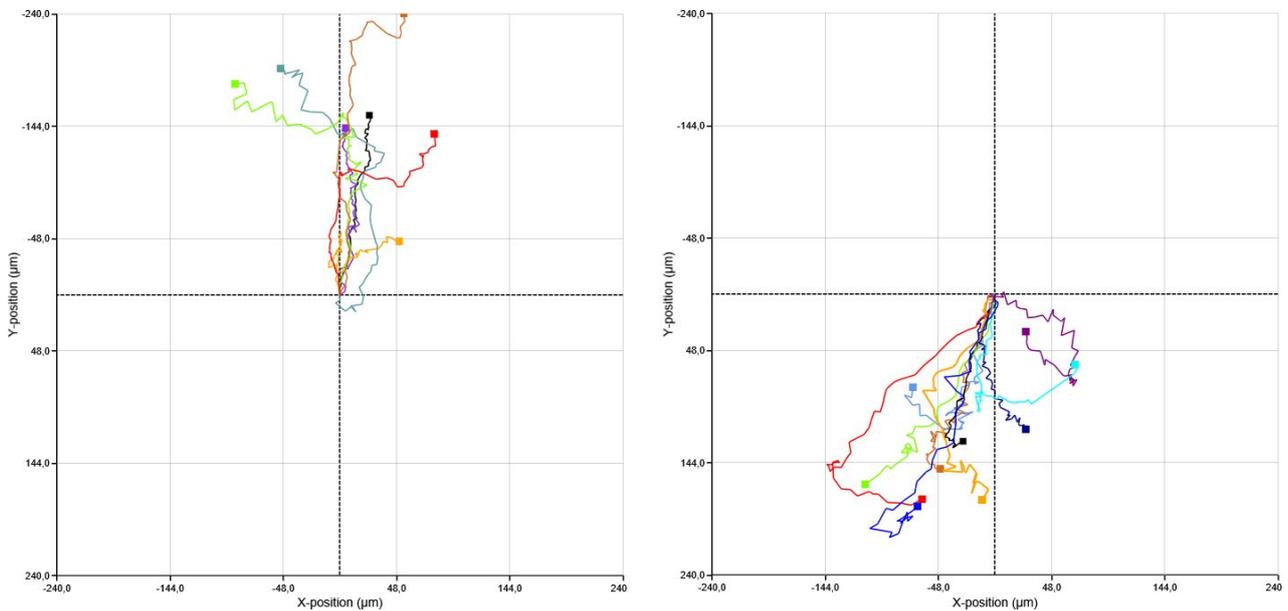


5. To be able to resume the analysis later, save each sample analysis. Go to the top menu, click *Tracking* and then *Save as*.

## Exporting results

1. For further analysis in Excel or other spread sheet applications, export the analyses to XML-files, using the *Export Data button* in the *Track cells tab*.

2. When opening the XML-files with Excel, the data for each cell parameter is given in a separate tab. The values for each cell, at the end of the time lapse, is in the right most column. *Migration distance*, *Migration directness*, *Motility distance* and *Motility speed* are accessible in the spread sheet together with other exported morphological parameters:

| 1 | 2017-08-29 19:33:55 | 2017-08-29 19:41:18 | 2017-08-29 19:48:43 | 2017-08-29 19:56:08 | 2017-08-29 20:03:34 | 2017-08-29 20:11 |
|---|---|---|---|---|---|---|
| 2 | | | | | | |
| 3 | 206,6285 | 211,67 | 209,2998 | 210,698 | 211,2067 | |
| 4 | 94,48009 | 95,70036 | 94,50278 | 98,86482 | 95,00683 | |
| 5 | 111,1609 | 107,7554 | 112,4425 | 114,1745 | 114,4838 | |
| 6 | 199,7125 | 199,8474 | 197,5918 | 200,1986 | 195,679 | |
| 7 | 73,97952 | 64,7408 | 61,87542 | 57,24923 | 54,37477 | |
| 8 | 156,9479 | 169,8701 | 173,3764 | 173,0229 | 181,8883 | |
| 9 | 150,1221 | 152,59 | 155,3687 | 155,6876 | 154,3535 | |

◄ ► ... | Boxed breadth (µm) | Boxed length (µm) | Eccentricity | Irregularity | **Migration (µm)** | Migration di ... ⊕

For a description of the morphological parameters, see the HoloMonitor M4 Setup and Operation Manual.