Micropatterning Transmission Electron Microscopy Grids to Direct Cell Positioning within Whole-Cell Cryo-Electron Tomography Workflows

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Abstract

Whole-cell cryo-electron tomography (cryo-ET) is a powerful technology that is used to produce nanometer-level resolution structures of macromolecules present in the cellular context and preserved in a near-native frozen-hydrated state. However, there are challenges associated with culturing and/or adhering cells onto TEM grids in a manner that is suitable for tomography while retaining the cells in their physiological state. Here, a detailed step-by-step protocol is presented on the use of micropatterning to direct and promote eukaryotic cell growth on TEM grids. During micropatterning, cell growth is directed by depositing extra-cellular matrix (ECM) proteins within specified patterns and positions on the foil of the TEM grid while the other areas remain coated with an anti-fouling layer. Flexibility in the choice of surface coating and pattern design makes micropatterning broadly applicable for a wide range of cell types. Micropatterning is useful for studies of structures within individual cells as well as more complex experimental systems such as host-pathogen interactions or differentiated multi-cellular communities. Micropatterning may also be integrated into many downstream whole-cell cryo-ET workflows, including correlative light and electron microscopy (cryo-CLEM) and focused-ion beam milling (cryo-FIB).

Introduction

With the development, expansion, and versatility of cryo-electron microscopy (cryo-EM), researchers have examined a wide range of biological samples in a near-native state from

macromolecular (~1 nm) to high (~2 Å) resolution. Single-particle cryo-EM and electron diffraction techniques are best applied to purified macromolecules in solution or in a crystalline state, respectively[1,2]. Whereas cryo-electron tomography (cryo-ET) is uniquely suited for near-native structural and ultrastructural studies of large, heterologous objects such as bacteria, pleomorphic viruses, and eukaryotic cells[3]. In cryo-ET, three-dimensional (3D) information is obtained by physically tilting the sample on the microscope stage and acquiring a series of images through the sample at different angles. These images, or tilt-series, often cover a range of +60/−60 degrees in one-to-three-degree increments. The tilt-series can then be computationally reconstructed into a 3D volume, also known as a tomogram[4].

All cryo-EM techniques require the sample to be embedded in a thin layer of amorphous, non-crystalline, vitreous ice. One of the most commonly used cryo-fixation techniques is plunge freezing, where the sample is applied to the EM grid, blotted, and rapidly plunged into liquid ethane or a mixture of liquid ethane and propane. This technique is sufficient for the vitrification of samples from <100 nm to ~10 μm in thickness, including cultured human cells, such as HeLa cells[5,6]. Thicker samples, such as mini-organoids or tissue biopsies, up to 200 μm in thickness, can be vitrified by high-pressure freezing[7]. However, due to increased electron scattering of thicker samples, sample and ice thickness for cryo-ET is limited to ~0.5 – 1 μm in 300 kV transmission electron microscopes. Therefore, whole-cell cryo-ET of many eukaryotic cells is limited to the cell periphery or extensions of cells unless additional sample preparation steps are used, such as cryo-sectioning[8] or focused-ion beam milling[9,10,11].

A limitation of many whole-cell cryo-ET imaging experiments is data collection throughput[12]. Unlike single-particle cryo-EM, where thousands of isolated particles can often be imaged from a single TEM grid square, cells are large, spread-out, and must be grown at low enough density to allow for the cells to be preserved in a thin layer of vitreous ice. Often the region of interest is limited to a particular feature or sub-area of the cell. Further limiting throughput is the propensity of cells to grow on areas that are not amenable for TEM imaging, such as on or near TEM grid bars. Due to unpredictable factors associated with cell culture on TEM grids, technological developments are needed to improve sample accessibility and throughput for data acquisition.

Substrate micropatterning with adherent extra-cellular matrix (ECM) proteins is a well-established technique for live-cell light microscopy to direct the growth of cells on rigid, durable, and optically transparent surfaces such as glass and other tissue culture substrates[13,14]. Micropatterning has also been performed on soft and/or three-dimensional (3D) surfaces. Such techniques have not only allowed for the precise positioning of cells; they have also supported the creation of multicellular networks, such as patterned neural cell circuits[15]. Bringing micropatterning to cryo-ET will not only increase throughput, but it can also open up new studies for exploring complex and dynamic cellular microenvironments.

Recently, several groups have begun using micropatterning techniques on TEM grids through multiple approaches[16,17].Here, the use of a maskless photopatterning technique for TEM grids is described using the Alvéole PRIMO micropatterning system, which

features high-resolution and contactless patterning. With this micropatterning system, an anti-fouling layer is applied on the top of the substrate, followed by the application of a photocatalyst and ablation of the anti-fouling layer in user-defined patterns with a UV laser. ECM proteins can then be added to the patterns for the appropriate cell culture. This method has been used by several groups for cryo-ET studies of retinal pigment epithelial-1 (RPE1), Madin-Darby canine kidney-II (MDCKII), human foreskin fibroblast (HFF), and endothelial cell lines[16,17,18]. This micropatterning system is compatible with multiple anti-fouling layer substrates as well as either a liquid or gel photocatalyst reagent. A variety of ECM proteins can be selected from and adapted for the specificity of the cell line, conferring versatility for the user.

Micropatterning has been successfully applied to a number of projects within the laboratory[19]. Here, a micropatterning protocol is presented, including specific adaptations to study cultured HeLa cells, respiratory syncytial virus (RSV)-infected BEAS-2B cells, and primary larval *Drosophila melanogaster* neurons[20].

Protocol

The protocol described here is a compilation of the cell culture, micropatterning, and imaging methods used by the Wright lab and the Cryo-EM Research Center at the University of Wisconsin, Madison. The workflow is presented in Figure 1. Additional training and instructional materials are available at the following sites: https://cryoem.wisc.edu or https://wrightlab.wisc.edu

1. **Preparation of grids for patterning**

    1. Transfer the TEM grids onto a clean glass slide, carbon side up (the standard thickness of the carbon foil is 12 nm). Using a carbon evaporator, ACE600, evaporate 5–8 nm of additional carbon onto the grids to increase overall carbon film durability.

       NOTE: This step is not necessary for SiO_2 grids. This step may also be done in advance; store the coated grids in a low humidity environment such as a vacuum desiccator.

    2. Transfer the grids to a grid prep holder and glow discharge the grids carbon side up. Using a glow discharge system, glow discharge the grids for 60 s at 10 mA with an 80 mm working distance and vacuum pressure of 1.0×10^{-3} mbar. Do this within 15–30 min of the next step.

       NOTE: Grid prep holders can be commercially bought or homemade with a piece of filter paper on a small Petri dish.

2. **Application of the anti-fouling layer**

    NOTE: Proper sterile technique should be used when handling the grids, and all solutions should be sterile and/or filter sterilized.

    1. Transfer the grids (carbon side up) to a clean glass slide or coverslip with at least 1 cm of separation between the grids. Pipette 10 μL of 0.05% poly-L-lysine

(PLL) onto each grid. Incubate the grids in a humid chamber, such as an enclosed plastic box with moist paper towels, for at least 30 min.

NOTE: This step can be extended to overnight. Be sure the humidity level in the chamber is sufficient to prevent the grids from drying out.

2. Wash each grid three times with 15 μL of 0.1 M HEPES pH 8.5. For each wash, remove most of the liquid from the grid with a pipette without letting the grid dry. Add 15 μL of fresh buffer, incubate for at least 30 s and repeat. Leave each grid in 15 μL of 0.1 M HEPES after the final wash.

    NOTE: In this step and future steps, it is important to keep the grid wet and to avoid contact between the pipette and the grid.

3. Prepare 10 μL of 100 mg/mL polyethylene glycolsuccinimidyl valerate (PEG-SVA) in 0.1 M HEPES pH 8.5 for each grid. The PEG-SVA will dissolve quickly with gentle mixing resulting in a clear solution.

    NOTE: Do not prepare the PEG-SVA solution in advance. PEG-SVA has a half-life of 10 min at pH 8.5. Avoid exposing the PEG-SVA stock to excessive moisture by storing it in a desiccator or dry environment at −20 °C and warming to room temperature before opening.

4. Immediately after preparing the PEG-SVA solution, remove the 15 μL drop of HEPES pH 8.5 from each grid (taking care not to dry the grid) and add a 10 μL drop of the PEG-SVA solution. Incubate the grids in a humid chamber for at least 1 h.

    NOTE: This step can be extended to overnight. Be sure the humidity in the chamber is sufficient to prevent the grids from drying out.

5. Wash each grid three times with 15 μL of sterile water. For each wash, remove most of the liquid from the grid with a pipette without letting the grid dry, add 15 μL of fresh water, incubate for at least 30 s and repeat. Leave each grid in 15 μL of water after the final wash.

3. **Applying PLPP gel**

    1. Prepare a clean microscope coverslip for each grid. Complete the following steps for each grid, one grid at a time, to minimize the chance of the grid drying out.

    2. Place a 1.0 μL drop of water on the center of the coverslip to help with placing the grid on the coverslip and keeping the grid wet. Carefully transfer the grid from the 15 μL water drop to the 1.0 μL water drop on the coverslip. Be sure to place the grid carbon side up.

    3. Carefully place a polydimethylsiloxane (PDMS) stencil over the grid, taking care to keep the grid centered and to minimize stencil contact with the carbon foil of the grid.

    4. Add 1.0 μL of 4-benzoylbenzyl-trimethylammonium chloride (PLPP) gel onto the grid. Pipette gently to mix (do not touch the grid with the pipette tip).

5. Move the coverslip with the grid to a dark location to dry. The gel will dry in approximately 15–30 min.

4. Calibration and design of the micropattern

1. Color one side of a glass coverslip with a highlighter. Add black lines from a fine-tipped permanent marker to make focusing easier. Place the coverslip on the microscope such that the colored side faces the objective lens. Using brightfield mode, focus on the highlighter.

2. Ensure the microscope and the micropatterning system is powered on, and the correct light path is set. Open Micromanager and the Leonardo software (**Plugins > Leonardo**) on the microscope computer.

3. Select calibrate and follow the on-screen instructions. Adjust the microscope focus so that the image projected onto the slide is in focus. The exposure time may need to be decreased. After calibration, select **Pattern Now**.

4. Record the micrometer/pixel (μm/px) ratio reported under calibration data in the top left window of the program (Figure 2, area 1). Use this ratio to determine the number of pixels to use per micrometer when designing a pattern.

5. After calibration, ensure that the software is now open with a live brightfield view from the microscope. Load a prepared grid on a coverslip (section 3) onto the stage with the grid facing the objective lens. Position the stage and adjust the focus so that the grid is visible in the software window.

6. Measure the size of the grid squares and grid bars in micrometers. The software includes a ruler activated by the button near the bottom left corner to measure the grid (Figure 2, area 2). For example, the patterns used here for a 200 mesh grid correspond to ~87 × 87 μm grid squares and ~36 μm grid bars.

   NOTE: The software offers flexibility in resizing patterns on the fly, so minor inaccuracies in measurement can be tolerated.

7. Based on the measurements and ratios above, create pattern(s) with any image creation software. The minimum feature size with a 20× objective is 1.2 μm. Patterns should be saved as uncompressed 8-bit .tiff files.

   1. Ensure the software does not rescale images to a different pixel size when saving. The pattern should fit within an 800 × 800 pixel box, sufficient to cover four grid squares.

      NOTE: Pixels with a value of 255 (white) will be patterned at the highest intensity (total dose of the laser) and pixels with a value of zero (black) will not be patterned. Any pixels with an intermediate value will be patterned with a dose of approximately (X/255)*total dose. In Figure 3A, pixel values of 255 and 129 were used for the greyscale patterns. Once the pattern is designed it can be saved and reused without modification.

5. Micropatterning

1. After calibration, ensure that the software is now open with a live brightfield view from the microscope. Load a prepared grid on a coverslip (section 3) onto the stage with the grid facing the objective lens. Position the stage and adjust the focus to see the grid in the software.

2. For an initial run, design a new template. In the software, select **Add ROI** (not shown, in the location of Figure 2 area 3) and choose a 3,000 μm circle. Position the circle ROI over the grid using the brightfield image on the screen as a guide. Press lock to secure the ROI.

3. After locking the ROI in place, select **Add Pattern** (not shown, in the location of Figure 2 area 3). Choose the pattern designed in section 4. Divide the grid into six regions to allow independent focusing and positioning in each region to factor for uneven grids. An 8×8 grid square region for each corner of the grid and a 2×8 grid square region on each side of the center, leaving the center four grid squares unpatterned (Figure 2, center image).

4. Use the replication options (Figure 2, area 4) to generate copies of the initial pattern to reach the desired number of total copies of the pattern. Adjust the spacing between copies to match the spacing between grid squares if necessary.

5. Set the **Total Dose** for the pattern. 30 mJ/mm^2 is a good starting point. See the discussion section for more details.

6. Under **Expert Options** (Figure 2, area 4) adjust the angle of the region to match that of the grid squares. Regions can be repositioned using the mouse. The ratio (size) of the patterns can also be adjusted. Iterate through adjusting the angle, position, space between, and ratio of the pattern until the patterns line up with the grid squares. Move the microscope stage to change the region of the grid in the live brightfield display.

7. Press **Lock** to save the changes made to the region.

8. To copy a region, click the **Duplicate** button (Figure 2, area 5, two sheets of paper icon) next to its name in the **Actions** panel on the left. To reposition, rename, or edit the copy, click on its name in the **Action panel**.

9. Repeat steps 5.4–5.9 as necessary to fill all of the desired regions.

10. Once the complete template is designed and positioned, **Save** the template file within the software (Figure 2, area 6, bar with up arrow icon in top toolbar).

11. When loading a previously saved template (bar with down arrow icon) center the ROI over the grid and press **Lock**. Click on each region in the **Action Panel** to change the angle, position, dose, and/or pattern file.

12. Once the template and patterns are positioned, uncheck all but one of the regions in the **Action Panel** in the software.

13. Use the microscope stage to navigate to that region and focus on the carbon foil. Clicking the **Eyeball** icon in the **Action panel** (Figure 2, area 5) will toggle the display of the pattern overlay on or off.

14. Once the grid is in focus, close the brightfield shutter and press the **Play** icon in the bottom right corner of the software to begin the patterning process, which can be monitored live.

15. In the action panel, select the box for the next region. Open the brightfield shutter so that the grid is visible and center that region using the microscope stage. Repeat steps 5.13–5.14 for each region in the **Action Panel**.

16. Remove the coverslip with the grid from the microscope, and immediately pipette 10 μL of sterile phosphate-buffered saline (PBS) onto the grid.

17. After 10 min, remove the stencil with tweezers, then wash the grid 3x with 15 μL of PBS. After the final wash, place each grid in 15 μL of PBS and move the grids to a dark location.

6. Deposition of ECM proteins

1. For cultured cells, follow steps 6.2–6.5; for primary *Drosophila* neurons, follow steps 6.6–6.10.

2. Prepare at least 15 μL of ECM for each grid. For BEAS-2B cells, prepare a final concentration of 0.01 mg/mL bovine fibronectin and 0.01 mg/mL fluorophore-conjugated fibrinogen in sterile PBS. For HeLa cells, prepare 0.01 mg/mL bovine collagen I and 0.1 mg/mL fluorophore-conjugated fibrinogen in sterile PBS.

3. Remove most of the PBS from each grid and apply 15 μL of the ECM. Incubate the grid in a humid chamber at room temperature for at least 1 h.

NOTE: This step may be extended to overnight at 4 °C.

4. After incubation in ECM, wash each grid 5x with sterile PBS. For each wash, remove most of the liquid with a pipette without letting the grid dry, add 15 μL of fresh PBS, incubate for at least 30 s, and repeat. Leave each grid in PBS after the final wash.

NOTE: Grids can be stored for up to a week in PBS at 4 °C with no observed deterioration in quality.

5. Use a fluorescence microscope to detect the fluorophore in the ECM to confirm patterning and that the carbon foil remained intact. A few broken squares are generally tolerable.

6. For primary *Drosophila* neurons, move the patterned grids to a 30 mm glass bottom dish containing sterile PBS.

7. Aspirate the PBS from the dish and apply 2 mL of 0.5 mg/mL fluorophore-conjugated concanavalin A. Incubate overnight at 25 °C in a sterile environment.

8. Remove the concanavalin A solution from the dish (without drying the grids) and wash the grids 3x with PBS. For each wash, add and remove 2 mL PBS from the dish.

9. Use a fluorescence microscope to detect the fluorophore in the ECM to confirm patterning and that the carbon foil remained intact. A few broken squares are generally tolerable.

10. After the final wash, remove the PBS from the glass bottom dish and add 2 mL of freshly-prepared, sterile-filtered supplemented Schneider's *Drosophila* media[21], containing 20% heat-inactivated fetal bovine serum (FBS), 5 μg/mL insulin, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL tetracycline. Incubate at 25 °C in a sterile environment until the neurons are ready to be plated.

7. Preparation of primary *Drosophila* cells prior to seeding

1. Sterilize a 55 mm dissection dish with 70% EtOH, and then add to the dish 2–3 mL of sterile-filtered 1x dissection saline (9.9 mM HEPES pH 7.5, 137 mM NaCl, 5.4 mM KCl, 0.17 mM NaH_2PO_4, 0.22 mM KH_2PO_4, 3.3 mM glucose, 43.8 mM sucrose)[21].

2. Pick 30–40 3rd instar larvae gently from the food using a pair of tweezers.

3. Place the larvae into the tube with 1x PBS, then transfer them into the second tube with 1x PBS to wash the larvae.

4. Transfer the larvae into the tube with 70% EtOH to wash off the PBS, then transfer them into the second tube with 70% EtOH. Leave the larvae in the second tube for 2–3 min to sterilize the larvae.

5. Transfer the larvae into a tube with 1x dissection saline, then immediately transfer them into the second tube with 1x dissection saline.

6. Transfer individual larvae onto the dissecting dish containing 1x dissection saline. With a pair of forceps and a dissection microscope, quickly tear each larvae to extract the brain and transfer it to the third tube with 1x dissection saline. Repeat until all brains are extracted.

7. Centrifuge the tube containing the brains at $300 \times g$ for 1 min.

8. Discard the supernatant, and wash with 1 mL of 1x dissection saline and centrifuge the tube at $300 \times g$ for 1 min. Repeat this step one more time.

9. Discard the supernatant until 200–250 μL is left in the tube and add 20 μL of 2.5 mg/mL Liberase in 1x dissection saline.

10. Rotate the tube on a rotator for 1 h at room temperature; during this hour, pipette the solution 25–30 times every 10 min. By the end, the solution should be slightly opaque.

11. Centrifuge the cells at $300 \times g$ for 5 min.

12.   Discard the supernatant, then add 1 mL of supplemented Schneider's media. Pipette the solution 30 times to mix.

13.   Centrifuge the cells at $300 \times g$ for 5 min.

14.   Discard the supernatant and wash the cell pellet by adding 1 mL of supplemented Schneider's media. Pipette the solution 30 times to mix.

15.   Centrifuge the cells at $300 \times g$ for 5 min.

16.   Discard the supernatant, then resuspend the cell pellet with 300 μL of supplemented Schneider's media. Pipette the solution 30–40 times to mix.

8.   Culture and RSV infection of BEAS-2B and HeLa cells

1.   Maintain HeLa cells and BEAS-2B cells in T75 flasks at 37 °C and 5 % CO_2. Passage cells every 3–4 days once reaching approximately 80% confluency. Maintain HeLa cells in DMEM + 10% FBS + 1x Antibiotic-Antimycotic. Maintain BEAS-2B in RPMI + 10% FBS + 1x Antibiotic-Antimycotic[6,22,23].

2.   For seeding of uninfected cells, skip to section 9. BEAS-2B and HeLa cells are susceptible to RSV infection; BEAS-2B cells were used for all experiments involving RSV shown here.

NOTE: Perform all BSL-2 steps in compliance with institutional protocols using an appropriate biosafety cabinet (BSC) and personal protective equipment (PPE).

3.   Prior to RSV infection of cells, passage 5×10^4 cells per well into a 6-well plate (surface area ~9.6 cm^2) with 2 mL of growth media and incubate overnight.

4.   Trypsinize and count one well of cells. To trypsinize, aspirate media from one well and wash with 2 mL of sterile PBS without Mg^{2+} and Ca^{2+} to remove residual media. Add 500 μL of 0.25% trypsin solution. Incubate at 37 °C for 5–10 min. Periodically check the cells to see if they are released from the surface. Once the cells are released, add 1.5 mL of culture media.

5.   Mix 100 μL of trypsinized cells with 100 μL of trypan blue. Pipette 10 μL of diluted cell mix into a hemocytometer. Count the cells and calculate the number of cells per well. Use this number to calculate MOI below.

6.   Prepare a dilution of RSV-A2mK+[24] in growth media to achieve an MOI of 10 per-well in 750 μL of media. The MOI of RSV-A2mK+ can be calculated from fluorescent focus units (FFU) titers of the stock (For example: for 1.0×10^5 cells per well and an RSV stock of 1.0×10^8 FFU/mL, dilute the viral stock 1:75 to 1 $\times 10^6$ FFU/750 μL or 1.33×10^6 FFU/mL).

7.   Aspirate the media from the cells in the 6-well dish and add 750 μL of the viral solution from above to each well.

8.   Rock the plate at room temperature for 1 h.

9. After 1 h, bring the total volume per well up to 2 mL with growth media pre-warmed to 37 °C and place the plate in an incubator set to 37 °C with 5% CO_2 for 6 h.

10. Trypsinize the cells to release them and proceed to seeding as described below. After seeding, incubate the grids for an additional 18 h before plunge freezing (for a total 24 h post-infection).

9. Cell seeding onto micropatterned grids

1. For cultured cells, follow steps 9.2–9.8; for primary *Drosophila* neurons, follow 9.9–9.11.

2. Trypsinize the cells to release them (see step 4 in section 8 above). To reduce cell aggregation, trypsinize the cells at 60 % or less confluency.

3. Mix 100 μL of trypsinized cells with 100 μL of trypan blue. Pipette 10 μL of the diluted cell mix into a hemocytometer and count the cells.

4. Dilute the cells in media to 2×10^4 cells/mL.

5. Add 1 μL of media to the center of a 30 mm glass bottom dish to assist in placing the grid and prevent it from drying. Transfer the grid from the PBS on the coverslip to the center of the glass bottom dish. Add 10 μL of cell solution to the grid.

6. Using a brightfield microscope, observe cell adhesion to the grid after 5 min. If a majority of the patterns remain unoccupied, add an additional 10 μL drop of the cell solution. Keep the grids and cell solution at 37 °C during the incubations.

7. Repeat step 9.6 until most patterns are occupied or many occupied patterns begin to have multiple cells. Incubate the grid for 2 h in the incubator (37 °C, 5% CO_2).

8. Flood the dish with 2 mL of pre-warmed media and incubate overnight (37 °C, 5% CO_2).

9. For primary *Drosophila* neurons, remove the media from the grid-containing dish and plate the cells onto the dish.

10. Wait 30–60 min for the cells to attach, then add 2 mL of supplemented Schneider's media.

11. Culture the neurons in a 25 °C incubator for a minimum of 2–3 days before plunge-freezing.

10. Imaging and vitrification of patterned grids

1. Place the glass bottom dish containing the patterned grid and cultured cells on the fluorescence microscope.

2. Acquire images of the grid using brightfield and the appropriate fluorescent channels to detect the pattern and any other labeling in the cells. Ensure the cell density and positioning is suitable for downstream imaging and analysis.

NOTE: Brightfield and fluorescent images were processed in the FIJI software package[25].

3. Prepare a cryo-plunge freezer; the type of freezing device will depend on availability, cost, and features that are most suitable for the sample.

NOTE: Primary *Drosophila* neurons were prepared on an automated plunge-freezer, and the BEAS-2B cells were prepared using a semi-automated plunge-freezer.

4. Apply gold fiducials to the samples for proper alignment of the tilt series. Blot samples to remove excess media, then plunge-freeze the samples into a cryogen, such as liquid ethane cooled by liquid nitrogen. For primary *Drosophila* neurons, blot for 4 s from the backside. For HeLa and BEAS-2B cells, blot from both sides for 4–6 s. The frozen grids can then be stored in liquid nitrogen until further use.

5. Image vitrified cells in a cryo-electron microscope, operated at 300 kV with a direct electron detector camera. Set up tilt-series collection for each region of interest with software such as SerialEM[26] for cryo-EM/cryo-ET data collection.

NOTE: Tilt-series of primary *Drosophila* neurons were collected on a direct electron detector from −60° to 60° bidirectionally at 2° increments at −8 μm defocus with a pixel size of 4.628 Å for a total dose of 70–75 e$^-$/Å^2. Tilt-series of RSV-infected BEAS-2B were collected on a direct electron detector with an energy filter (20 eV slit) at −5 μm defocus with a pixel size of 4.603 Å and a total dose of ~80 e$^-$/Å^2.

6. Process the tilt series to reconstruct tomograms.

NOTE: Tomograms presented here were reconstructed using the IMOD package[27]; lowpass filtering was done using the EMAN2 software package[28].

Representative Results

This procedure was used to pattern EM grids for whole cell cryo-ET experiments. The entire workflow presented in this study, including initial cell culture preparations, micropatterning (Figure 1), and imaging, encompasses 3–7 days. A two-step procedure was used to generate the anti-fouling layer by applying PLL to the grid and subsequently linking PEG by addition of the reactive PEG-SVA. The anti-fouling layer can also be applied in a single step by adding PLL-g-PEG in one incubation. The PLPP gel is a catalyst for the UV micropatterning, which is also available as a less concentrated liquid. The gel allows for patterning at a significantly reduced dose compared to the liquid, which results in much faster patterning. With this system, the actual patterning time of a full TEM grid was ~2 minutes. The micropatterning workflow alone generally spans 5–6 hours and allows an individual to pattern eight grids for standard cell-culture on TEM grids.

A number of the steps during the micropatterning process require long incubation times (see steps 2.1, 2.3, 6.4). Conveniently, some of these steps, such as PLL passivation (2.1) or PEG-SVA passivation (2.3), may be extended to an overnight incubation. Additionally,

grids may be patterned in advance and stored in a solution of the ECM protein or PBS for later use. In our study, these options were valuable in instances where the timing of cell preparation and seeding is critical, such as primary *Drosophila* neurons and RSV-infection of BEAS-2B cells.

Grids were prepared in a general biosafety-level 2 (BSL-2) lab setting using clean tools, sterile solutions, and included antibiotics/antimycotics in the growth media[6,22,29,30]. For samples particularly sensitive to microbial contamination, the anti-fouling layer and ECM can be applied in a tissue culture hood or other sterile environment. Additionally, the grid could be washed in ethanol between patterning and ECM application. If working with infectious agents, it is important to adapt the procedure to comply with appropriate biosafety protocols.

This workflow and the procedures presented (Figure 1) allowed HeLa cells (Figure 4), RSV-infected BEAS-2B cells (Figure 3, Figure 5), and primary *Drosophila* larval neurons (Figure 6, Figure 7) to be seeded onto patterned EM grids for optimal cryo-ET data collection.

HeLa cells seeded onto micropatterned TEM grids remain viable as determined by fluorescent staining using a calcein-AM and ethidium homodimer-1 based cell viability assay (Figure 4A,**B**). Using a mixed ECM of collagen and fibrinogen, HeLa cells readily adhere to patterns across the grid (Figure 4A,C). The overall morphology of cells that expand along the pattern is similar to that of cells grown on unpatterned grids (Figure 4C,D). In the case of HeLa cells, the total cell thickness remains ~< 10 μm with significantly thinner areas ~< 1 μm thick near the cell periphery (Figure 4E,F).

For RSV studies, we patterned entire grid squares using a gradient, with a low-dose exposure on the edges and a higher dose pattern towards the center (Figure 3A). Gradient patterns yielded better results when searching for released viruses present near the periphery of cells. With these patterns, cells were found to preferentially adhere to the higher ECM concentration, but are also able to adhere to and grow on the lower ECM concentrations. The relative dose between areas will need to be optimized when using patterns that require multiple doses. If the doses and thus ECM concentrations are too similar or too disparate to one another, the effect of using multiple doses will be lost.

In Figure 3, a TEM grid was patterned and subsequently seeded with RSV infected BEAS-2B cells and used for cryo-EM data collection. Figure 4A is a fluorescent image of ECM patterned onto a TEM grid using a gradient pattern. Cell adhesion and growth along the central region of the pattern can be seen in Figure 3B as a brightfield image of the cells 18 hours post-seeding. In Figure 3C, fluorescent signal (red) from replication of RSV-A2mK+ is overlaid with signal from the ECM. The majority of the infected cells are positioned along the higher density central region of the gradient pattern. A low-mag TEM map of the grid post cryo-fixation reveals a number of cells, including RSV-infected cells, positioned on the carbon foil near the center of the grid squares. As previously shown for cells grown on standard TEM grids[22], tilt-series were located and collected of RSV virions in close proximity to the periphery of infected BEAS-2B cells grown on micropatterned grids (Figure 5A,B). Many of the RSV structural proteins can be identified within the

tomograms including nucleocapsid (N) and the viral fusion protein (F) (Figure 5C, blue and red arrows respectively).

For primary *Drosophila* neuron studies, it was found that the narrow pattern, near the resolution limit offered by the software (where the thickness of the pattern was 2 μm), allowed from one to a few cells to be isolated within a grid square (Figure 6). The neuronal soma was able to extend its neurites over a period of several days within the pattern. This allowed easy identification and tilt series acquisition of the neurites compared to neurons cultured on unpatterned grids (Figure 7). It was also found that fluorescently-labeled concanavalin A, a lectin that has been used as an ECM for *in vitro Drosophila* neuronal cultures[20,21], is amenable for patterning.

Drosophila neurons from third instar larvae were isolated according to previously published protocols[20,21,31]. The neuronal preparations were applied to micropatterned cryo-EM grids where concanavalin A was deposited on the pattern to regulate cell placement, spreading, and organization. The neurons on patterned or unpatterned grids were allowed to incubate for a minimum of 48–72 hours, and the grids were then plunge frozen. A representative image of a micropatterned EM grid with several *Drosophila* neurons distributed across the patterned regions is shown in Figure 6A. These neurons, derived from a transgenic fly strain that has pan-neuronal GFP expression in the membrane, can be easily tracked by light microscopy not only due to its fluorescent labeling, but also because of its location within the micropatterns. While neurons cultured on unpatterned grids can also be tracked through its GFP signaling by light microscopy (Figure 7A, yellow circle), locating them in cryo-EM became substantially more difficult due to the presence of cellular debris and contamination from the media (Figure 7B, yellow circle). Such presence was lessened for neurons on patterned grids, likely due to the PEG in the anti-fouling layer of the non-patterned regions repelling the cell debris from adhering. Due to the dimensions of the neuron cell body and the extended neurites (Figure 6A,B, yellow circle), cryo-ET tilt series were collected along thinner regions of the cells (Figure 6C,D, red circle). The neuronal cell membrane, a mitochondrion (cyan), microtubules (purple), actin filaments (blue), vesicular structures (orange and green), and macromolecules such as ribosomes (red) were well resolved in higher-magnification image montages and slices through the 3D tomogram (Figure 6E). While similar sub-cellular features can be seen from 3D tomograms of unpatterned neurons (Figure 7E), the difficulty in locating viable cellular targets for data collection decreased throughput substantially.

In Figure 8, representative images from grids with some of these issues have been assembled to assist in their identification and troubleshooting. Once optimal conditions are determined, micropatterning is a reliable and reproducible method for the positioning of cells on grids for cryo-TEM.

Discussion

Modern, advanced electron microscopes and software packages now support streamlined automated cryo-EM and cryo-ET data collection where hundreds to thousands of positions can be targeted and imaged within a few days[32,33,34,35]. One significant limiting factor

for whole-cell cryo-ET workflows has been obtaining sufficient numbers of collectable targets per grid. Recently, a number of groups have developed protocols for micropatterning grids for cryo-EM, with one advantage being improved data collection efficiency[16,17,18]. Here a protocol is presented for using a commercially available micropatterning system to micropattern TEM grids for cryo-ET studies of primary *Drosophila* neurons and cultured human cell lines (uninfected or RSV-infected). This micropatterning system is versatile and many steps can be optimized and tailored to fit specific experimental goals. A user with TEM and fluorescence microscopy experience can quickly become skilled in grid preparation and micropatterning. With careful practice, good results should be achievable after a few iterations. Below, some of the options available, user considerations, potential benefits, and future applications of micropatterning for cryo-EM are discussed.

One of the important considerations for whole cell cryo-ET is EM grid selection. EM grids are composed of two parts: a mesh frame (or structural support) and the foil (or film), which is the continuous or holey film surface on which cells will grow. Copper mesh grids are commonly used for cryo-EM of proteins and isolated complexes. However, they are unsuitable for whole-cell cryo-ET due to the cytotoxicity of copper. Instead, a gold mesh is commonly used for cellular tomography. Other options include nickel or titanium, which may provide benefits over gold such as increased rigidity[16]. EM grids are available with different mesh dimensions to support a range of applications. Larger mesh sizes provide more room for cells to grow between grid bars and more areas that are amenable for tilt series collection, though at the cost of increased overall specimen fragility. The most commonly used foil is perforated or holey amorphous carbon, such as Quantifoils or C-flat grids. Biological targets can be imaged either through the holes in the carbon or through the electron-translucent carbon. Grids such as R 2/1 or R 2/2, where the holes are 2 μm wide that are spaced 1 and 2 μm apart respectively, provide a large number of holes and thus a large number of potential areas for data collection. However, some cells may grow and expand better on more uniform surfaces such as R 1.2/20 grids or continuous carbon. For downstream sample processing by focused-ion beam milling (cryo-FIB), the foil is removed through milling, reducing concerns over the continued presence of the underlying film. As with the mesh, foils from other materials are also available, with the patterning protocol presented here being equally suitable for SiO_2 grids. Commonly used grids include gold Quantifoil, continuous carbon, or SiO_2 film 200-mesh grids (~90 μm spacing between grids bars) for whole-cell cryo-ET.

There are a number of considerations when designing a pattern. A majority of these decisions are guided by the cell type and purpose of the experiment. A good starting point is to choose a pattern that approximates the shape and dimensions of the cells in culture. Many studies have demonstrated significant effects of pattern shape on cell growth and cytoskeletal arrangement[13,36,37]. Special care should be taken during pattern design if this could alter the target of interest. Several patterns for each cell type were tested to determine which patterns promoted cellular adhesion and growth. The flexibility of the micropatterning system permits the testing of multiple patterns on a single grid and changing patterns for different grids within a single experiment. Larger patterns (~50–90 μm), such as those used here, increase the likelihood that multiple cells adhere to a single region of the pattern and allow cells to expand and extend after adhesion. More constrained patterns (20–30 μm) may

be appropriate in experiments where cell isolation is more critical than cell expansion, such as for focused-ion beam milling (cryo-FIB) experiments. For tomography applications, one may need to consider the impact of the tilt-axis. If a pattern is positioned such that all cells grow parallel to one another in a single direction, it is possible that all of the cells will be perpendicular to the tilt-axis when loaded onto the microscope stage, resulting in a lower quality of data.

On unpatterned grids, cells often preferentially adhere to the grid bars, where they cannot be imaged by TEM. Even on patterned grids, cells are often observed to be positioned in the corners of grid squares partially on both the patterned carbon foil and grid bar. Recently, micropatterning was used to intentionally position part of the cell over the grid bar[18]. This could be considered for experiments where it is not critical to have the entire cell periphery on the foil. This can be especially important for cells that can grow larger than a single grid square, such as primary neurons growing over multiple days.

There are many tools that can be used to design a pattern. Here, the pattern was limited to less than 800 pixels in any dimension such that the pattern can be rotated to any angle and still fit within the maximum area that can patterned in a single projection by this micropatterning system. This allows the user to rotate the pattern to be properly oriented with the grid regardless of the orientation of the grid on the microscope. Here, the grid was divided into six patterning areas. Primarily, this allows focus adjustment between different regions of the grid. Gold grids, in particular, are very malleable and may not lay down completely flat on the glass. Proper focus is essential for clean, refined patterning results. By using segmented patterns, only minor adjustments to the pattern position need to be made if the grid shifts slightly during patterning process, though this is usually not an issue when using the PLPP gel with the PDMS stencils. Finally, the central four grid squares of the grid remained unpatterned. This supports a user being able to clearly identify the center of the grid, which is very useful for correlative-imaging experiments.

The patterning software for this micropatterning system, Leonardo, also has more advanced features such as stitching and the ability to import patterns as PDFs, which are beyond the scope of this protocol. This software also includes microstructure detection and automated pattern positioning that can be used on TEM grids. This feature is most useful when the grid is very flat and can be patterned without the need to adjust focus between different areas.

Selection of an ECM protein can have a significant impact on cell adhesion and expansion. Some cells are known to undergo physiological changes when grown on specific substrates[38]. Multiple ECM proteins and concentrations were tested for any new cell type based on prior work reported in the literature. Laminin, fibrinogen, fibronectin, and collagen are widely used for cultured cells and can be used as a starting point if other data is not available. However, other ECM proteins must also be considered if the commonly used ECM proteins fail to confer proper adherence properties for the cells. This was particularly true for primary *Drosophila* neurons, as a high-concentration of the plant lectin concanavalin A was necessary for proper cellular adherence. The compatibility of cellular adhesion and growth with the ECM can be tested by patterning on glass dishes or slides prior to transitioning to TEM grids. This pre-screening approach is time and cost-effective if a large

number of combinations need to be examined. The inclusion of a fluorescently conjugated ECM protein is valuable for assessing the success and quality of patterning.

Cell seeding is one of the most important steps for whole cell cryo-ET, either with or without micropatterning[6,16,39]. For primary *Drosophila* or other neurons, which are fragile, unstable in suspension, and may be limited in quantity, single seeding approaches are preferred over monitored, sequential cell seeding. A single seeding step at an optimized cell density, as described in the protocol for *Drosophila* neurons, is a viable option for most cell types. However, it is also possible to seed cells onto the substrate at a lower initial concentration and add more cells in a monitored fashion as described here and in other literature[18]. This sequential seeding can provide more consistent results in some cases. Similar to standard cell culture, care should always be taken to maintain cell viability and minimize cell clumping during isolation.

When first starting with micropatterning, there are a few potential pitfalls that are detrimental to the final result. Careful grid handling and sterile technique, a uniform distribution of the PLPP gel, proper dose and focus during patterning, and maintenance of cell viability prior to seeding are among the most important considerations for success. A list of some of the potential issues as well as solutions were assembled in Table 1.

Micropatterned grids can be used to help position cells to establish a consistent cell density across the grid and to position regions of interest in areas suitable for tilt-series collection[16,18]. The placement and positioning of cells can be used as fiducial markers for correlation in cryo-CLEM experiments, reducing the need for fragile finder-grids and fluorescent fiducial markers. However, it should be noted that such fiducial markers may still be useful for sub-micrometer accuracy correlation[29,40]. Furthermore, an even distribution of isolated cells is also highly beneficial for focused-ion beam milling (cryo-FIB) experiments to maximize the number of cells from which lamella can be cut[16].

The addition of micropatterning to cryo-EM workflows will result in measurable improvements in data throughput and potentially enable new experiments. As the technique is further adopted and developed, more advanced applications of micropatterning including ECM gradients, multiple ECM depositions, and microstructure assembly will further expand the capabilities of cryo-ET to study biological targets and processes in full cellular context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Prepare grids

Carbon coat and glow discharge grids
Incubate grid in PLL and wash
Incubate grid in PEG-SVA and wash
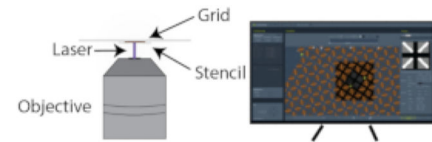Add PDMS stencil over grid on coverslip
Add PLPP gel and dry

Approx. 3 hours

Grids incubated in PEG-SVA after PLL

Grid with stencil, dried with PLPP gel
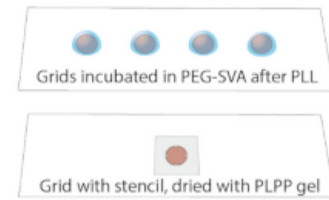
Micropatterning

Load grid onto microscope
Load/design pattern template
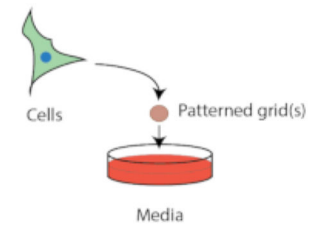Position pattern on grid
Pattern grid
Wash grid in PBS

Approx. 20 min/grid

Grid
Laser
Stencil
Objective

ECM and cell seeding

Incubate grid in ECM and wash
Prepare cells
Add cells to grid
Add media and grow in incubator

2-3 hours

Cells
Patterned grid(s)
Media

Cryo-preparation and data-collection


Screen grids by light microscopy
Plunge-freeze grids
Cryo-CLEM or FIB-SEM (optional)
Cryo-electron tomography

Time varies significantly based on workflow

Plunge freeze
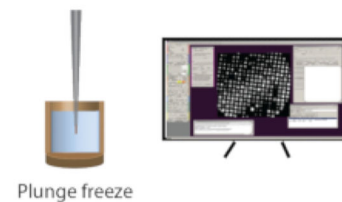
Figure 1: General workflow of micropatterning for cryo-EM.
The workflow can be roughly divided four parts: Grid preparation, micropatterning, ECM and cell seeding, and cryo-preparation and data collection. Major steps of each section are listed below the headings and the approximate time to complete each section is shown to the left.
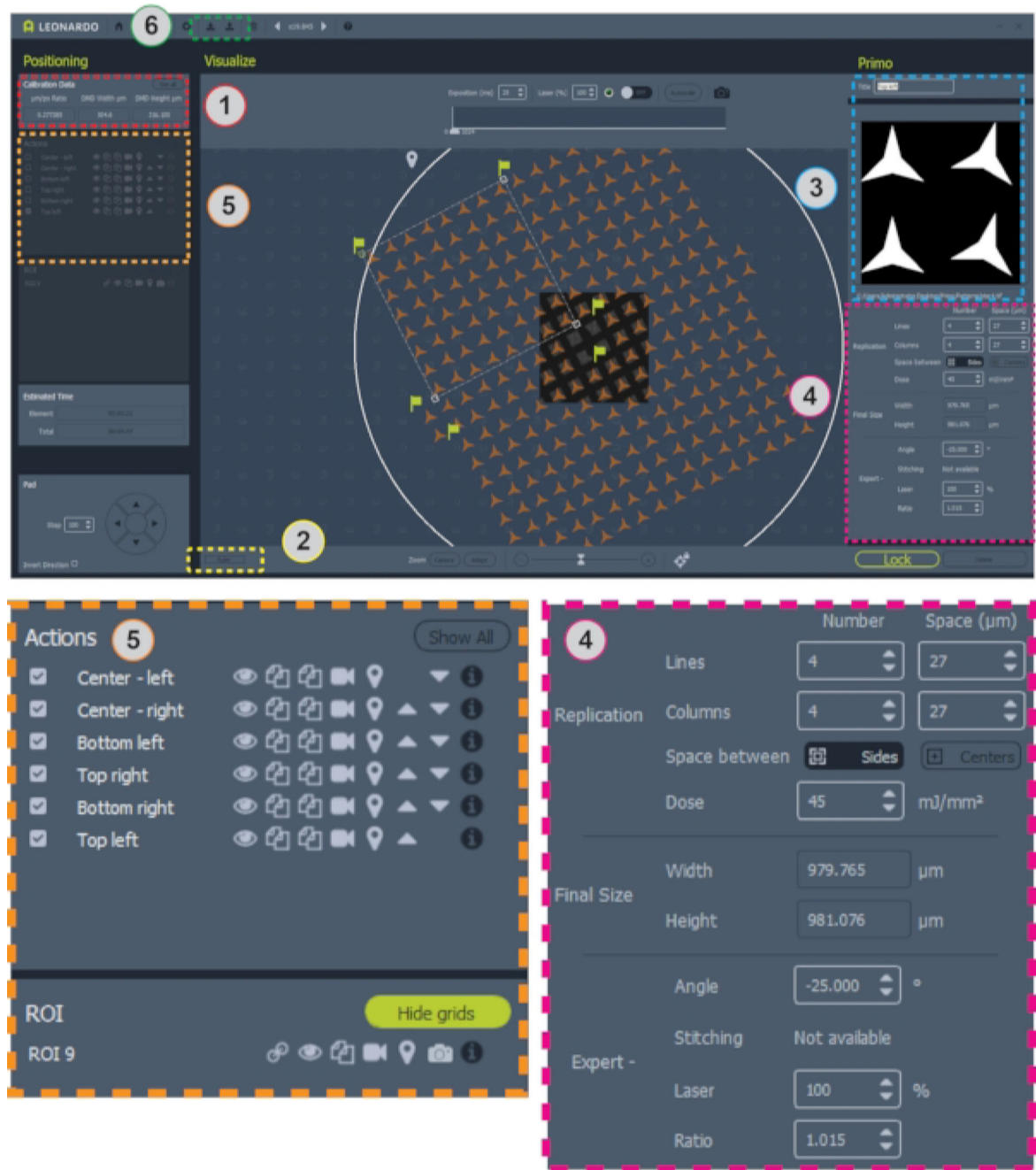
Figure 2: Screen shot of the software with pattern positioned on grid.
Area 1 contains the μm/pix ratio for pattern design. Area 2 is the ruler for measuring a grid. Area 3 is where to add or change patterns and ROIs. Area 4 contains all of the information for pattern positioning and dose. Area 5 contains options for patterns, including toggling overlays, copying or deleting patterns, and selecting patterns for micropatterning. Area 6 is where templates can be saved and loaded. Larger views of areas 4 and 5 are shown below for clarity.
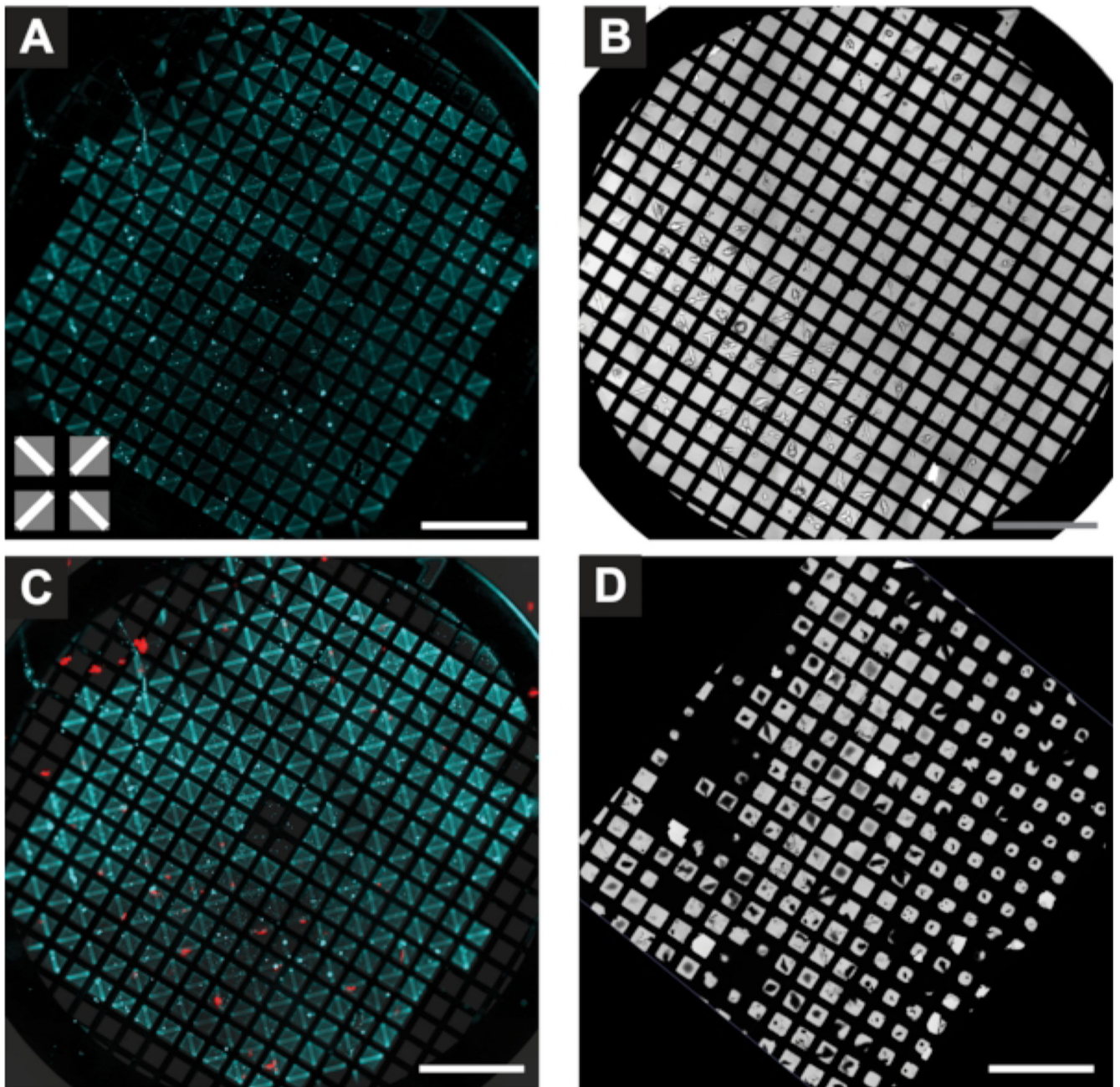
Figure 3: RSV-infected BEAS-2B cells on the patterned cryo-TEM grid.
(**A**) Fluorescent image of the patterned grid after addition of fluorescently labeled ECM. The input pattern is shown in the lower left corner. (**B**) Brightfield image of BEAS-2B cells grown on the grid in A. (**C**) Merge of the image in A (cyan) and B (grey) with fluorescent image of RSV-infected cells (red) immediately prior to plunge-freezing; infected cells express mKate-2. Scale bars are 500 µm. (**D**) Low-magnification cryo-TEM map of the grid in B after plunge-freezing. Fluorescent images are pseudocolored. Scale bars are 500 µm.
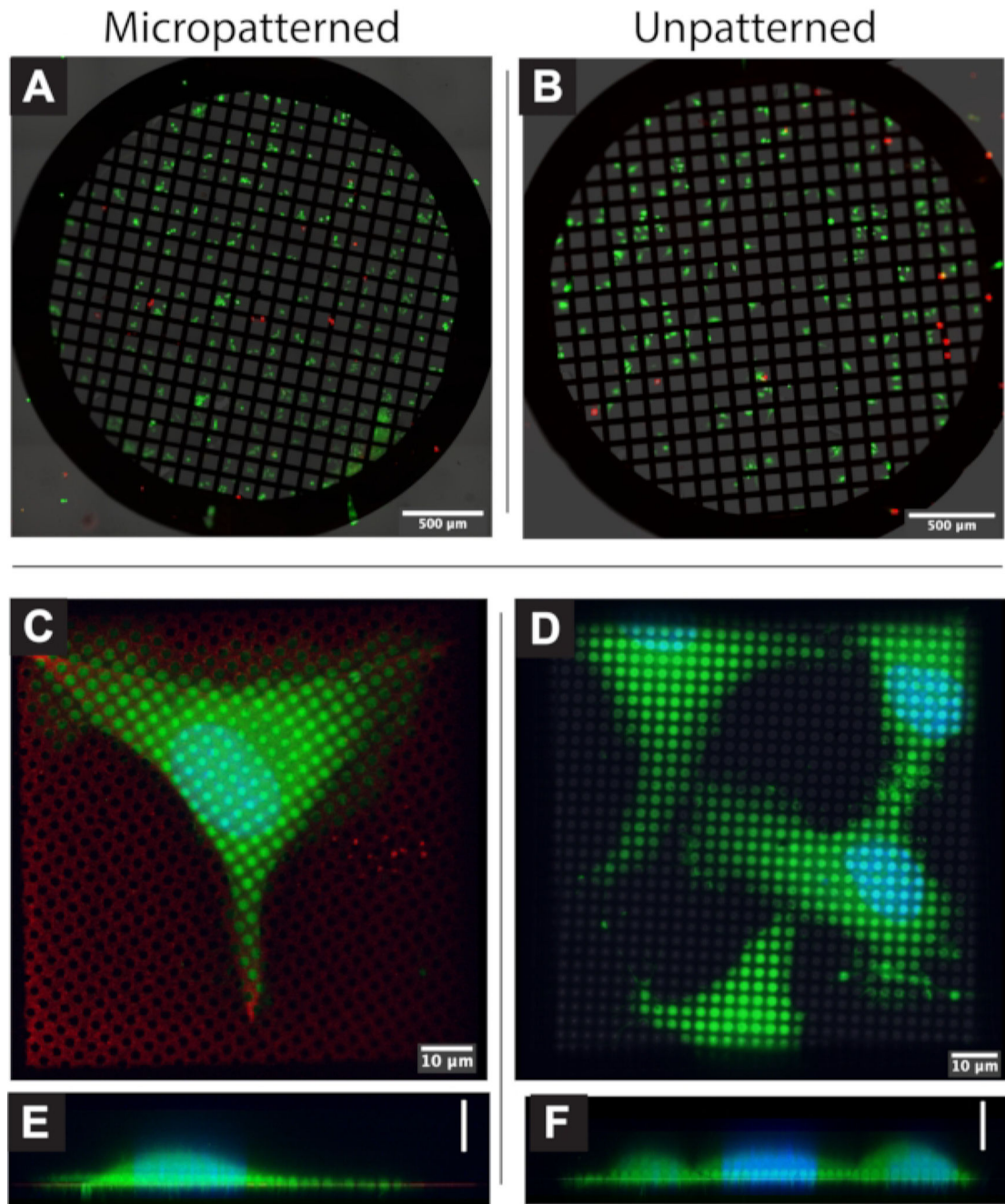
Figure 4: Live/Dead staining of patterned and unpatterned cells.
(**A**) Fluorescent image of HeLa cells grown on a patterned grid and stained with calcein-AM (live cell stain, green) and ethidium homodimer-1 (dead cell stain, red). (**B**) HeLa cells grown on an unpatterned grid and stained as in A. (**C**) Projection of confocal z-stacks of a HeLa cell on a patterned Quantifoil R2/2 grid with 0.01 mg/mL collagen and fibrinogen 647 ECM (red). Cell was stained with calcein-AM (green) and Hoechst-33342 (blue). (**D**) HeLa cells on unpatterned grid incubated with 0.01 mg/mL collagen and fibrinogen 647 ECM, incubated and stained with calcein-AM and Hoecsht-33342. The fluorescent images were merged with transmitted light (grayscale). (**E**) X,Z projection of C. (**F**) X,Z projection of D.
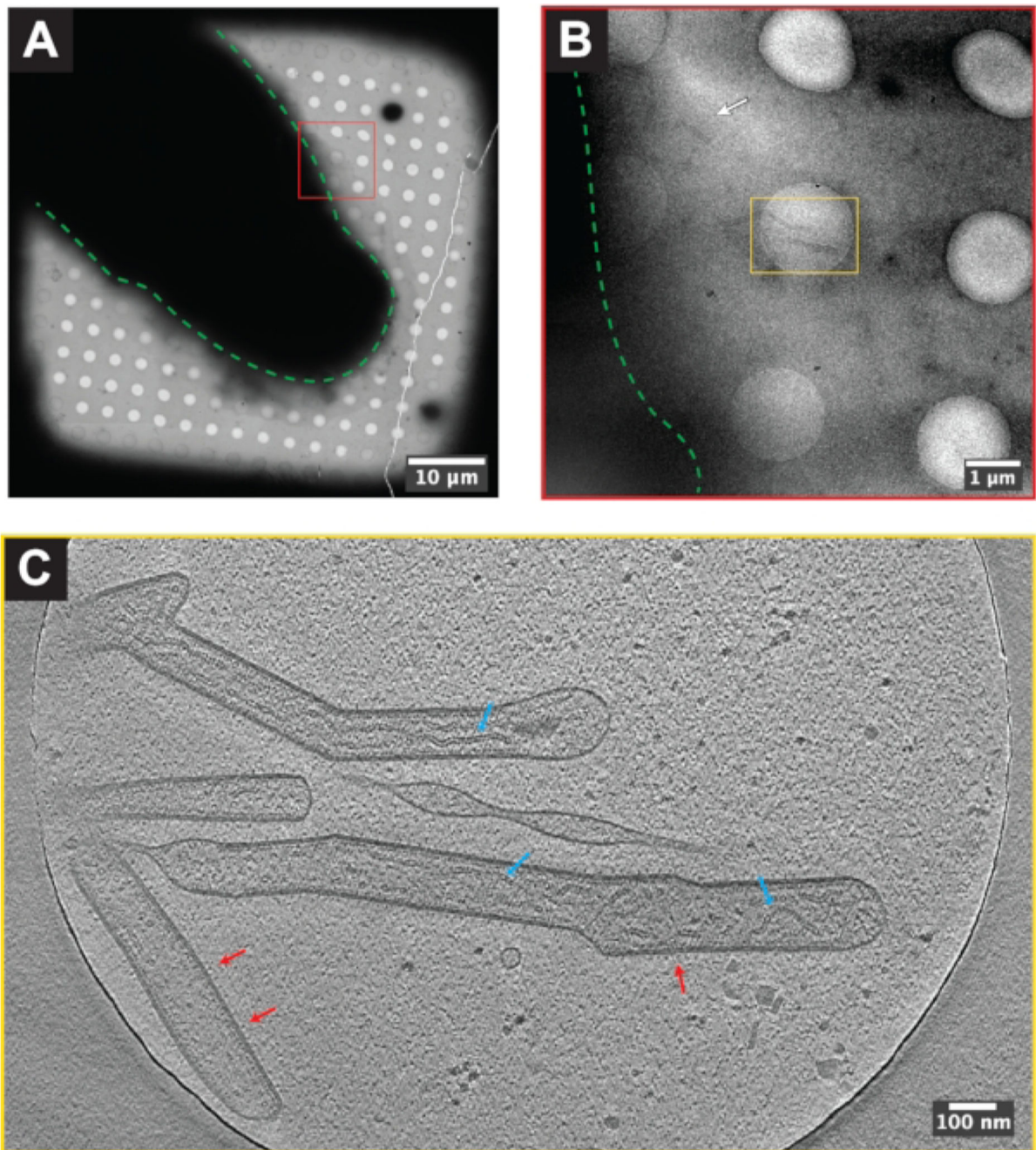
Images are pseudocolored. Scale bars in (A) and (B) are 500 μm; scale bars in (C) - (F) are 10 μm.

Figure 5: Cryo-ET of RSV-infected BEAS-2B cell on the patterned cryo-TEM grid.
(**A**) Cryo-EM grid square map of RSV infected BEAS-2B cell. Approximate cell boundary is indicated by the dashed green line. (**B**) Higher resolution image of area boxed in red in (A). Approximate cell boundary is indicated by dashed green line. RSV virions can be seen near the cell periphery (white arrow and yellow box). (**C**) Single z-slice from tomogram collected in the area of the yellow box in (B). Red arrows point to RSV F fusion protein, blue arrows point to the ribonucleoprotein (RNP) complex. The scale bars in (A)-(C) are embedded in the image.
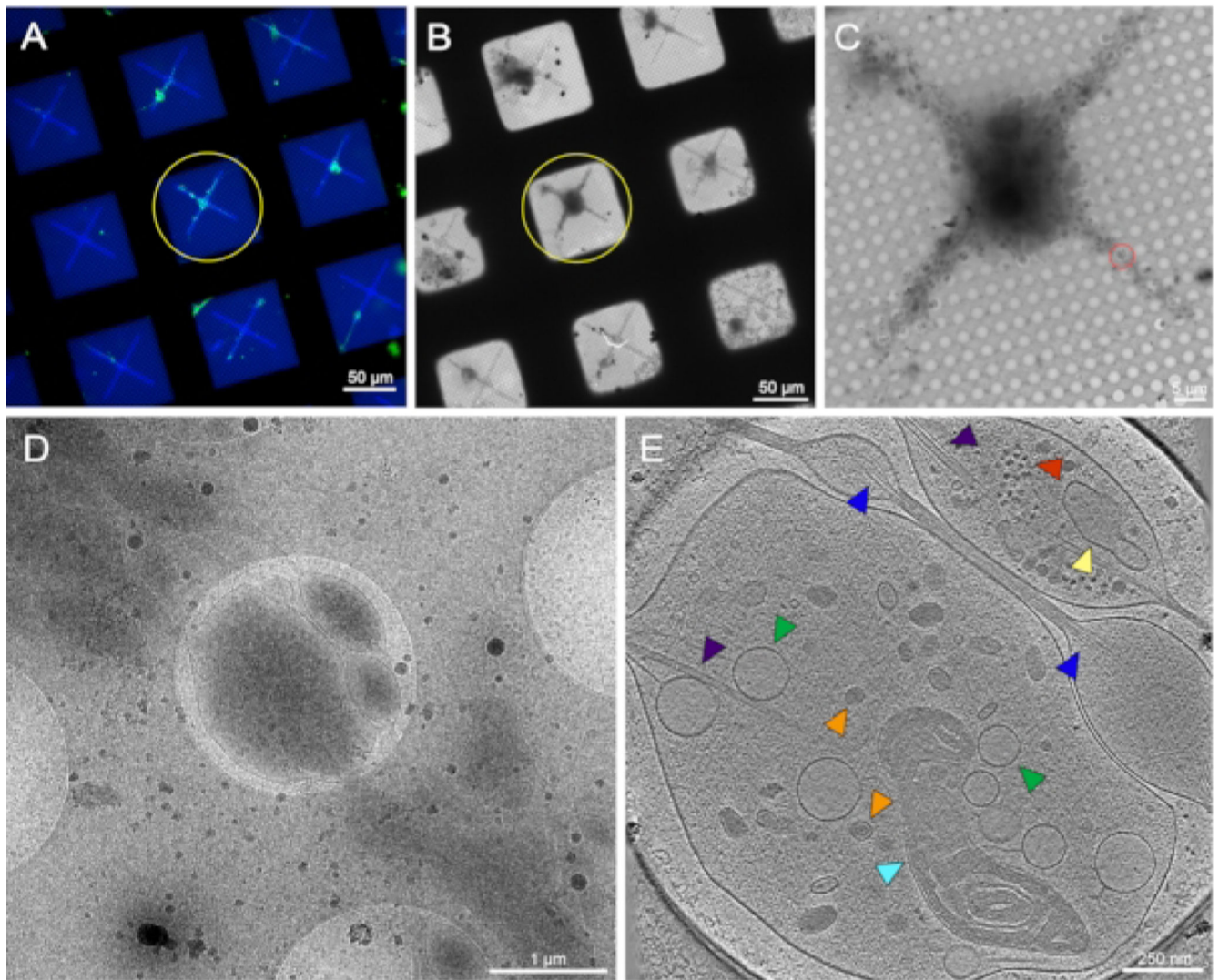
Figure 6: Primary neurons derived from the brains of 3rd instar *Drosophila melanogaster* larvae on the patterned cryo-TEM grid.

(**A**) Overlaid live-cell fluorescence microscopy grid montage of *Drosophila* neurons expressing membrane-targeted GFP on patterned grid squares with 0.5 mg/mL fluorescent concanavalin A. Green: *Drosophila* neurons. Blue: Photopattern. (**B**) Cryo-EM image montage of the grid in (A) after cryo-preservation. Yellow circle notes the same grid square as in (A). (**C**) Cryo-EM image montage of the square highlighted by the yellow circle in (A) and (B). (**D**) Higher magnification image of the area bounded by the red circle in (C), where a tilt series was collected on the cell's neurites. **E.** 25 nm thick slice of a tomogram reconstructed from the tilt series that was acquired from the red circle in (C). Various organelles can be seen in this tomogram, such as the mitochondria (cyan), microtubules (purple), dense core vesicles (orange), light vesicles (green), the endoplasmic reticulum (yellow), and actin (blue). Macromolecules, such as ribosomes (red), can also be seen in the upper right corner. Fluorescent images are pseudocolored. The scale bars in (A)-(E) are embedded in the image.
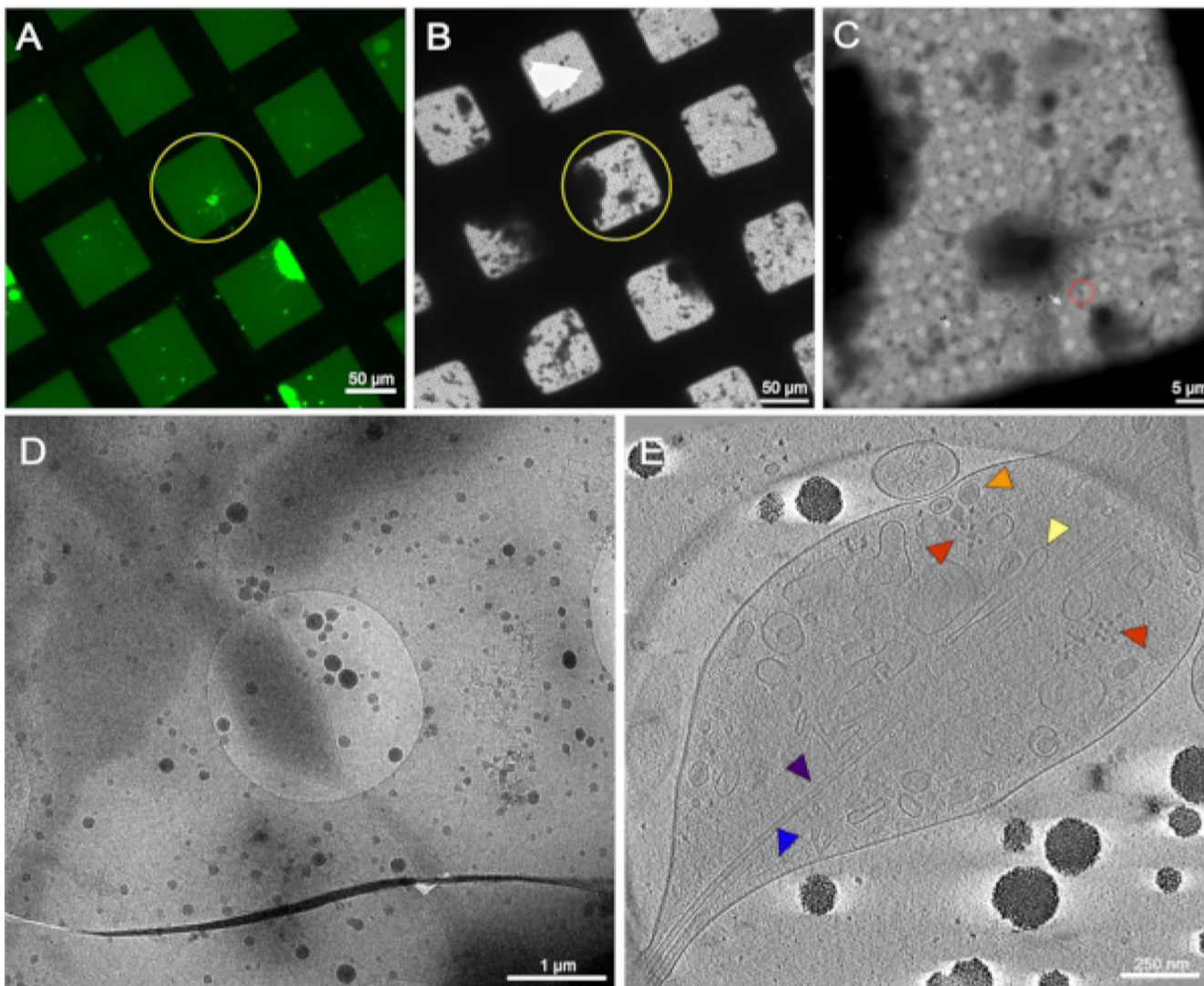
Figure 7: Primary neurons derived from the brains of 3rd instar *Drosophila melanogaster* larvae on unpatterned grids.

(**A**) Live-cell fluorescence microscopy grid montage of *Drosophila* neurons expressing membrane-targeted GFP on grid squares with 0.5 mg/mL concanavalin A. Green: *Drosophila* neurons. (**B**) Cryo-EM grid montage of the same grid in (A) after plunge-freezing. Yellow circle shows the same grid square as in (A). Note the presence of cellular debris and media contamination, which made target identification difficult compared to patterned grids. (**C**) Cryo-EM image montage of the square highlighted by the yellow circles in the (A) and (B) maps. (**D**) Higher magnification image of the area bounded by the red circle in (C), where a tilt series was collected on the cell's neurites. (**E**) 25 nm thick slice of the reconstructed tomogram from the tilt series from (C) and (D). A number of organelles are visible in this tomogram, such as microtubules (purple), actin (blue), the endoplasmic reticulum (yellow), and dense core vesicles (orange). Macromolecules, such as ribosomes (red), can also be seen. Fluorescent images are pseudocolored. The scale bars in (A)-(E) are embedded in the image.
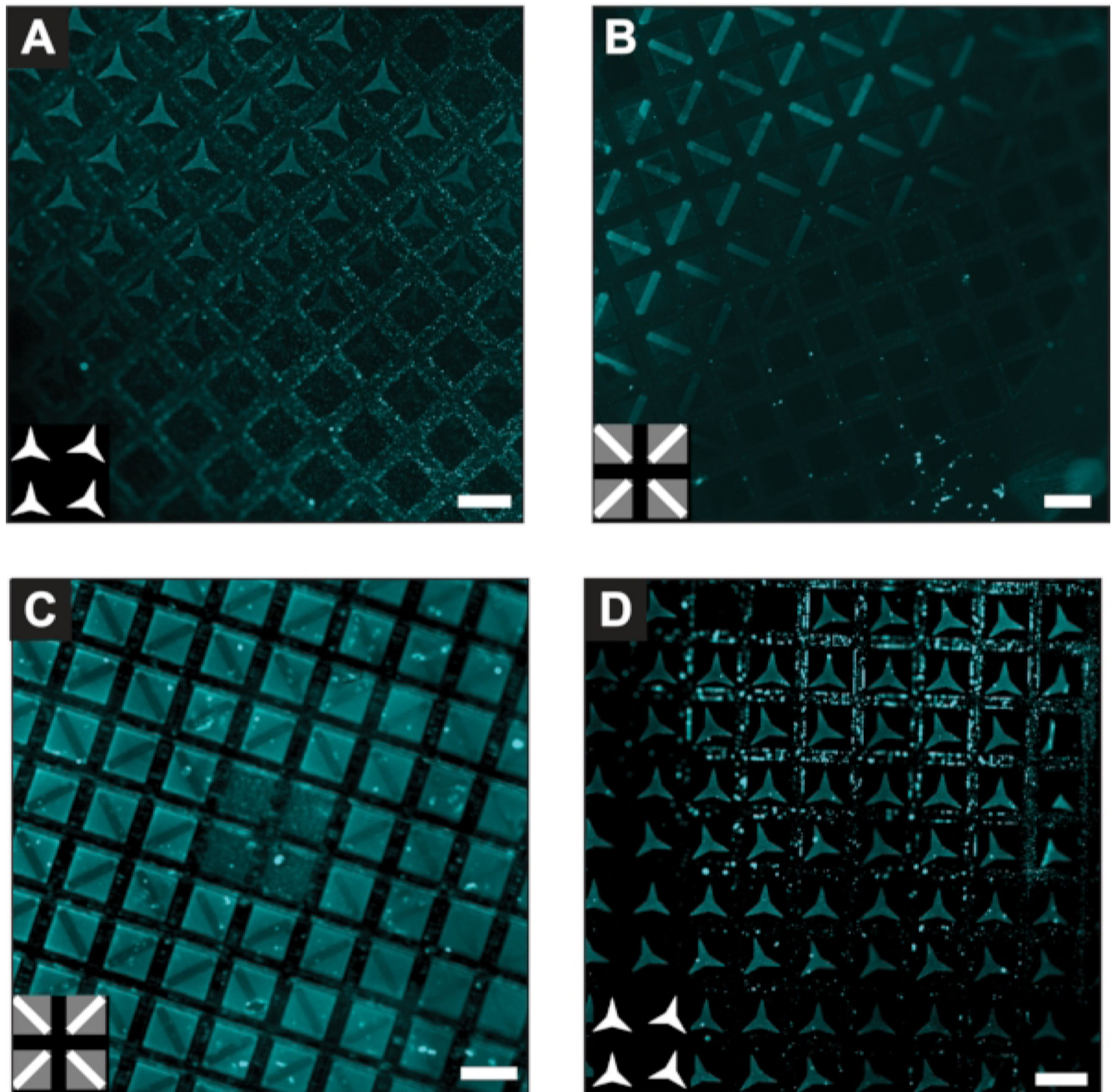
Figure 8: Examples of possible problems with patterning.

Fluorescent images of labeled ECM deposited on micropatterned grids. (**A**) Uneven patterning across the grid due to uneven distribution of PLPP gel. (**B**) ECM cannot adhere to areas covered by the PDMS stencil during patterning. (**C**) Saturated gradient pattern (right side) or inverted pattern (left) on a grid patterned with too high total dose. (**D**) ECM is adhering to areas on the grid bars as well as patterned area due to reflections of the UV laser during patterning. Images are pseudocolored; input pattern is shown in the lower left; scale bars are 100 μm.

Table 1:

Potential issues during micropatterning.

This table describes some issues a user may experience during micropatterning or cell-seeding. Potential causes and troubleshooting are provided for each issue. Representative images of some problems can be seen in Figure 8.

Issue	Potential cause(s)	Troubleshooting
Micropatterning		
Cannot see illumination from PRIMO laser	• Light path is not set up correctly	• Check that the microscope light path is set up properly
	• PRIMO laser is not on or laser is interlocked	
Many broken grid squares	• Touching grid foil with tweezers or pipet while handling	• Handle grids with care
	• Grid dried out during incubations or washing	• Do not allow grid to dry during washes and incubations
Large unpatterned areas	• Insufficient gel coverage	• Ensure gel spreads evenly over grid while adding
	• Grid foil out of focus during patterning	• Add an additional microliter of gel
	•Area covered by stencil	• Check focus before patterning each region
		• Carefully center grid in stencil
Saturated or inverted pattern	• Incorrect dose	• Try a range of total doses for pattern
	• Insufficient gel coverage	• Ensure grid is evenly covered with gel
		• Try different values for grayscale patterns
Blurry pattern	• Poor focus during patterning	• Repeat PRIMO calibration at same height as sample
	• Incorrect calibration	• Focus on grid foil before patterning
		• Divide pattern into additional regions for patterning
ECM adhereing outside of pattern	• Reflections from gel or dust	• Ensure gel is dry before patterning
		• Make sure coverslip and objective lens are clean
ECM not visible after patterning	• Photo bleaching	• Minimize light exposure to ECM prior to imaging
	• Incorrect dose during patterning	• Try a range of total dose values for pattern
	• Insufficient ECM incubation time	• Increase incubation time for ECM
Cell seeding		
Cells clumping	• Over digestion	• Use lower percentage of trypsin or time for release of adherent cells
	• High cell density	• Passage and/or digest cells at lower confluency
		• Do not agitate cells during release
		• Gently pipet cell solution or use cell strainers
Cells not adhering to patterned areas	• ECM is not suitable for cell type	• Try different ECM concentrations and composition
	• Cells viability is decreased prior to seeding	• Ensure cell culture and cell release conditions are not damaging cells
Cells not expanding after adhesion	• ECM or pattern not suitable for cell type	• Try different patterns and ECM
		• In some cases a more continuous foil (R1.2/20 vs R2/1) may promote cell expansion