Tailoring cryo-electron microscopy grids by photo-micropatterning for *in-cell* structural studies

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Abstract

Micromachining by cryo-focused ion beam (FIB) milling coupled to cryo-electron tomography (ET) enables visualization of macromolecules directly inside cells. Yet, spatial control of cell adhesion on electron microscopy (EM) grids remains a bottleneck in the specimen preparation pipeline. This protocol describes a contactless and mask-free photo-micropatterning of EM grids for site-specific deposition of extracellular matrix-related proteins. We achieved accurate and reproducible cell positioning, leading to optimized preparations for cryo-FIB milling. We tested HeLa and RPE1 cell lines on various grid types (gold or titanium mesh coated with SiO_2, gold or carbon films). Briefly, grids were passivated with an anti-fouling agent, followed by controlled ablation of the passivation layer, and further functionalization with fibronectin. The micropatterning procedure takes ~3 h. Employing micropatterning to produce complex shapes generated a predictable intracellular organization, allowing direct correlation between cellular architecture and *in-cell* 3D-structural characterization of the underlying machinery at molecular resolution.

Introduction

A standard cryo-EM carrier is a 3 mm diameter metal mesh (grid) coated with a perforated (holey) thin film, commonly made of amorphous carbon. For *in situ* cryo-ET, cells are typically allowed to adhere and spread over such grids to be subsequently arrested by vitrification[1]. Most cells are too thick for direct analysis by cryo-ET. To render the biological specimen electron transparent, cells must be micromachined by cryo-FIB to generate a thin lamella[2-8]. To this end, cells must be (i) positioned roughly at the center of an individual grid square[9], and (ii) in the central region of the grid. The first requirement is essential for proper ablation of cellular material by cryo-FIB milling, while the second constraint is posed by the subsequent requirement of stage tilt in the transmission electron microscope (TEM) for collection of tomographic tilt-series. Currently, optimization of grids for cellular cryo-ET is carried out primarily by adjusting the concentration of the cell suspension during seeding. However, adherent cells settle randomly on grids, often in the vicinity or directly on the metal grid bars, making them inaccessible to the FIB or to cryo-ET. Here, we describe a method to overcome these issues by photo-micropatterning of EM supports to generate areas preferable for cell adhesion that meet the requirements of subsequent steps in the cryo-ET pipeline. At the technical level, the micropatterning of EM grids contributes to refined, routine and user-friendly specimen preparations for *in-cell* structural biology. Furthermore, it aids to solve technical challenges that have, thus far, hindered high-throughput FIB thinning preparations.

The proof of principle and broader applications of this protocol in the context of biological studies by means of state-of-the-art cryo-electron microscopy pipeline (i.e., sample vitrification, cryo-correlative light and electron microscopy, cryo-FIB milling and cryo-electron tomography) are discussed in detail in the associated manuscript[10].

Reagents & Materials

Reagents for micropatterning:

- Fibrinogen-Alexa546 (ThermoFischer Scientific, Schwerte, Germany. Cat. no. F-13192).
- Fibronectin (ThermoFischer Scientific, Schwerte, Germany. Cat. no. F1141-1MG).
- Hepes 10 mM pH 7.4.
- $NaHCO_3$ 100 mM pH 8.4.
- PEG-sva (Laysan Bio, Arab, US. Cat. no MPEG-SVA-5000).
- PLL(20)-g[3.5]-PEG(5) (SuSoS AG, Dübendorf, Switzerland).
- PLPP (14.5 mg/ml Alvéole Lab, Paris, France).
- Poly-L-lysin 0.01% solution, suitable for cell culture (Sigma Aldrich, St. Louis, MO. Cat no P4707).
- Phosphate-buffered saline (PBS), pH 7.4.

Additional reagents for cell culture:

- DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (ThermoFisher Scientific, Schwerte, Germany. Cat. no. 31966021).
- DMEM/F-12, GlutaMAX™ supplement (ThermoFisher Scientific, Schwerte, Germany. Cat no. 10565018).
- Fetal Bovine Serum, FBS Superior (Biochrom, Berlin, Germany. Cat. no. S 0615).
- FluoroBrite™ DMEM (ThermoFisher Scientific, Schwerte, Germany. Cat. no. A18967-01).
- Penicillin-Streptomycin (10,000 U/ml) (ThermoFischer Scientific, Schwerte, Germany. Cat. no 15140122).
- Trypsin-EDTA (0.05%), phenol red (ThermoFischer Scientific, Schwerte, Germany. Cat. no 25300054).

Electron microscopy grids:

- Gold (Au) or Titanium (Ti) 200-mesh grids with either a holey 12 nm thick film made of SiO_2, 50 nm gold or 12 nm amorphous carbon. The grids employed in this study were either R2/1 (2 µm holes separated by 1 µm spacing, with or without a continuous 2 nm layer of carbon), R1/4 or R1.2/20. All grids were purchased from Quantifoil Micro Tools, Jena, Germany.

Equipment

- Plasma cleaners (successfully tested):
    - Diener Femto Plasma (Diener electronic, Ebhausen, Germany).
    - Diener Zepto Plasma (Diener electronic, Ebhausen, Germany).
    - Pelco EasyGlow Glow Discharge Unit (Ted Pella, Redding, USA).
    - Nano Clean Plasma cleaner Model 1070 (Fischione Instruments, Hanau, Germany).

- Confocal microscope (Olympus FluoView 1200) with UPLSAPO 63x (NA 1.35) oil objective, equipped with a 355 nm laser line.

- UV pulsed laser source of 355 nm laser wavelength (PNV-001525-140, Teem Photonics, Meylan, France).

- PRIMO™ micropatterning module (Alvéole Lab, Paris, France) connected to a compatible microscope (Nikon Ti-E microscope. Other compatible brands are Leica and Olympus).

- Olympus CKX41 widefield microscope.

- Glass bottom ibidi µ-Dish 35 mm low and high (ibidi, Martinsried, Germany - Cat. no. 80136 and 81156, respectively).

- Glass slides (ThermoFisher Scientific, Schwerte, Germany Cat. no. 12114682).

- Bunsen burner, portable Labogaz® 206 (GEYER GmbH, Stuttgart, Germany. Cat. no. 9018510).

- Bunsen burner cartridge C206 (GEYER GmbH, Stuttgart, Germany. Cat. no. 6261010).

- Nitrile Examination Gloves, Powderfree (ThermoFisher Scientific, Schwerte, Germany. Cat. no. 112-0998).

- Brand Parafilm M 10 cm wide, 38 m per roll (neoLab Migge GmbH, Heidelberg, Germany. Cat. no. 3-1011).

- Kimberly-Clark Professional™ Kimtech Science™ Precision Wipes (ThermoFisher Scientific, Schwerte, Germany. Cat. no.7552).

- Bacteriological Petri Dishes with Lid 150 mm and 100 mm (Corning, Kaiserslautern, Germany, Cat. no. 351038 and 351029, respectively).

- Cell culture flasks 175 cm^2 and 25 cm^2 (ThermoFisher Scientific, Schwerte, Germany. Cat. no. 178905 and 136196, respectively).

- Tweezers n° 55 (Dumont, Montignez, Switzerland. Cat no. 0209-55-PO).

- TC20™ Automated Cell Counter (BioRad, Feldkirchen, Germany. Cat. no. 1450102)

- Cell Counting Slides for TC10™/TC20™ Cell Counter, Dual-Chamber (BioRad, Feldkirchen, Germany. Cat. no. 1450021)

- TC10 Trypan Blue Dye, 0.4% solution (BioRad, Feldkirchen, Germany. Cat. no. 1450003)

- Test Tube with Cell Strainer Snap Cap 35 µm mesh (Corning, Kaiserslautern, Germany, Cat. no. 352235)

- Softwares:
  Inkscape (open source, http://www.inkscape.org/).
  Olympus FV 10-ASW software v04.02.03.02.
  µmanager software v1.4.22 (open source, https://micro-manager.org/).
  Leonardo software v4.12 (Alvéole Lab, Paris, France).
  CellSens Entry 1.9 (Olympus)

Procedure

Grid passivation

PLL-g-PEG solution:
Prepare 1 mg/ml solution in Hepes 10 mM pH 7.4 as recommended by the manufacturer. Aliquot a 100 µl volume (or a convenient amount), which would serve to treat ~20 grids, and store at -20°. Dilute the solution in the same buffer to 0.5 mg/ml immediately before use.

> **Note:** PLL-g-PEG can be stored for one week at 4°. Nevertheless, we recommend to use it immediately after its preparation or thawing.

Plasma cleaning:
Use low-pressure plasma cleaning to oxidize the grids and render them hydrophilic.

- Place grids onto a glass slide and plasma clean both sides. For SiO_2 film use a 100W power with a 10 cm^3/min flow rate of oxygen gas for 30-40 s (each side), while for carbon films a 30W power for 10-30 s should be used.

> **Note:** we have also employed successfully different equipment for this step, e.g., plasma cleaning under atmospheric air at 30W for 45 sec at 0.039 mbar (i.e., Pelco EasyGlow Glow Discharge Unit. Ted Pella, Redding, USA, Cat. no. 91000) for any grid, or 0.03 mbar (Diener Zepto Plasma, Diener electronic, Ebhausen, Germany) using a 100W for SiO_2 and 30W for carbon films for 30 s. We additionally tested a mixture of oxygen/argon 75/25% (Nano Clean Plasma cleaner. Fischione Instruments, Hanau, Germany) at 100W for SiO_2 and 30W for carbon films for 30 s.

One-step passivation:
- Prepare a humid chamber: 100 mm culture dish, containing water-soaked filter paper and a parafilm on top.
- Place 20 µl droplets of PLL-g-PEG (0.5 mg/ml) on the parafilm. Each droplet is used to passivate a single grid.
- Place the plasma cleaned grids on the PLL-g-PEG droplets, and incubate in the humid chamber for 1h at room temperature or overnight at 4°C (sealed with parafilm).

***CRITICAL*:** do not let the PLL-g-PEG dry out on the grid during the passivation.

Alternative two-step passivation:

- Incubate the grids on 20 µl droplets of 0.01 % PLL overnight on parafilm in a humid chamber at room temperature.
- Wash the grids five times in 100 mM $NaHCO_3$ pH 8.4, and incubate for 1-2 h with 50 mg/ml PEG-sva dissolved in the same solution.
- Wash the grids five times in 100 mM $NaHCO_3$ pH 8.4.

> **Note:** the two-step passivation is a convenient alternative in the absence of a plasma cleaner.

Design of micropatterns

The design of micropatterns depends on the microscope set-up used. For example, if nanoablation with a 355 nm pulsing laser is performed, the micropattern design will be limited by the features and

performance of the confocal microscope software. If using the PRIMO module (Alvéole Lab, Paris, France), patterns can be designed in Inkscape (http://www.inkscape.org/) as 8-bit binary files and exported as png files according to the PRIMO manufacturer's instructions, which can be loaded into the Leonardo software v4.12 (Alvéole Lab, Paris, France).

> **Note:** when designing the patterns in Inskape, it is important to consider the calibration of the equipment, i.e., pixel to µm ratio, as well as the size of the DMD mirror, for proper sizing of the pattern and the estimated number of expositions. These parameters will dictate the patterning time.

Micropatterning of grids (two methods)

1. Nanoablation by a 355 nm pulse laser:

An inverted confocal Olympus FluoView 1200 (Olympus, Hamburg, Germany) microscope was used, equipped with a UV pulsed laser source of 355 nm, a UPLSAPO 63x (NA 1.35) oil objective, and a standard PMT or GaAsP PMT detectors.
The 355 nm laser had an average power of 50 mW, 500 ps pulse width, 1kHz repetition rate, and a maximum energy per pulse of 30 µJ.

- After passivation with PLL-g-PEG, blot-dry the grid from the back on a Kimtech wipe, and quickly place the grid with the SiO_2 film facing down (towards the objective) on a 1-3 µl drop of either Hepes 10 mM pH 7.4 or PBS in a sealed glass bottom low 35 mm ibidi µ-Dish.

  > **Note:** high humidity should be kept using water-soaked filter paper inside the dish to avoid buffer evaporation.

  > **Note:** only grids with SiO_2 films were micropatterned with this technique. For carbon and gold films, the laser conditions need to be adjusted, and especially tuned to a lower power for carbon.

- Use transmission and/or reflection (e.g., 488 nm laser) to focus on the grid film.

- Draw patterns (ROIs) of square or circular shape (between 10 to 40 µm diameter, depending on the aim of the experiment) using the Olympus FV 10-ASW software.

- Focus on the film and generate micropatterns by applying a 10-11 % laser power, 40 µs per pixel and 10 iterations.

- Move to the next grid square, focus on the film, and apply the laser. Repeat this step until the desired area is covered (e.g., 6x6 grid squares at the central area of the grid).

2. PRIMO™ (DMD-based illumination + Photo-activator):

Micropatterning is performed using a 375 nm laser (4.5 mW) and a digital mirror device (DMD) to generate a spatially controlled laser illumination of the sample (PRIMO™, Alvéole Lab, Paris, France). Resolution limit: ~1.2 µm. In this case, an inverted Nikon microscope Ti-E equipped with a CFI Super Plan FLuor 20x ELWD (NA 0.45) lens with high UV-transmission, a Perfect Focus System 3, an ORCA-Flash 4.0 LT CMOS camera (Hamamatsu, Japan), a motorized stage (Märzhäuser, Wetzlar, Germany), and the PRIMO micropatterning module (Alvéole Lab, Paris, France) was used.

- After passivation with PLL-g-PEG, blot-dry the grids from the back on a Kimtech wipe, and quickly place it (with the film facing towards the objective) on a 1-3 µl of PLPP (4-benzoylbenzyl-trimethylammonium chloride, 14.5 mg/ml) drop in a sealed glass bottom ibidi µ-Dish 35 mm low.

CRITICAL: do not let the PLPP solution dry out on the grid.

Note: high humidity should be kept using water-soaked filter paper inside the dish to avoid PLPP evaporation.

Note: the microscope stage and photo-patterning were controlled with the μmanager software by the Leonardo plugin software.

Note: although PLPP solution is stable (as per manufacturers), it is recommended to aliquot a convenient volume and freeze at -20°.

- Load the *png* file with the design of the micropattern in the Leonardo software, and accommodate (rotate and translate) the pattern to the grid position. Select the stitching mode, and use a final dose of 800-1000 mJ/mm^2 (equivalent to ~30 s per DMD exposition).

  Note: continuous focusing on the film is required during the patterning period. The automated hardware-based focus system of the microscope employed here is unable to accurately and reliably focus on the grid film. Therefore, on the fly continuous and manual focusing on the film is required while the automated patterning is running. The focusing step will highly depend on the quality and flatness of the grid. Focusing could be further optimized by generating a tool (e.g., script) to keep the focus on the grid film while patterning.

- Retrieve the grid from the PLPP solution and wash in a 1000 μl drop of water, followed by two consecutive washes in 300 μl drops of PBS.

  Pause point: at this point, one-step passivated and micropatterned grids can be stored in PBS at 4°C in a humid chamber, remaining functional for at least 30 days. Alternatively, grids can be stored dried, after the water washing step, remaining functional for up to ~7 days in atmospheric conditions. However, although grids were functional after 1 h – overnight rehydration, their performance appears superior when stored wet.

  General remarks and guidelines for micropatterning:

  Minimize the time that the grids are kept in the PLPP solution. Grids were typically retrieved from the PLPP solution immediately after patterning, washed in abundant water and placed into the corresponding buffer for the next step.

  Patterning of 4 grids (using the Primo module) with a 6 x 6 or 8 x 8 grid squares area will take approximately 30 min or 1 h, respectively. To pattern 4 grids, 40 μl of 1 mg/ml PLL-g-PEG is required, and takes a minimal time of 3 hours, including grid functionalization.

  Optional: a silicon stencil can be used to keep the grid on place[11].

Grid functionalization

- Incubate grids in a 20 μl drop of either 50 μg/ml fibronectin (prepared in PBS), or 30 μg/ml of fibrinogen-Alexa546 (prepared in 100 mM $NaHCO_3$ pH 8.4) on parafilm for 1h at room temperature.

  Note: fibrinogen-Alexa546 is used to test for proper micropatterning, while fibronectin is meant for cell adhesion. A combination of both can also be used in a 1:5 or 1:10 ratio (fibrinogen:fibronectin).

- Wash the grids three times in 300 μl drops of the corresponding buffer (PBS for fibronectin and, 100 mM $NaHCO_3$ pH 8.4 for fibrinogen).

Pause point: fibronectin-treated grids can be stored in PBS in a humid chamber at 4°C, remaining functional for at least 10 days. The maximum active life time of protein-functionalized grids remains unknown. This will depend on the protein stability itself.

- Check for micropatterned areas by fluorescence microscopy (detection of Alexa546) if applicable.

Cell seeding

- Retinal pigment epithelium (RPE1) cells were cultured in 5 ml of DMEM F-12 + GlutaMAX, and HeLa cell lines in DMEM + GlutaMAX, in 50 ml culture flasks. Both were supplemented with 100 mg/ml of penicillin and streptomycin. Incubate the cultures at 37° with 5% CO_2.

  Note: Cells should ideally be at maximum 60% confluency.

- Transfer the grids (film facing upwards) to a 35 mm ibidi dish high, filled with 1 ml of medium.

- Detach cells from flask by trypsinization using 2 ml of Trypsin-EDTA (0.05%), retrieve the solution and incubate for 2 min at 37°. Check under the microscope for cells rounding, and resuspend cells in 1 ml of medium.

- Count cells by mixing 10 µl of TC10 Trypan Blue Dye with 10 µl of the cell suspension. Mix by pipetting up and down gently 20 times.

- Pass the cells trough a strainer and prepare 80,000 RPE1 cells or 200,000 HeLa cells in 1 ml of medium. Add the cells to the 35 mm ibidi dish with the grids, equivalent to a density of 8×10^3 cells/cm^2 and 2×10^4 cells/cm^2, respectively.

***CRITICAL*:** perform the cell strainer step immediately before seeding.

- Incubate the cells on the grids for 20-35 min for RPE1 cells or 1.5-2 h for HeLa cells, to allow initial attachment.

  Note: cell number and timing of seeding needs to be optimized for each cell type.

- Transfer the grids to a new cell-free dish with medium. Briefly, check grids for seeding by transmitted light microscopy (e.g., Olympus CKX41 widefield microscope equipped with the CellSens Entry 1.9 software).

  Note: the transfer to a new dish is beneficial to avoid attachment of multiple cells per pattern, and also to avoid eventual non-specific attachment of cells on non-patterned areas.

- Incubate the dish at 37°C with 5 % CO_2 to allow full cell adhesion to the grids.

  Note: Seeding should be performed in a sterile laminar flow hood.

Vitrification, Cryo-FIB milling and Cryo-ET

- Vitrify cells after 4-6 h post-transfer for RPE1 cells (to attain a higher number of grid squares with a single cell) or after overnight incubation for HeLa cells.

- Cells can be plunge-frozen in a Leica GP EM. Settings: 1.5 s blotting for R2/1 and R1/4 grids, and 2.5 s blotting for R1.2/20 holey and continuous film. For further details of vitrification, cryo-FIB milling, and cryo-ET please refer to[5,10].

- Grids should be stored in sealed boxes in liquid nitrogen until use.

Final remarks:

All micropatterning steps and grid treatments were performed under sterile conditions using a Bunsen burner. Grids were handled with a tweezer n° 55. FluoroBrite™ medium was used for live cell imaging.

Troubleshooting

Grids moving during patterning

Reduce buffer/PLPP volume. If the volume is already reduced to 1 µl and the issue persists, after placing the grid in the drop, drag/move the grid aside of the drop. This will reduce the volume between the grid and the glass bottom of the dish, better fixing the grid. Evaporation of the solution under these conditions should not be an issue while high-humidity is kept as recommended. Alternatively, a silicon stencil can be used[11].

No apparent micropatterning

- Case 1: PLPP solution dried out on the grid during the patterning process.
- Case 2: the grid dried out after protein functionalization.
- Case 3: PLPP solution is compromised. Although the PLPP solution is stable (as reported by the manufacturers), it may be worth trying a new batch of PLPP solution.
- Case 4: PLL-g-PEG solution is compromised. Try using a fresh solution. Keep the chemical dry, close it under neutral atmosphere (argon or nitrogen gas), and store at -20°.
- Case 5: laser not working properly. When using the 355 nm pulsing laser technique, this can be validated by visualizing the presence or absence of the engraving pattern on the film by transmitted light microscopy (or grid reflection). For the PRIMO module, one can simply check the power (mW) of the laser in the software and, by placing a white paper on top of the sample, one should observe the laser illumination.
- Case 6: fluorescence reporter not working properly.

Multiple cells per pattern

- When aiming for single cells per pattern, progressively reduce the time and/or number of cells upon seeding.
- Reducing the pattern size also aids to obtain single cells per pattern, however, it should be considered that not all cell types may perform well (fitness) when strongly constrained to a small area.
- Pipetting medium directly over the grid (20 µl volume) in the dish allows to detach agglomerated cells and thus obtained single cells per grid square or patterns. While RPE1 and HeLa cells are robust, it should be noted, however, that some cell types are highly sensitive to such mechanical stress, affecting subsequent performance.

Time taken

- One-step passivation: 1 h or overnight.

- Two-step passivation: 20-24 h.

- Micropatterning with a 355 nm pulse laser:
  Micropatterning of a 4x4 grid squares area (200-mesh grid: ~260,000 μm^2) takes approximately 8-10 min for an experienced user familiar with the microscope set-up.

  Note: a disadvantage of this method, compared to the PRIMO module, is that it takes considerable time to manually move the stage between grid squares, positioning at the center of the square, and refocusing on the film. This may vary from microscope to microscope and their ability to autofocus on the grid film in an automated and quick manner. To optimize time, potentially a lower magnification objective can be used to pattern more grid squares simultaneously, provided that the film is sufficiently flat to keep the targeted areas in focus. Titanium grids had a consistent film flatness aiding quick focusing on each grid square, facilitating the micropatterning using this technique.

- Micropatterning with the PRIMO module:
  This device can generate a patterned area of:
  - 6x6 grid squares (200-mesh grid: ~560,000 μm^2) in ~3 min
  - 8x7 grid squares (200-mesh grid: ~900,000 μm^2) in ~6 min
  - 10x10 (200-mesh grid: ~1,500,000 μm^2) in ~10 min.

  Note: Timings correspond to a dose of 800-1000 mJ/mm^2. Times may vary depending on the grid positioning with respect to the DMD mirror illumination.

  Note: a disadvantage of this method, compared to the micropatterning using a 355 nm pulsing laser, is the lower spatial resolution. While the PRIMO module provides a resolution limit of ~1.2 µm, the 355 nm pulsing laser is limited by the point spread function (PSF). Therefore, achieving a lateral resolution of ~250 nm. Continuous on the fly focusing on the film (during patterning) is likely necessary.

- Protein functionalization: 1 h

- Cell seeding: 3 h

- Vitrification: 1 h

- Cryo-FIB milling: 10 h

- Cryo-ET: 24 h

Anticipated results

1. Plasma cleaning
Make sure to check that the grids are rendered hydrophilic. This can be observed by immediate wetting of the grids when placed on the passivation PLL-g-PEG solution. The grids should not exhibit resistance to be immersed in the drop of solution.

2. Patterning with 355 nm laser
Micropatterning can be directly observed by the engraving of the grid film immediately after the patterning by transmission or reflection modes.

3. Pattern visualization
Successful grid micropatterning can be judged from fluorescence light microscopy imaging of fibrinogen-Alexa546-adsorption (or other fluorescent reporter, e.g., GFP protein) that should be restricted to the PEG-free (patterned) areas.
Differential functionalization with several proteins is possible by performing a stepwise micropatterning of several areas, followed by protein functionalization after every run of micropatterning.

4. Cell seeding
After the transfer of the grids to a new cell-free dish, cells should be observed in the middle of the grid square (in the putative patterned area) most likely starting to acquire the shape of the designed micropatterned.

For visual material of steps 2 to 4, please, refer to the associated publication of this protocol[10].

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