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

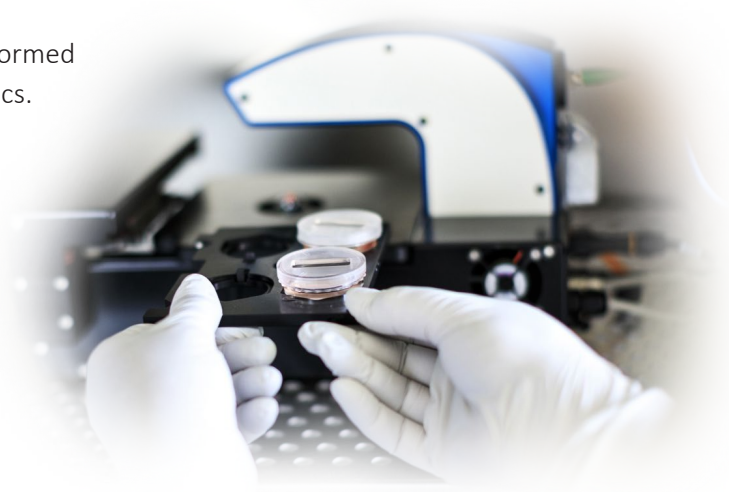
  HoloLid product information and protocol is available here.

- 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/cm^2 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.

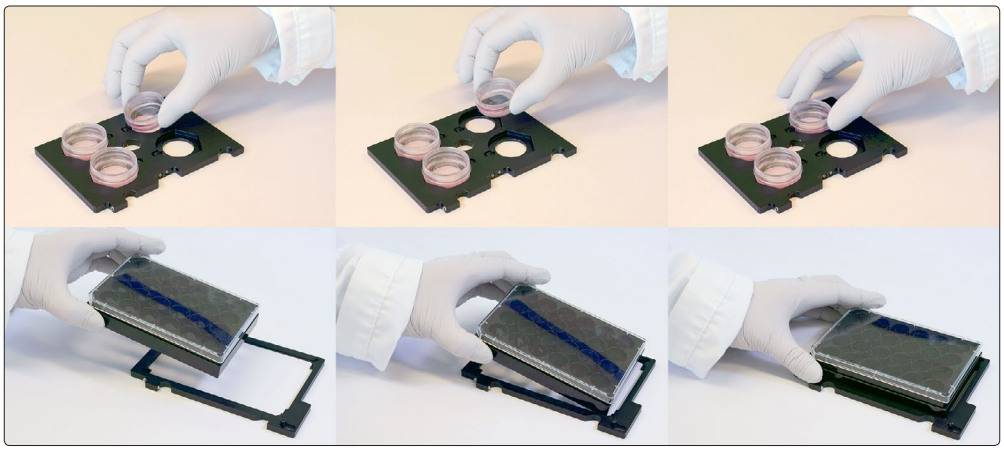
IMAGING

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

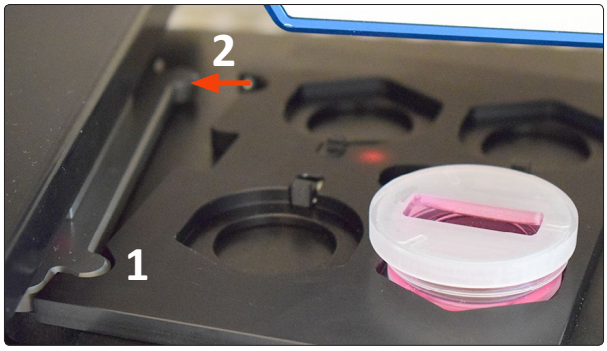
1. Wipe off the ***Vessel holder*** with alcohol and put it in to the LAF-bench, the grips facing down.

2. Place the cell samples on to the ***Vessel holder***:



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

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



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

Vessel	Template
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

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

7. Focus the images at a position close to the center of the plate/vessel.

8. Check ***Timelapse*** and type the total time and interval of the time-lapse imaging. 6-12 hours between captures is recommended.

9. Check ***Capture pattern*** and select the wells and positions to be captured, as described in the Setup and Operation Manual.

10. Select at least 5 capturing positions to capture for each sample. The more heterogenous the cell distribution, the more captures are needed.

11. Go to capturing positions at the four corners of the vessel and ensure they are well focused (left-click the red squares in the stage position window).

12. Click ***Advanced setup*** and check ***Multiple destination groups***.

13. Make sure ***One group per well*** is checked.

14. Click ***Save and close***.

15. Click ***Capture***. Go to the ***View image tab*** and review the images for quality.

16. 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:

Vessel	Distance plate
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 software focus, if required.

4. Create one ***Group*** per sample, i.e. “Control1 Day 1” and “Treated Day 1”.

5. Capture at least 5 images per sample, the more heterogeneous the cell distribution, the more captures are needed.

6. Repeat capturing (steps 4-5) for as many time points as the experiment requires.

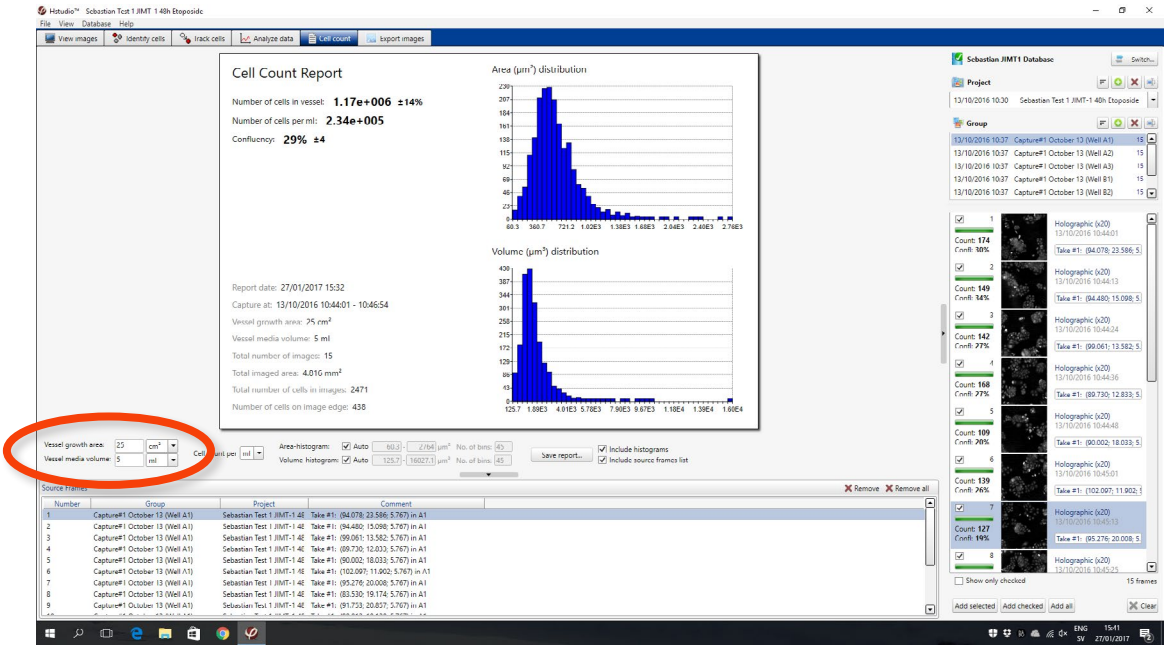
ANALYSIS

1. Go to ***Identify cells***. Check the segmentation and, if needed, adjust the ***Threshold*** and ***Min object size*** settings to fit the cells for all ***Groups***. The settings can be applied for all images within each ***Group*** but need to be validated and possibly adjusted for all ***Frames***. Discard bad frames.

2. Go to ***Identify cells***. Adjust the ***Threshold and minimum object size*** for all frames to fit most of the cells for each ***Group***. Use the same settings for each sample. Discard bad ***Frames***.

3. Go to ***Cell count***. Add the images corresponding to one time-point and one treatment, e.g. control at 0 hours (see image below).

4. Adjust the ***Vessel growth area*** (below, red arrow) to correspond to the vessels used.



5. Note the ***Number of cells in vessel***, the ***Confluence*** and ***Confidence interval*** (the number to the right of the cell number). If the number is above 10 %, more frames ought to be added to the cell count to achieve sound statistics.

6. A ***Report*** (PDF) can be created by pressing the ***Save report*** button.

7. Repeat the cell count for all treatments and time-points.

8. Create graphs using the values from the cell count reports using e.g. ***Excel***.