

HoloMonitor[®] M4

MITOSIS LENGTH PROTOCOL

MATERIAL

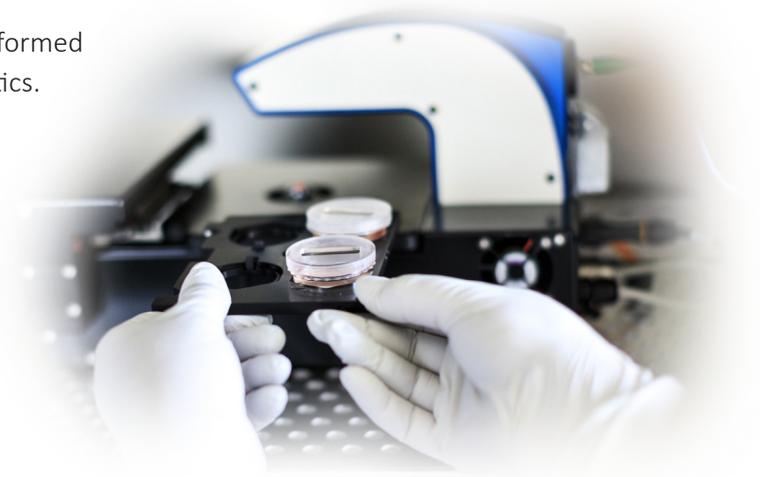
- **HoloMonitor[®] M4**, placed inside a cell incubator.
- **Hstudio software**, version 2.7.1 or later.
- **Culture vessel** by choice:
 - Sarstedt TC Dish 35, Standard (cat. # 83.3900)
 - Sarstedt TC Plate 6 Well, Standard, F (cat. # 83.3920.005)
 - Sarstedt lumox[®] multiwell, 24 Well (cat. # 94.6000.014)
 - Sarstedt lumox[®] multiwell, 96 Well (cat. # 83.3924.005)
- PHI **HoloLid** for selected vessel:

Vessel	HoloLid
Sarstedt TC Dish 35, Standard	71110
Sarstedt TC Plate 6 Well, Standard, F	71120
Sarstedt lumox [®] multiwell, 24 Well	71130
Sarstedt lumox [®] multiwell, 96 Well	71140

HoloLid product information and protocol is available [here](#).

- PHI **Vessel holder** for the selected vessel For information regarding vessel holders contact PHI at support@phiab.se.
- **Cells** suspended to reach a confluence of 2-5 % when seeded (approx. 6 000-11 000 cell/cm² for L929, A375, and Jimt-1 cells). Other cell types may require a different seeding concentration.
- [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 to a confluence of 2-5 % in preferred vessel. The final working volumes, essential for using **HoloLids** are:

Volume	Vessel
3.0 ml	Sarstedt TC Dish 35, Standard
3.0 ml/well	Sarstedt TC Plate 6 Well, Standard, F
1.8 ml/well	Sarstedt lumox® multiwell, 24 Well
170 µl/well	Sarstedt lumox® multiwell, 96 Well

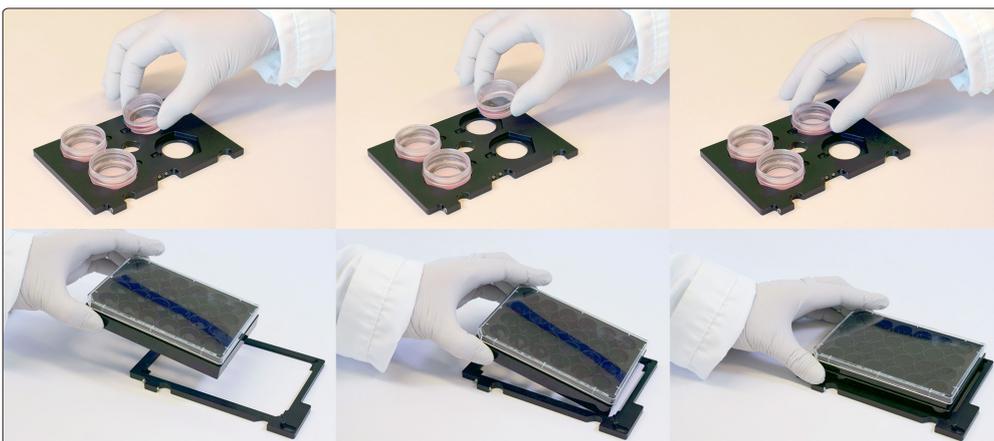
2. Put the vessel into the cell incubator and let the cells attach for 2-24h.
3. Sterilize the **HoloLids** according to the **HoloLid protocol**.
4. Add the treatment, if stated in the experimental setup.
5. Put on the standard lid.

IMAGING

Start up the **HoloMonitor** and proceed with the calibration. The values achieved should lie within the green area of the calibration results bar.

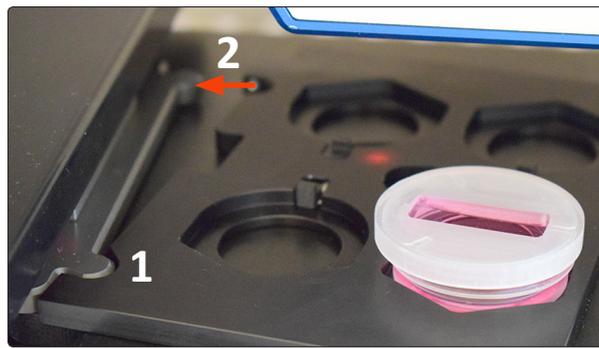
For imaging with a motorized stage

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**:



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

- Thereafter place the **Vessel holder** with the samples on the HoloMonitor stage:



- Go to the **Live capture tab** in the Hstudio software and select the appropriate vessel template:

<u>Vessel</u>	<u>Template</u>
Sarstedt TC Dish 35, Standard	Petri dishes 40 mm
Sarstedt TC Plate 6 Well, Standard, F	Sarstedt 6 well plate with PHI lid
Sarstedt lumox® multiwell, 24 Well	Lumox 24 well plate
Sarstedt lumox® multiwell, 96 Well	Lumox 96 well plate with PHI lid

- Create a **Project** for image storage.
- Focus the images at a position close to the center of the plate/vessel.
- Check **Timelapse** and type the total time and interval of the time-lapse imaging. 5 minutes between captures is recommended. However, for very fast cells an even shorter interval may be required.
- Check **Capture pattern** and select the wells and positions to be captured, as described in the [Setup and Operation Manual](#). Alternatively select capture positions and click the **Remember button** for as many positions as required. However, short interval between image captures (< 5 min) limits the number of positions that can be captured.
- Click **Advanced setup** and check **Multiple destination groups**.
- Make sure **One group per well** is not checked.
- Click **Save and close**.
- Click **Capture**.
- Go to the **View image tab** and review the images for quality.
- Await the time-lapse capturing to finish.

For imaging with a fixed stage

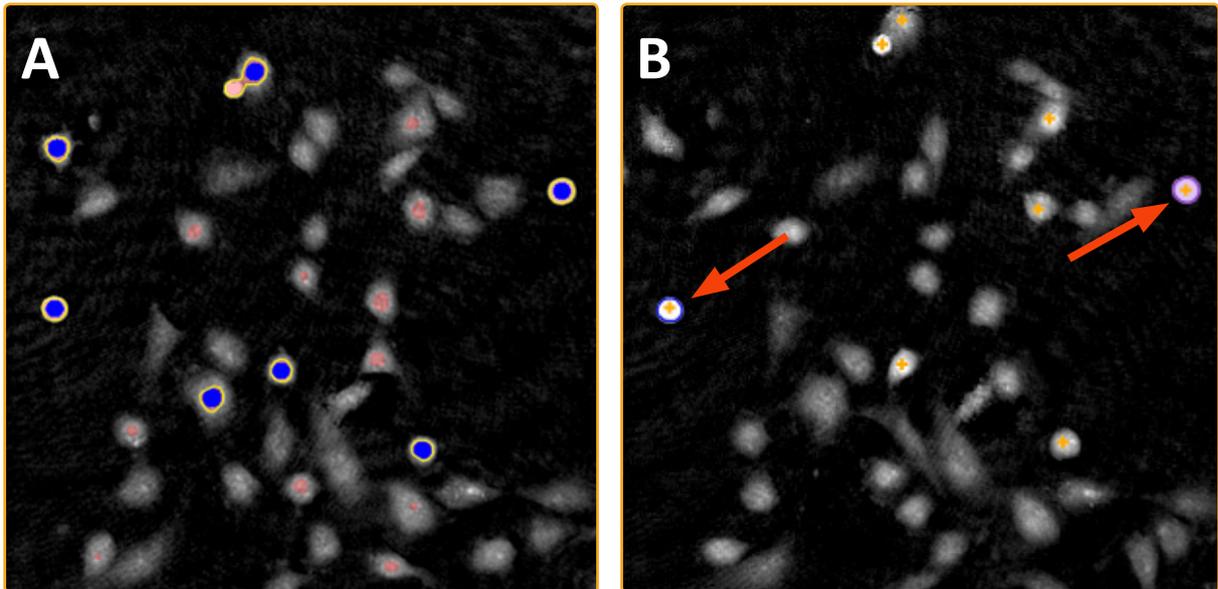
1. Replace the standard lid with the appropriate **HoloLid**, following the HoloLid protocol.
2. Place the sample on the **HoloMonitor stage** using the appropriate distance plate:

<u>Vessel</u>	<u>Distance plate</u>
Sarstedt TC Dish 35, Standard	# 2
Sarstedt TC Plate 6 Well, Standard, F	None
Sarstedt lumox® multiwell, 24 Well	# 1
Sarstedt lumox® multiwell, 96 Well	None

3. Go to the **Live capture tab** and ensure that the images are well focused. Adjust the software focus, if required.
4. Create a **Group**.
5. Check **Timelapse** and type the total time and interval of the time-lapse capture. 5 minutes between captures is recommended. However, for very fast cells an even shorter interval may be required.
6. Click **Capture**.
7. Go to **View image** and review the images for quality.
8. Await the time-lapse capturing to finish.

ANALYSIS

1. Go to **Identify cells**. Start by segmenting the cells in such a way that only rounded up cells will be red, with a border and a blue center. To achieve this, both the threshold and the cell size needs to be set rather high. This step can be performed with low precision. It does not have to be perfect, it is better to have a few extra cells than to have too few cells (Figure A below).
2. Go to **Track cells**. Add all frames from one position to the tracking analysis. Go through all the frames to manually select each newly rounded cell. Each selected cell acquires individually colored outlines (Figure B, red arrows).

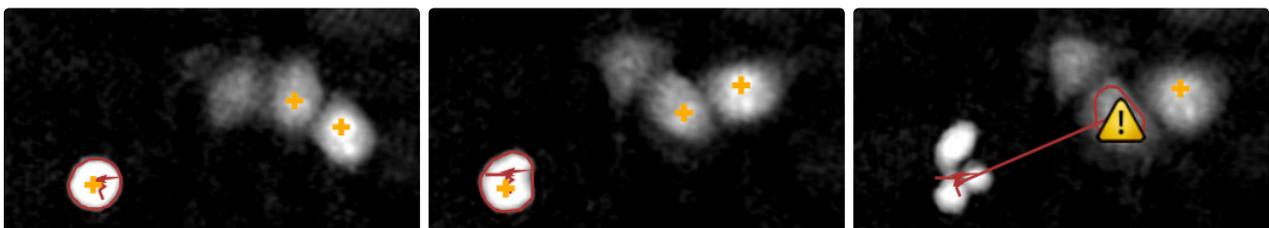


3. When the cells divide, a warning sign appears in the frame. This indicates a cell division. When the warning appears (right image), it is time to unset that cell. If the cell is covered with the warning sign, zoom in to be able to unselect it (otherwise the warning message appears when hovering over it).
4. Continue to select and unset cells until the end of the time-lapse.



Tip

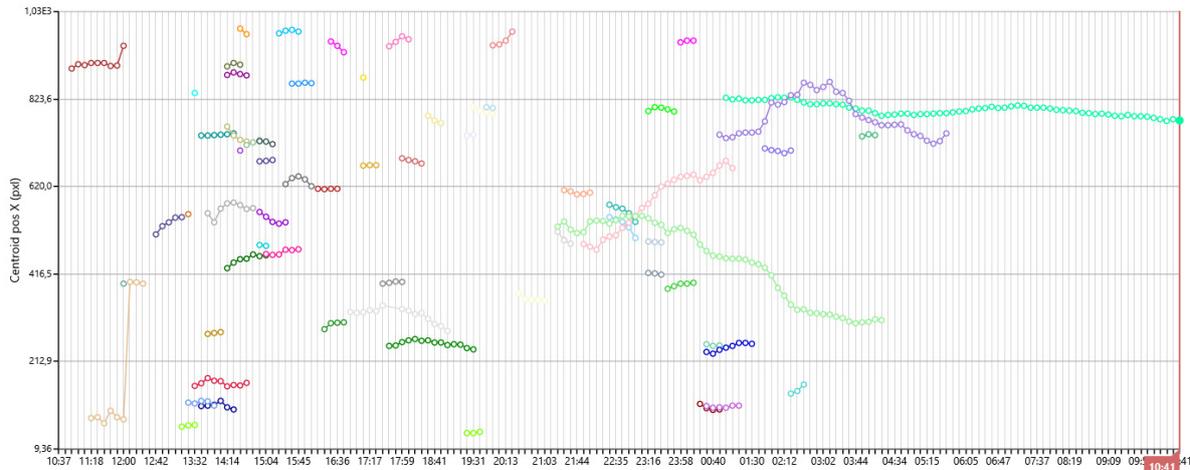
In some cases, e.g. after asymmetrical cell divisions, the software loses the cell and the tracking jumps to the closest suitable cell. The user must select that cell and then unset it.



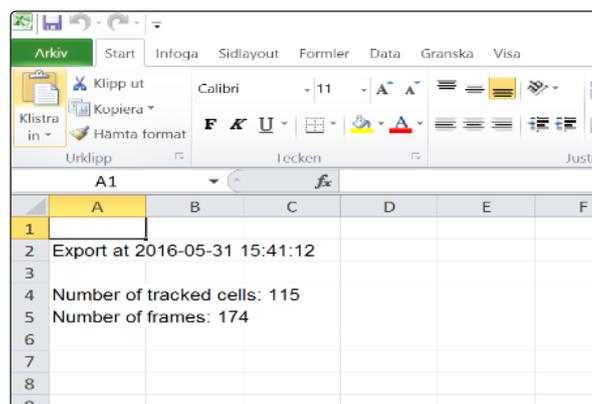
Imperfectly tracked cell after asymmetric division.

RESULTS

1. Click **Plot features**. A graph showing the length of the mitosis for each cell over time appears:

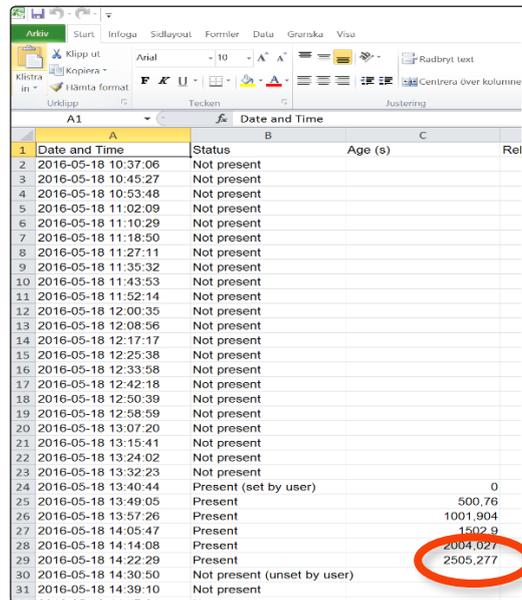


2. Check each box to display all individual cells. Each colored line corresponds to one mitotic cell. Set the Y-axis parameter to **Center position x**. The x-axis denotes the time. Each time-point corresponds to a captured frame. In the example below, some cells stay in mitosis only for one frame, and can be seen as a single marker. The longest mitosis lasts until the end of the movie, and is seen as a long string of markers. That cell is stuck in mitosis. If the line bends, it means that the cell has changed position in the image frame (i.e. a change of Center position). This diagram is a highly visual way to see the data.
3. To be able to recall the analysis later, save each sample analysis. Go to the top menu, click **Tracking** and then **Save As**.
4. For further analysis, export the tracking data to an XML-file. Open the file in **Excel**. In the first worksheet you will see the number of tracked cells and the number of frames:



	A	B	C	D	E	F
1						
2	Export at 2016-05-31 15:41:12					
3						
4	Number of tracked cells: 115					
5	Number of frames: 174					
6						
7						
8						
9						

- Each tracked cell has its own tab (see bottom of calculation sheet). Click the individual cell tabs. You will see in which frames the cells were in mitosis and for how long (in seconds) the cells were in mitosis by the numbers found in the “Age” column, or the column C (red circle):



	A	B	C	Rel
1	Date and Time	Status	Age (s)	
2	2016-05-18 10:37:06	Not present		
3	2016-05-18 10:45:27	Not present		
4	2016-05-18 10:53:48	Not present		
5	2016-05-18 11:02:09	Not present		
6	2016-05-18 11:10:29	Not present		
7	2016-05-18 11:18:50	Not present		
8	2016-05-18 11:27:11	Not present		
9	2016-05-18 11:35:32	Not present		
10	2016-05-18 11:43:53	Not present		
11	2016-05-18 11:52:14	Not present		
12	2016-05-18 12:00:35	Not present		
13	2016-05-18 12:08:56	Not present		
14	2016-05-18 12:17:17	Not present		
15	2016-05-18 12:25:38	Not present		
16	2016-05-18 12:33:58	Not present		
17	2016-05-18 12:42:18	Not present		
18	2016-05-18 12:50:39	Not present		
19	2016-05-18 12:58:59	Not present		
20	2016-05-18 13:07:20	Not present		
21	2016-05-18 13:15:41	Not present		
22	2016-05-18 13:24:02	Not present		
23	2016-05-18 13:32:23	Not present		
24	2016-05-18 13:40:44	Present (set by user)	0	
25	2016-05-18 13:49:05	Present	500,76	
26	2016-05-18 13:57:26	Present	1001,904	
27	2016-05-18 14:05:47	Present	1502,9	
28	2016-05-18 14:14:08	Present	2004,027	
29	2016-05-18 14:22:29	Present	2505,277	
30	2016-05-18 14:30:50	Not present (unset by user)		
31	2016-05-18 14:39:10	Not present		
32	2016-05-18 14:47:31	Not present		

Mitosis length data presented in a spread sheet.

The total mitosis time is the last and largest number in the column, as the numbers for each frame is added to the previous frame from the onset of the cell. If the cell status is denoted **Not Present**, the cell is not in mitosis and not selected by the user. **Present** denotes that the cell is selected and therefore in mitosis.

- To collect the mitosis length of all cells, create a new tab in **Excel**.
- Type **Cell 1** in A1 in the tab you just created.
- Use the small rectangle in the bottom-right corner of the cell and expand it to the right for as many cells as there are cell tabs in the exported file.
- Add the formula = MAX(INDIRECT("'"&A1&"'!C2:C175")) to A2. The interval which is exemplified here with C2:C175, is meant to include all time points of your experiment. You might have to extend the interval C2:C175 to cover all time points in your experiment, i.e. type the correct last number for column C (instead of C175 as in this example).
- Use the small rectangle in the bottom-right corner of the cell again, but now in the cell where you just typed the formula. Expand it to the same length as in step 6.