PRIMO PROTOCOL: TEM grid photopatterning

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This protocol is a guideline and you might need to optimize it for each different application.

This protocol was established using Quantifoil Cu an Au grids. It may need some optimization on different substrates.

1. Reagents and Equipment

Equipment

- PRIMO® setup
- TEM grid PDMS stencil (if using PLPP Gel)
- Plasma cleaner or glow discharge system (optional depending on the passivation)

Reagents

Specific reagents depending on the passivation. Please refer to the next paragraph to look for the passivation you are going to use.

- Protein solution of interest
- PBS 1 X

PLL-g-PEG passivation:

- PLL-g-PEG (*Alvéole,* PLL(20)-g[3.5]-PEG(2) 100 µg.mL^{-1} in PBS 1 X. Storage : -20°C, 10x solution)
- PLPP 1 X

PEG-SVA passivation :

- mPEG-SVA (mPEG-Succinimidyl Valerate, MW 5 000, *Laysan bio*)
- Poly-L-Lysine (PLL, *Merck/ Sigma-Aldrich*, ref: P8920)
- HEPES 8,3 < pH < 8,5 at 0,1 M
- PLPP Gel (*Alvéole*)
- EtOH (70 % or more)
- H_2O mQ

1. Choosing the right protocol

We developed two protocols.

- The first one is using the **PLPP Gel**. This protocol requires several drying of the grid.
- The second one uses the **PLPP liquid**. The grid stays wet from the first step to the last step.

PLPP Gel is 10 to 30 times faster than the PLPP liquid depending on the passivation

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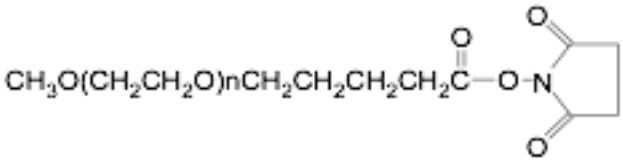
2. Patterning using PLPP Gel photoinitiator and PEG-SVA passivation

2.1. Passivation protocol with PEG-SVA



mPEG-SVA is a PEG (5kDa) coupled with a Succinimidyl Valerate (SVA) ester. This ester is used for PEGylation of primary amine.
The strategy for glass passivation with PEG-SVA is the following:

1) Coating of the substrate with polylysin (PLL)
2) Coupling of the amines of the polylysin with the SVA ester

- Glow discharge your TEM grid (optional but highly recomended).
- Place your TEM grid PDMS stencil on your grid
- Incubate your substrate with PLL at 100 µg.ml^{-1} for 30 minutes.
- Rinse 3 times with HEPES (8,3 < pH < 8,5).
- Make a solution of PEG-SVA in a buffer 8,3 < pH < 8,5 at 70 mg.ml^{-1}. If the passivation is not strong enough, you can increase the concentration up to 100 mg.ml^{-1}.

As the half-life of the SVA ester is 10 minutes at pH 8,5, the solution must be prepared just before use.

- Incubate your grid with 10-20 µl of the PEG-SVA solution for 1 hour.
- Rinse with DI water. The passivated grid can be kept dried for few weeks at 4°C.

2.2. Photopatterning with PRIMO using PLPP Gel photoinitiator

- Deposit 1 µl of PLPP gel at the center of the stencil.
- Add 2 µl of DI water. Make sure that the solution spread on all the grid, if not add more mQ water.
- Wait for the complete drying of the solution, a transparent gel will form.
- Place the sample upside down on the microscope holder
- Focus on the surface
- Load and lock your pattern on Leonardo software. We can use the automatic structure detection functionality or the TEM grid functionality (Check tutorial video at https://www.alveolelab.com/tutorials/micropatterning-tem-grid/)
- The typical UV doses are listed below. It might depend on the density of PLPP Gel, substrate, etc… **dose adjustment might be needed.**

Primo (laser, 375 nm)	Primo 2 (LED, 365 nm)
40 mJ/mm^{-2}	20 mJ/mm^{-2}

- Launch patterning sequence
- Rinse profusely with PBS.
- Rehydrate the slide with PBS for 5 minutes

- Incubate 5 minutes with a solution of 20 µg/ml of protein in its dedicated buffer. You can increase the concentration to 100 µg.ml^{-1} and the incubation time to 15 min.
- Rinse profusely with PBS without drying

3. Patterning using PLPP photoinitiator and PLL-g-PEG passivation

3.1. Passivation with PLL-PEG

- Glow discharge your TEM grid. You can also use a plasma cleaner with low power.
- For each grid, put a drop of 20 µl of PLL-PEG at 100 µg.ml^{-1} on a glass coverslip. Insert the grid inside the drop. Put the coverslip in a humid chamber to avoid evaporation. **Be careful not to dry during all the process.**
- Incubate 1 hour.
- Rinse 3 times with PBS 1X.

3.2. Photopaterning with PRIMO using PLPP photoinitiator

- Put your grid face down on a glass coverslip
- Put a 15 µl drop of PLPP on your grid.
- Focus on the surface.
- Load and lock your pattern on Leonardo software. We can use the automatic structure detection functionality or the TEM grid functionality (Check tutorial video at https://www.alveolelab.com/tutorials/micropatterning-tem-grid/)
- The typical UV doses are listed below. It might depend on the density of PLPP Gel, substrate, etc… **dose adjustment might be needed.**

Primo (laser, 375 nm)	Primo 2 (LED, 365 nm)
800 mJ/mm^{-2}	400 mJ/mm^{-2}

- Launch patterning sequence
- Rinse profusely with PBS.
- Rehydrate the slide with PBS for 5 minutes
- Incubate 5 minutes with a solution of 20 µg/ml of protein in its dedicated buffer. You can increase the concentration to 100 µg.ml^{-1} and the incubation time to 15 min.
- Rinse profusely with PBS without drying

4. Cell seeding

This step is really cell type dependent and will need some adjustment.

- Rince your sample with media without dewetting the substrate.
- Add 1.10^4 to 1.10^5 cells per cm²
- Carefully put your sample in the incubator and let them adhere for 15 min to 1 hour (or more) depending on your cell type
- Rinse gently with cell culture media to remove the non-adherent cells.
- If cells are attaching outside the patterns, wait one more hour and pipet over the cells until the one outside of the pattern detach.

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5. Publication

- Toro-Nahuelpan, M., Zagoriy, I., Senger, F. *et al.* **Tailoring cryo-electron microscopy grids by photo-micropatterning for in-cell structural studies**. *Nat Methods* **17,** 50–54 (2020).

- Leeya Engel[1,6] et al. **Extracellular matrix micropatterning technology for whole cell cryogenic electron microscopy studies.** *Journal of Micromechanics and Microengineering,* Volume 29, Number 11 (2019).