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Protocol – Micropatterning TEM grids using PLPP gel

- Grids used: Quantifoil R1/4 holey carbon 200 mesh / gold
- Take care to not let the grid dry out – use a humidified chamber for incubations and never aspirate all the liquid during washes. If the grid dries, grid squares will break and the passivation and anti-fouling will fail and allow cells to adhere non-specifically.
- Take care to not touch the pipet tip to the grid to avoid damaging the squares
- No need to do the entire protocol in the TC hood for sterility - just observe the 70% ethanol wash step after patterning and before coating with protein
- The full process can be done in two separate days, make sure to read ahead to determine where to stop as some steps require immediate action.

1. Glow discharge grids
    - Place grids carbon side up onto a glass microscope slide. Take care to grab the grids by the edge and avoid bending the grids (4 grids fit comfortably on a microscope slide)
    - Place slide into the Pelco Easiglow instrument
    - Use default run parameters, but change time to 40 sec (25 mA, negative)
    - Place glass top onto instrument and hit 'Auto run'
    - Glow discharge within 30 minutes before the start of the experiment

2. Place PDMS stencil on grids
    - Remove PDMS stencil from its sheet using tweezers. Gently pick away at a corner until it peels off.
    - Remove the center circle. Use a second set of tweezers to gently hold down the circle while gently pulling the outer stencil up and away from the center. Repeat until the circle is completely separated.
    - Without moving the glow discharged grid off the glass slide, carefully try to orient grid bars horizontal and perpendicular to the slide; place the PDMS stencil directly over the edge of the grid to ensure a tight, secure fit.

3. Coat grids with poly-l-lysine (PLL) for 30 min in a humidified chamber
    - Place 10 uL drop of PLL (100 ug/mL) on the center of the grid
    - Incubate for at least 30 min in a humidified chamber, such as a 10 cm TC dish with wet kimwipes.

4. Wash grid 3x with 0.1 M HEPES (pH 8.3-8.5)
    - Remove 8 uL of PLL, replace with 8 uL of HEPES (so the grid doesn't completely dry)
    - Remove 8 uL of HEPES, replace with 8 uL of HEPES
    - Repeat for a third wash.

5. Coat grid with PEG-SVA for 1 h
    - Prepare a solution of PEG-SVA fresh just before use at 100 mg/mL in 0.1 M HEPES (pH 8.3-8.5). PEG-SVA has a halflife of 10 min in solution, so reconstitute it JUST before using. Gently pipet or vortex to mix. It dissolves easily.
    - Remove most of the HEPES wash buffer still on the grid, and pipet 10 uL of PEG-SVA solution onto grid.

- Incubate in the dark in a sealed, humidified chamber for at least 1 h.

6. Wash grid 3x with sterile water
    - Remove 8 uL of the PEG-SVA solution and wash with 8 uL sterile water.
    - Remove 8 uL of water wash, replace with 8 uL water.
    - Repeat for a third wash.
    - This is a good stopping point. After washing, grids can be stored in DI water in a humidified chamber at 4 C for several days before proceeding.

7. Coat grid with PLPP gel photoinitiator
    - Prepare 5 uL of PLPP gel for each grid by combining 4.2 uL of 70% ethanol with 0.8 uL PLPP gel. Gently mix.
        - Note: If you're micropatterning many grids, it's best to perform this step in batches of ~4 grids so the PLPP gel doesn't dry for too long.
    - Remove most of the water from the grid and pipet 5 uL of PLPP gel onto the grid. Store in the dark and allow to completely dry (about 15-30 min).
    - Do not stop at this step with the grid coated in PLPP gel! You must proceed with patterning the same day. It's good for a few hours kept in dark, but not longer.

8. Set up microscope and calibrate Primo
    - Turn on microscope power strips, then microscope, then primo.
    - **Important! Make sure the dichoric mirror position is IN (or "on") and PFS is "Off"!**
    - Open Micromanager 1.4 software on the desktop
        - Click "ok" at prompt, make sure camera is set at 16-bits
    - Navigate to plugins → Leonardo → Leonardo
    - In Leonardo software, click 'calibrate'
        1. Preparation
            - Click Remove
            - Type in '20.0' under magnification approx., and set laser power (mW) to 3.80
            - Set microscope to 20x lens
            - Click next

        2. Alignment
            - Place a slide with grids on it either face up or face down
                - If face down (recommended), be sure the focus adjuster ring on the objective body is set to 0 (no thickness)
                - If face up, be sure the focus adjuster ring on the objective body is set to 1.0 – corresponding to the thickness of the microscope slide (or whatever the thickness of your sample is in mm)
            - Find focal plane for the grid holes. Note the Z-position in microns.

        3. Focus
            - Remove grids, place a glass slide with highlighter on it for calibration. Have the highlighter face the same direction that you intend to pattern the grids (if grids will face objective, have highlighter face the objective, i.e., face down)
            - Turn on bright field (left side of microscope body, top round button)

- Find a black round dust speck, move the spot into the red rectangle, and double click on it.
- Once complete, turn off bright field and turn on EPI2 (push the left/right toggle switch inward on right side of microscope body) this opens shutter for UV
- Can also adjust EPI1 to increase contrast and intensity of the image
    - Make sure the EPI2 and EPI1 turrets are in the correct position (EPI1 should be 'EMPTY', EPI2 should be the Primo laser, appearing blue when you turn the laser on in the Leonardo interface)
- On screen, change exposition to 10(ms)

4. Calibration Data
    - adjust the focal plane using the Z-position adjuster on the microscope if necessary. The goal is to get the text in as good of focus as possible. Aim to get the tiny text in the bottom right corner in focus.
    - Once complete, note the calibration values (um/pix should be around 0.28, DMD positions should be roughly: width = 0.500mm; height = 0.300mm)
    - Click on Pattern to load into main page

9. Micropattern grids with Leonardo (~10 minutes per grid)

<u>Method 1</u>

- Center the camera on the center of the grid and bring the mesh into focus
- Click the wand on the right toolbar then 'TEM grid'
- Select a pattern, size=30 and dose = 60 mJ/mm^2, then click 'add grid' then click rotate
- Adjust rotation axis until it lines up with the grid bars
- Select 'Lock', then 'Finish'
- The software will now scan the grid and automatically place your pattern in the middle of each grid square.

<u>Method 2</u>

- Turn on brightfield
- Histogram in the center of the page should be full range
- Use the joystick on the microscope to navigate to the edge of the grid
    - Open ROI
        - Adjust width 3000um; height 3000um (for grids)
- Focus onto mesh holes
- Adjust histogram to allow best contrast
- Open uPattern
    - Micromanager 1.4
    - Find patterns
    - Adjust how many copies of patterns, spacing, sizing (change ratio) and rotation if necessary
    - Dose 30 (do not go higher than 45 as the patterns will get more blurry)
    - Move pattern to grid squares
- Turn off brightfield
- Click PLAY to start patterning

10. Wash grid and coat with ECM protein

- As soon as possible, add 20 uL drop of water to the grid once done patterning and incubate for 5 min
- Wash 3 x water – do not allow the grid to dry!
    - Note: you can stop here after photopatterning and rinsing the grids. Store for up to a few days at 4 C in PBS in a humidified chamber.
- Remove stencil
- Transfer grids to 35 mm glass bottom dish with 2 mLs 70% ethanol in TC hood to sterilize.
- Pipette out most of the liquid and fill with sterile water. Repeat twice more. Don't let the grids dry!
- Wash once with PBS for 5 min
- Incubate grid in ECM protein (0.1 mg/mL fibronectin) for 15-60 min (15 min for fluorescent fibronectin, 60 min for normal fibronectin)
- Wash grid three times with PBS – do not allow the grid to dry!
- Seed cells or store in PBS at 4 C in humid chamber for up to a few days.
- Equilibrate grids in media before adding cells.