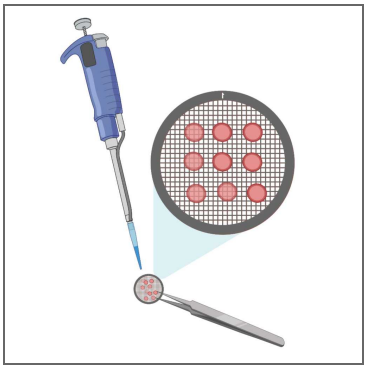

AUG 07, 2023

OPEN ACCESS

Protocol Citation: Farhaz Shaikh, Josh Hutchings, Tamar Basiashvili, Elizabeth Villa 2023. Grid patterning protocol for Cryo-FIB/ET workflow. **protocols.io** https://protocols.io/view/grid-patterning-protocol-for-cryo-fib-et-workflow-cyanxsde

Protocol status: Working
We use this protocol and it's working

Created: Aug 07, 2023

Last Modified: Aug 07, 2023

PROTOCOL integer ID: 86062

Grid patterning protocol for Cryo-FIB/ET workflow

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ABSTRACT

Cryo-Electron Tomography combined with Cryo-Focused Ion Beam milling provides a novel way to study the structure of proteins and the architecture of organelles in situ. Electron microscopy grid preparation is a key step toward a successful Cryo-FIB/ET workflow execution. Here we provide an optimized protocol to generate photo-micropatterns on the grids to enhance cellular deposition and accessibility for Cryo-FIB milling.

Keywords: Cryo-FIB/ET, Cryo grid preparation, Cryo-FIB milling, In situ structural biology, Grid, Photo-Micropatterning

MATERIALS

- Use Gold AU 200 R1/4 Grids for mammalian cells.
- Pelco Plasma cleaner.
- A 10ul PLPP aliquot in ice
- Tweezers
- 1.5 ml centrifuge tubes that each hold 1 mL of DPBS buffer
- Two P10 pipette with tips
- Blotting paper

1. Passivation

1 Place a PDMS stencil (14 mm with 2x2 4 mm wells)into a 35 mm confocal dish (MatTek P35G-1.5-20-C).

2 Glow-discharge both sides of the grids—standard settings suffice (20 mA, 1 min hold, 3-sec hold). Discharge gold side up first. Then discharge carbon film side up.

3 While grids are discharging you can place 1 uL of water in the center of each of the PDMS stencil sections. This is intended to keep grids in place.

4 Get PLL-PEG (100 ul of 1ug/ml) from the -20 freezer. Dilute two fold to working concentration with 100 ul of HEPES buffer (10 mM, pH 7.4).

5 After grids are discharged, add the grid to the center of each of the PDMS wells. Make sure the carbon film side is up.

6 Apply 10 uL of the PLL-PEG solution onto each grid. Start a timer for 1 hour. After 1 hour, you can begin patterning. 01:00:00 1h

2. Preparing Microscope

7 Switch on a light source, microscope, microscope light, Primo (button on the back and key to the right), PC, then PCO camera – in that order.

8 Switch objective to 20x lens with correction collar (S plan Fluor20x), set filter cube #6, and set condenser turret to #7.

9 Open Micromanager. Select Leonardo from plug-ins menu.

10 We recommend using a glass slide with yellow highlighter to verify and focus the Primo laser. The laser can be toggled on/off in Leonardo when autoscale is on.

3. Patterning

11 Remove PLL-PEG solution from the grid.

12 Wash with 10 uL of DPBS four times, try not to touch the grid. During each wash, pipette up and down four times.

13 Blot extra liquid around grids.

14 Add 1 uL of PLPP onto the grid.

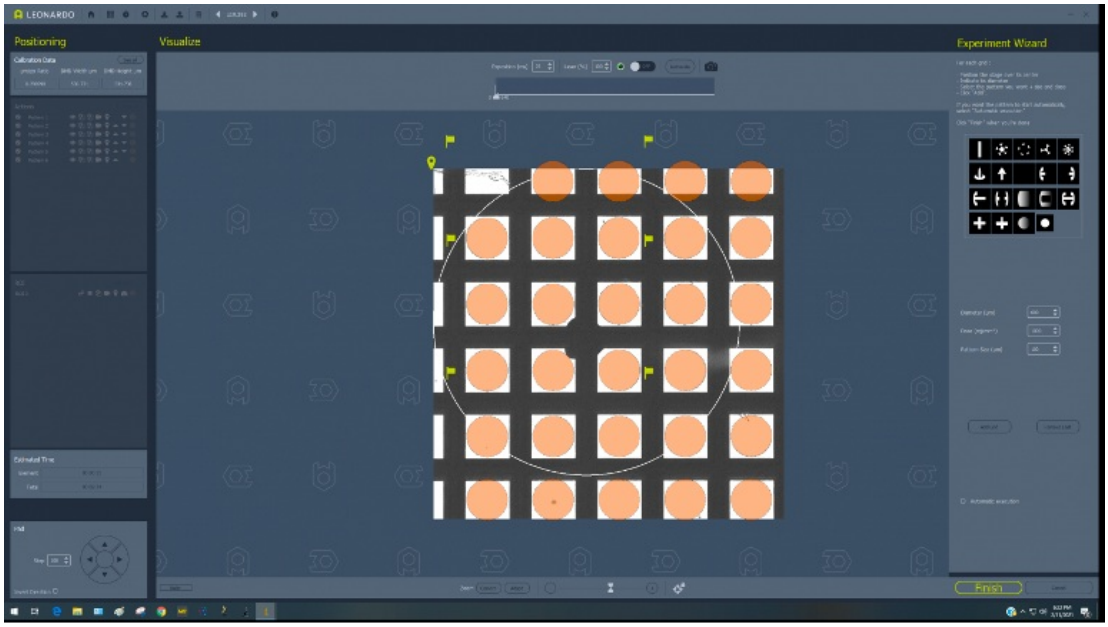
15 Place the dish onto the microscope stage, drive stage to grid center and

focus on the foil giving the sharpest view of holes in the foil mesh.

16 Set contrast by pressing and unpressing autoscale.

17 Automatic Patterning: Load TEM wizard and specify settings.

- Select "Add Grid"
- Select "Automatic execution"
- Select "Lock"
- Select "Rotate"
- Select "Finish



18 Manual Patterning:

- Select "MicroPatterning"
- Select desired pattern
- Set desired ration and angle
- Set dose to 1000
- Select "Lock"
- Select "Play"

4. Finishing up

19 Wash the grids with 10ul of DPBS four times, try not to touch the grid. During each wash, pipette up and down four times.

20 Leave 10ul of DPBS on the grids.

21 Repeat the patterning steps for each grid you have.