

# Micropatterning EM grids for cryo-electron tomography of cells

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Micropatterning of electron microscopy (EM) grids facilitates cryo-focused ion beam and cryo-electron tomography pipelines by optimally positioning cells and regions of interest in for milling and imaging (Engel, *et al.* 2019, Toro-Nahuelpan, *et al.* 2020, Engel and Vasquez, *et al.* 2021). Since EM grid micropatterning was introduced, we and other researchers have adopted new reagents for micropatterning EM grids using the Alveole PRIMO system which improve the efficiency of patterning and the stability of grid's surface chemistry (Swistak *et al.* 2021, Sibert and Kim, *et al.* 2021). We have also introduced a sterilization step to make the process compatible with cell culture systems that do not contain antibiotics. Here, we present our protocol for efficient micropatterning of EM grids, which we have successfully used to micropattern grids for neuronal, cardiomyocyte, melanoma, and endothelial cell cultures.

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- EM grids: Au Quantifoil grids or UltrAuFoil (R2,2, 200 mesh)
- Silicone sheeting (0.005" gloss silicone sheeting, Specialty Manufacturing Inc.)
- Laboratory wrapping film (Parafilm, Bemis™ PM999)
- 10 cm plastic petri dish (Corning)
- Negative pressure tweezers (Cat. # 0203-N3-PO, Dumont)
- Optional: Anti-capillary negative pressure tweezers (Cat. # 0203-N4AC-PO, Dumont)
- Glass coverslips (#1.5), 24 x 60 mm
- 35 mm Glass-bottom dishes(#1.5) (Cat. # D35-20-1.5-N, Cellvis)
- Pipettes + pipette tips (20 ul, 200 ul, 1 ml)
- PLPP gel (Alveole) - nanoscalelabs.com
- PBS 1x
- Deionized water (DIW)
- 0.1M HEPES at pH 8.5 ( $C_8H_{18}N_2O_4S$ ; Mw=238.30,CAS Number: 7365-45-9 )(e.g., 1.1915 g powder in 50 mL DIW)
- Ethanol (100%)
- 0.01% Poly-L-lysine (CAS Number 25988-63-0, p4707 Sigma)
- mPEG-Succinimidyl Valerate, MW 5,000 (PEG-SVA, Laysan Bio Inc.).

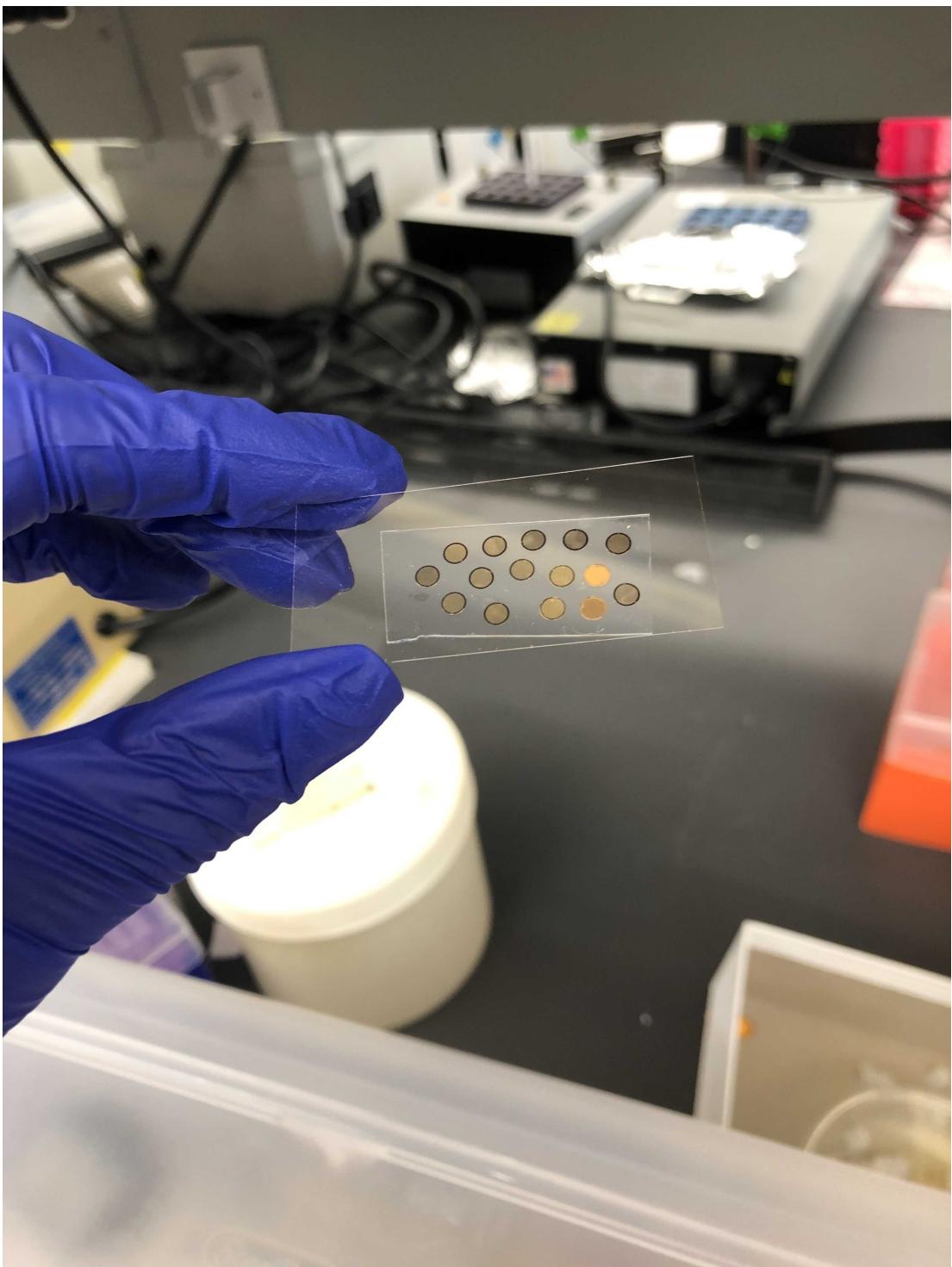
oNote: Has half life of ~10 min. Aliquot powder in a glove box filled with inert gas (e.g., argon) (~8-10 aliquots per bottle) and store in -20°C freezer as powder.

- Plasma etcher (Plasma etch P-50)
- Inverted tissue-culture microscope
- Optional: Fluorescently tagged extracellular matrix (ECM) protein. Examples: Oregon Green 488-gelatin, Rhodamine-fibronectin, Rhodamine-laminin.
- Optional: silicone stencil with 4mm diameter wells for holding micropatterned grids during sterilization, incubations steps, and cell culture.

Passivation

2h 40m 10s

- 1 Cut out a rectangle (<24 x 60 mm) of silicone sheeting so that it can be mounted on a glass<sup>2m</sup> coverslip.
- 2 Place grids gold mesh side down (holey Quantifoil or UltrAuFoil thin film-side up) on the silicone.<sup>15m</sup>



EM grids face-up on silicone sheet on glass coverslip.

3 Line the bottom of a 10 cm plastic petri dish with parafilm.

3m

The glass slide with the grids can be transported in this dish to the plasma etcher.

- 4 Place the glass coverslip containing the grids in center of the chamber of a plasma etcher <sup>5m</sup> (always do a dry-run first to season the chamber and set the power level). Expose the grids to atmospheric plasma at 30 W for 10-12 sec or 27 W for 12 sec. For silicon dioxide film grids, 15 sec at 30 W has also worked well. Note: always make sure to check that you can see plasma through the window on the chamber.

Always check to make sure that you can see plasma through the window on the chamber (atmospheric plasma should glow magenta while oxygen plasma has a bluer hue).



- 5 Remove glass coverslip from vacuum chamber and place it on the parafilm-lined 10 cm dish.<sup>10s</sup>
- 6 Add liquid PLL to the silicone surface until all the grids are covered. 1-2 mL should be sufficient to form a bubble over the whole coverslip surface that is repelled by the parafilm underneath.<sup>1h</sup>

Cover the dish and leave it overnight at 4 degrees Celsius.

Add the PLL to the surface as close to the plasma exposure as possible.



Parafilm beneath the glass coverslip contains the liquid PLPP to the coverslip.

- 7 Rinse at least three times in HEPES or DIW. To rinse, aspirate the liquid with a pipette then gently pipette on fresh solution. <sup>5m</sup>
- 8 Take an aliquot of PEG-SVA powder out of the -20 C freezer and allow it to reach room temperature before removing the cap. Weigh the PEG-SVA powder and then dissolve it in 0.1M HEPES to create a 100mg/ml solution. As you pipet up and down to mix, the solution will go from opaque to clear. <sup>5m</sup>
- 9 Aspirate off the remaining rinse solution from the grids, then immediately pipet the PEG-SVA solution onto the grids until they are covered. Leave at room temperature for 1 hr. <sup>1h</sup>

It is critical to use the solution immediately as the PEG-SVA solution has a half-life of 10 min.

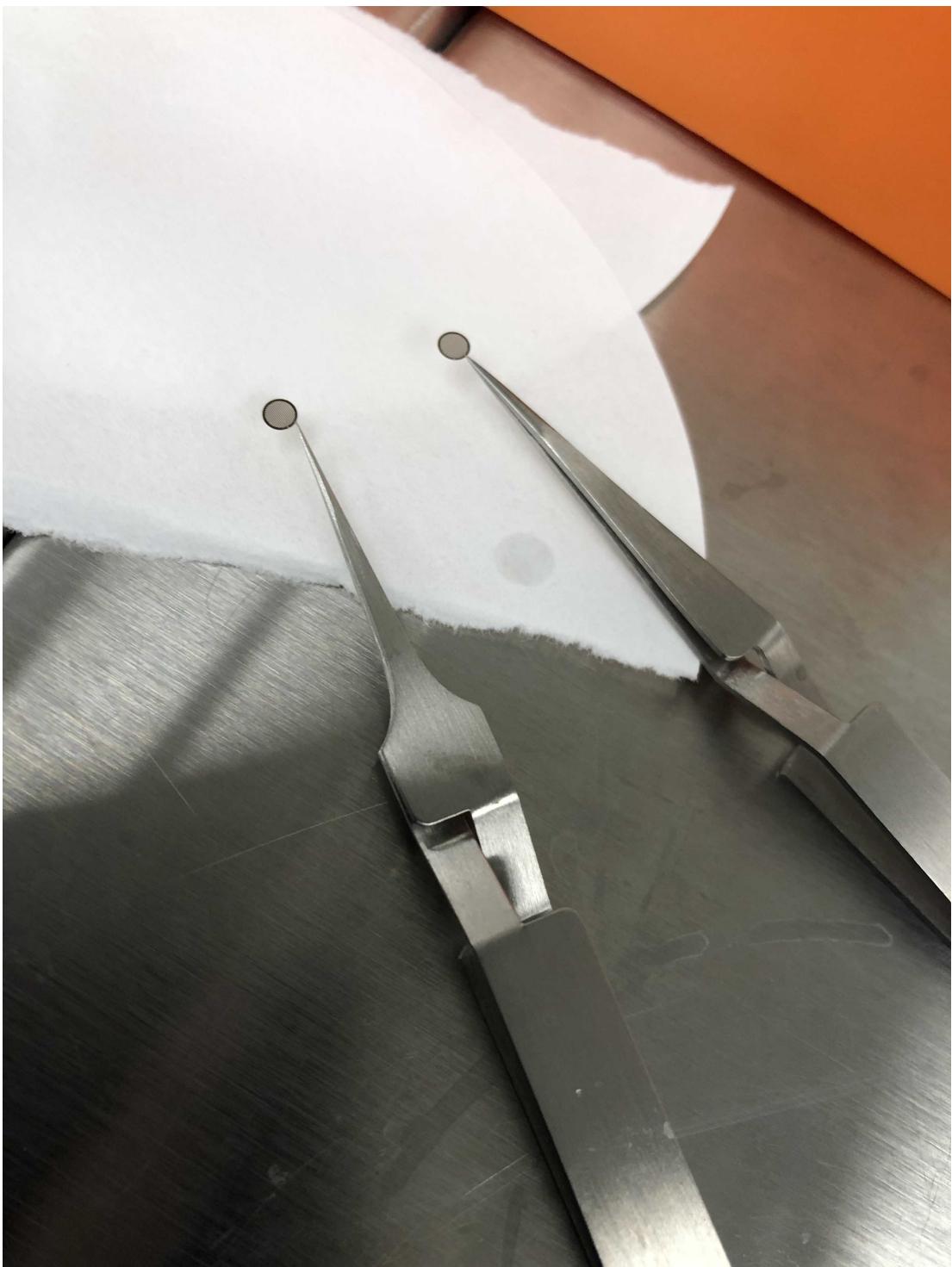
- 10 Aspirate off the PEG-SVA solution and carefully rinse at least three times in DIW by pipetting on and off. <sup>5m</sup>

This is a good stopping point as you can store the wet grids in DIW at 4 degrees C (in the refrigerator) for up to several days before photopatterning. You can store dry PEG-SVA coated glass surfaces for up to a month at 4 degrees C, but the delicate grids may be at risk of breaking due to stiction if they are left to dry on a flat surface such as glass. It's best to dry them in negative pressure tweezers.

## Photopatterning

2h

- 11 Gently lift an EM grid off of the silicone with negative-pressure tweezers and remove excess water by blotting vertically on filter paper.
- 12 While still holding the grid, set the tweezers down on your work surface so that the grid is film-side up with both sides exposed to the air. Allow the grid to air dry, this should take <5 min. <sup>5m</sup>



EM grids are held above the work surface with negative-pressure tweezers.

- 13 Prepare a 1:6 PLPP gel solution in pure ethanol in an Eppendorf tube. Pipette up and down to mix well.

Protect this solution from light when possible.

14 Add **3  $\mu$ L** of the 1:6 PLPP gel solution to the surface of each grid that is being held by the tweezers. 2m

15 Allow it to air dry while it is protected from ambient light (e.g., place under a box cover) 10m

It is essential that the grids are held elevated by tweezers at this stage. If the PLPP solution dries while the grid is in direct contact with a surface the holey thin film may tear. The wait time for drying is a good opportunity to get the PRIMO system calibrated.

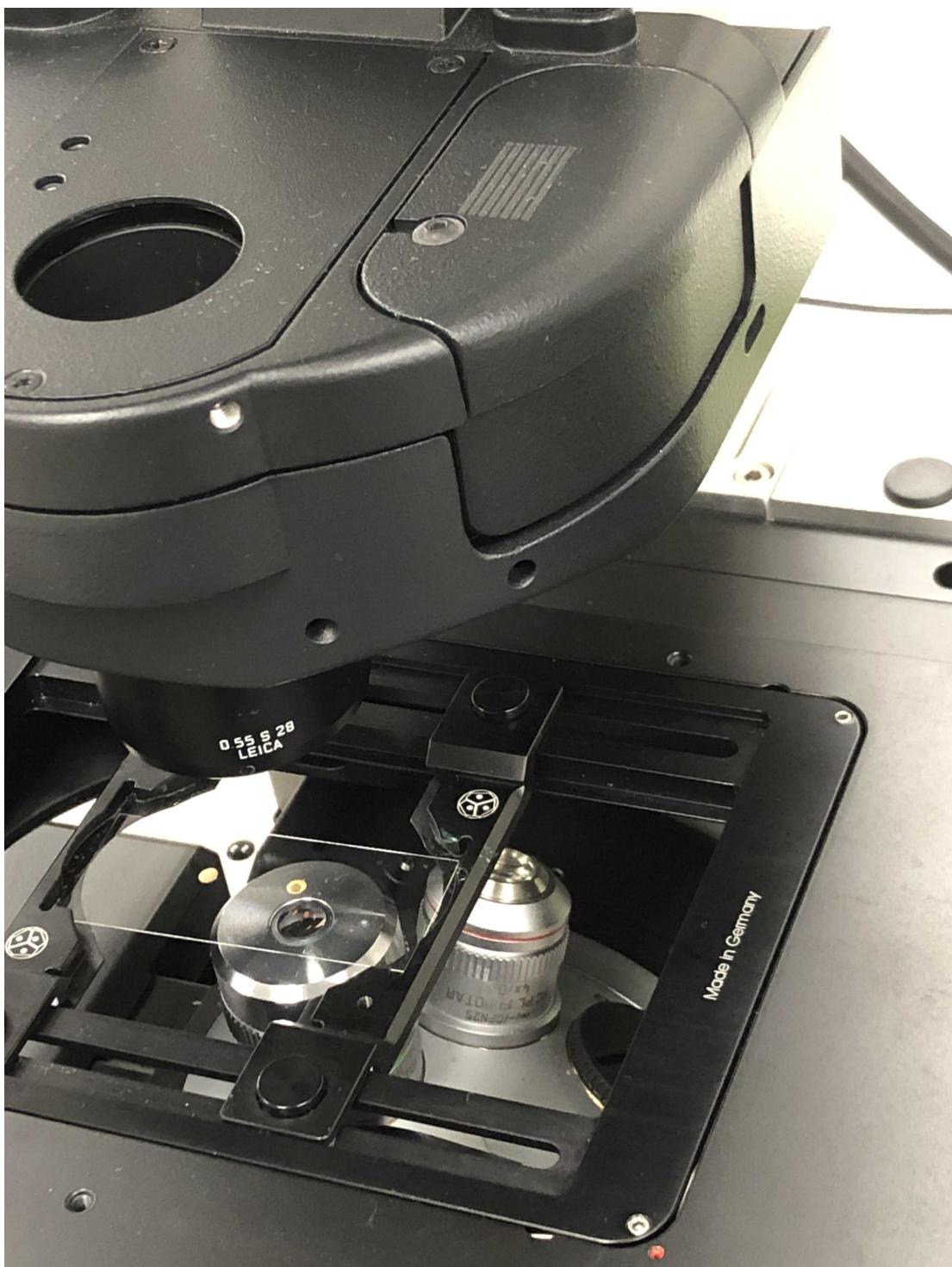
16 Turn on the Primo system and calibrate it using a #1.5 glass coverslip. 10m

17 Prepare a piece of parafilm with 3 droplets of deionized water per grid. These will be used to rinse the grids after exposure in step 22. 3m

If the parafilm is not lying flat on your work surface, add a droplet of liquid below the parafilm.

18 Prepare a 35 mm glass bottom dish with a stencil to create four 4mm wells. Fill each well with 25  $\mu$ L PBS. 7m

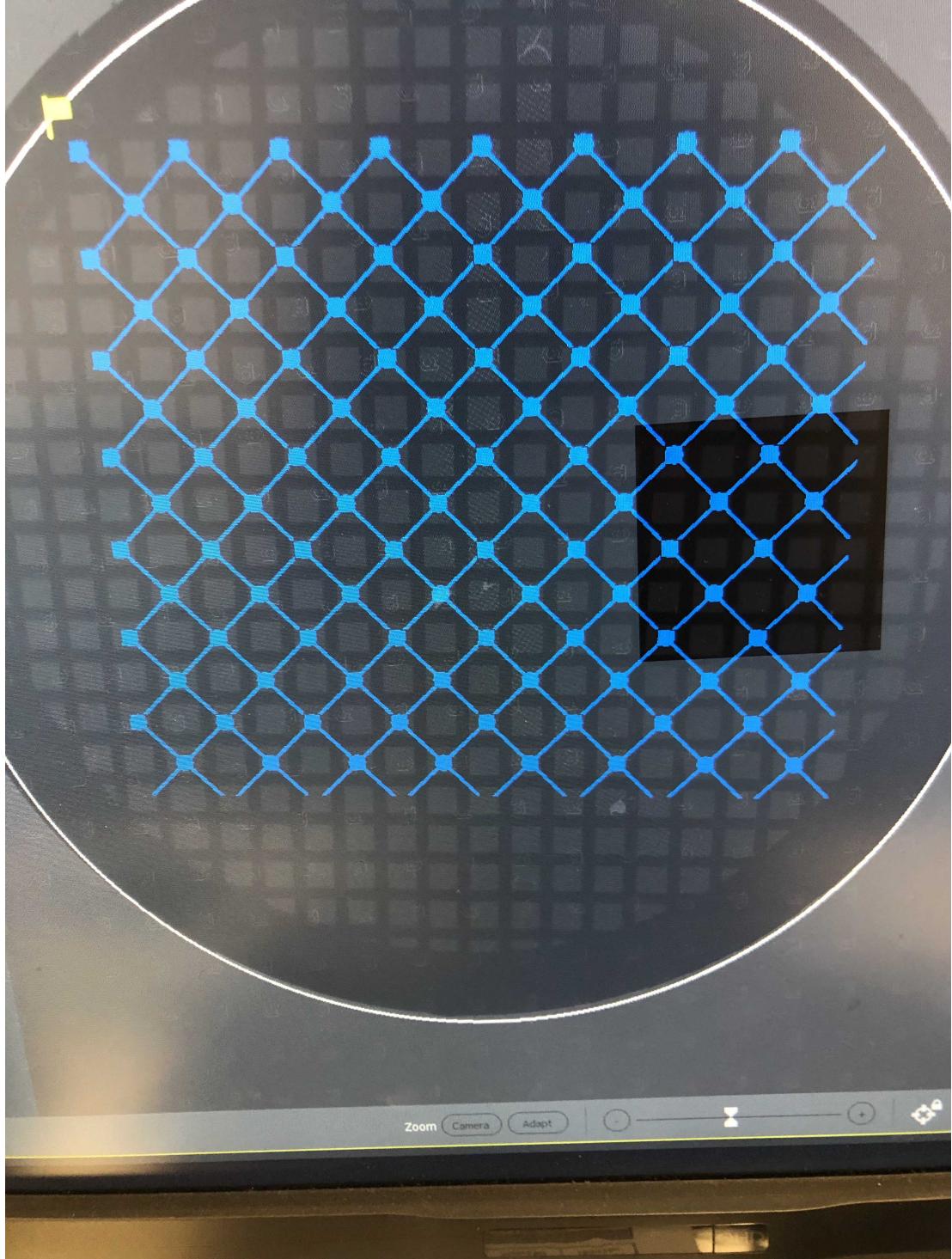
19 Once the PLPP has dried on the grids, place each grid holey film-side down on a clean #1.5 glass coverslip. Place the coverslip containing the grids (2-3) on the stage of a microscope outfitted with the Primo system. 5m



EM grids are face-down on #1.5 glass coverslip during UV exposure in the PRIMO system.

- 20 Locate the grid using the microscope and focus on the holes in the holey film that are closest to the center of the grid. Create a circular region of interest on the grid using the Leonardo software that is 2.5 mm in diameter and centered on the center of the grid. Select the "scan" option to get a preview of the grid. The preview will allow you to align your micropattern to the grid in the next step. <sup>2m</sup>

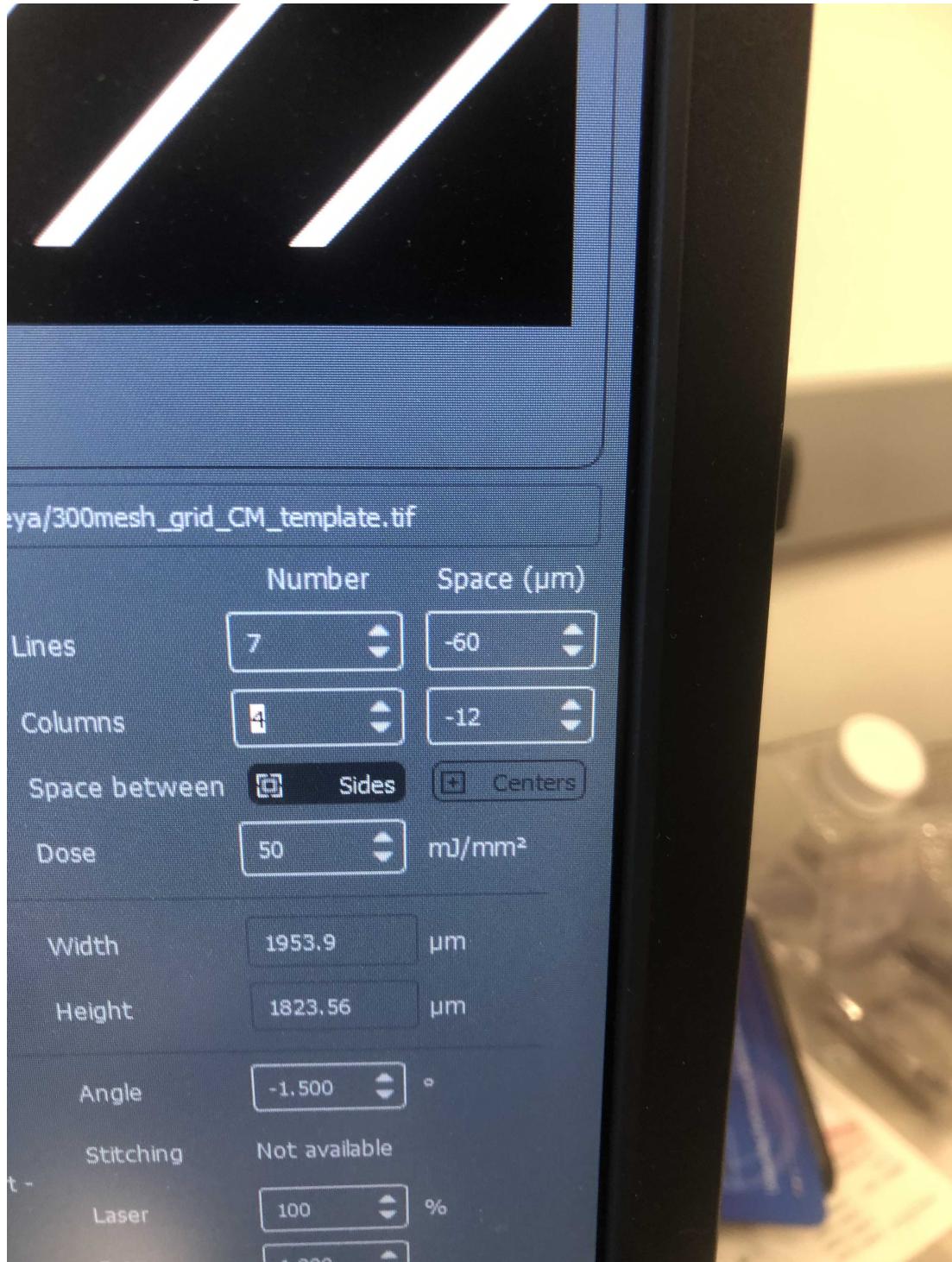
- 21 Load your digital pattern into the Leonardo software, and define the array spacing and number of repeats so that the pattern appears well aligned to the grid in the preview. Expose each grid using an exposure  $50 \text{ mJ/mm}^2$ .



The white circle defines the ROI. The blue represents a preview of the micropattern on the EM grid.

Depending on the orientation of the grid and whether it has undergone any bending due to

handling, you may need to adjust the angle and ratio of the digital pattern in the "expert" tab on the lower right side of the screen.



The "Lines", "Columns", and "Space" define the array. "Angle" and "Ratio" can be adjusted in the "Expert" tab.

- 22 Following exposure of all of the grids, remove the glass coverslip from the stage of the microscope, deposit a droplet of water onto each grid to dissolve the photoinitiator, and let it sit until the grid is detached from the glass (after ~1 min the grid should move when contacted by tweezers or the coverslip is tilted). Pick up the grid with tweezers and dip it in droplets of water on Parafilm three times to rinse off the photoinitiator, then place it carbon face up into a droplet of PBS contained in a well (prepared in step 18). Tip: insert grid into droplet at a 45 degree angle. 10m

This is a good stopping point. After exposure and rinsing, t can be kept in PBS in the refrigerator for a few days or dry at 4 °C, but delicate grids may be at risk of breaking due to stiction if they are left to dry on a flat surface such as glass. If drying, make sure to rinse in water first so as not to form salt crystals.



## Cell Culture

2h 49m

- 23 Before use, sterilize EM grids in 70% ethanol in the TC hood. If the grids are in a 35 mm glass bottom dish with a stencil you can wipe the outside of the dish with 70% ethanol, bring it into the hood, and fill it with 70% ethanol. 5m

For incubation times <48 hrs with cells that are cultured in media containing antibiotics,

the sterilization step may not be necessary.

- 24 After **⌚00:05:00**, dilute the ethanol in sterile water, pipette out most of the liquid, and repeat until you are confident that you have diluted out the ethanol. 5m
- 25 Replace the water with sterile PBS and make sure that grids are immersed in PBS for at least **⌚00:05:00** before adding ECM proteins or cells. 5m

This step is essential to re-hydrate the PEG. If omitted, the anti-fouling properties of the PEG may be compromised.

- 26 Aspirate the PBS and add a droplet (~25 uL) of ECM protein solution into each well containing an EM grid. 5m

The exact coating depends on the cell type and may not be required for some cell type. We have successfully used the following ECM proteins at the following concentrations on micropatterned grids: Oregon Green 488-gelatin at 1 mg/ml in PBS for 1 hr at room temp; a 1:400 solution of Matrigel in DMEM/F12 in incubator for 30 min, Rhodamine-fibronectin at a concentration of 100 ug/ml in PBS for 1 hr at RT.

- 27 Rinse each well three times by aspirating the ECM and and then pipetting in PBS or DMEM/F12. Aspirate gently from the edge of the well or use a pipette for more control. 7m
- 28 Add 10-20 uL cell culture media to each well. Optional: Store dish with micropatterned grids in incubator while resuspending cells. 5m
- 29 Resuspend cells as you would to replate them and then make a dilute cell suspension (~1:8). 10m
- 30 To seed cells, add 3-5 uL of a dilute cell suspension to each droplet of media above the grid in a well until observing 1-2 cells/square (close dish and observe in the tissue culture room) 7m

microscope).

- 31 Once the cells begin to spread on the micropatterns (30 min – 2 hr depending on the cell type), add 2 mL warm cell culture media to the dish and leave in incubator overnight or for as long an incubation time as necessary.

The added 2 mL media will wash away non-adherent cells. The timing will depend on how quickly your cell type attaches to the surface. For example, adding 2 mL media to HUVECs after 2 hr has been successful, while HFFs only need 30 min.

Vitrification

2h

- 32 Fix or plunge freeze the grids once the cells achieve the desired level of spreading on the grids.  
Example: 12-16 hrs for HUVECs, 14 days for neurons). Remove the silicone sticker prior to lifting the grids out of the dish with tweezers.