TITLE: Preparation of Primary Neurons for Visualizing Neurites in a Frozen-Hydrated State Using Cryo-Electron Tomography

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77 **KEYWORDS:** Neurobiology; brain; rat; primary neuron culture; morphological assay; cryo-
78 electron microscopy; cryo-electron tomography

79 **SHORT ABSTRACT:**
80 To preserve neuronal processes for ultrastructural analysis, we describe a protocol for plating
81 of primary neurons on electron microscopy grids followed by flash freezing, yielding samples
82 suspended in a layer of vitreous ice. These samples can be examined with a cryo-electron
83 microscope to visualize structures at the nanometer scale.

LONG ABSTRACT:

Neurites, both dendrites and axons, are neuronal cellular processes that enable the conduction of electrical impulses between neurons. Defining the structure of neurites is critical to understanding how these processes move materials and signals that support synaptic communication. Electron microscopy (EM) has been traditionally used to assess the ultrastructural features within neurites; however, the exposure to organic solvent during dehydration and resin embedding can distort structures. An important unmet goal is the formulation of procedures that allow for structural evaluations not impacted by such artifacts.

Here, we have established a detailed and reproducible protocol for growing and flash-freezing whole neurites of different primary neurons on electron microscopy grids followed by their examination with cryo-electron tomography (cryo-ET). This technique allows for 3-D visualization of frozen, hydrated neurites at nanometer resolution, facilitating assessment of their morphological differences. Our protocol yields an unprecedented view of dorsal root ganglion (DRG) neurites, and a visualization of hippocampal neurites in their near-native state. As such, these methods create a foundation for future studies on neurites of both normal neurons and those impacted by neurological disorders.

INTRODUCTION:

Neurons establish the complex circuitry essential for the function of the central and peripheral nervous systems by elaborating dendrites to receive information and axons, often quite lengthy, to communicate with downstream neurons. Neurite outgrowth plays a fundamental role during embryonic development and neuronal differentiation and maintenance of neurites supports critically the function of the nervous system. Neuritic processes also feature critically in neuronal injury and regeneration, as well as nervous system disorders. The study of neuronal architecture is crucial to understand both the normal and diseased brain. Fortunately, physiologically relevant neuronal cell culture systems exist that can recapitulate complex and heterogeneous cellular structures. Based on the elucidation of solid experimental platforms, effective visualization strategies that enable qualitative and quantitative analyses of neuronal morphology are needed. Especially useful would be a detailed methodology that provides a consistent platform for visualizing neurites, both axons and dendrites at the nanometer scale.

Traditional electron microscopy requires the use of organic solvent during dehydration and resin embedding, which can induce distortions in the specimens from their true state. To date, most structural characterizations at nanometer scale are based on larger cells or tissues that are subjected to such harsh chemicals — thus limiting the interpretation of the findings [4,9,25]. Moreover, for electron beam penetration, sectioning is required for organisms or cellular protrusions exhibiting a thickness greater than 1 µm [12]. Finally, sectioned or milled tissue results in collection of discrete slice-specific data sets, making cumbersome the definition of the elongated feature of neurites. Even for cryo-EM, in which sectioning a frozen-hydrated

specimen is possible, the method introduces compression artifacts [1].

In recent years, researchers have learned how to grow hippocampal neurons directly on EM grids and flash-freezing them in liquid ethane to subsequently visualize neurites using cryo-ET [8,10,18,23]. However, such studies either use a custom-made device [10,23], or lack details on the blotting step for generating thin enough vitreous ice for routine visualization [8,18]. For example, one study recommends the use of 30-40s for blotting the EM grid [10]; however, this value is optimized not for general use but is specific for that custom-made plunge-freezing device. Using a custom-made device rather than a commercially available one [17] for maintaining humidity prior to plunge-freezing the sample could pose a hurdle for widespread reproducibility.

While these studies have been groundbreaking in visualizing neurites by cryo-ET, we have taken a step further to explore the applicability of cryo-ET to a variety of neuronal specimens (hippocampal and dorsal root ganglion neurons). Additionally, we discuss both optimal and suboptimal results, as well as the potential artifacts that one could encounter using cryo-ET for such specimens.

Defining a detailed technique for preserving and visualizing whole neurites at the nanometer scale in a near-native state would enhance the ability for more researchers to carry out ultrastructural studies. To this end, we describe an effective and detailed protocol using commercially available equipment to prepare unfixed, unstained neurons to visualize neurites. This is an important first step toward detailing the ultrastructure of healthy neurites and to lay the foundation for understanding what structural differences are present in nervous system disease models. Since cryo-ET can resolve unfixed, unstained neurite features in 3-D at the nanometer scale, the method will make it possible as never before to define neuritic architecture [12].

PROCEDURE:

1. Preparing Dishes with EM Grids for Plating Primary Neurons

1.1) Examine the integrity of holey carbon on the gold EM grids using a light microscope at magnification of at least 25X. Make sure carbon holes are >98% intact.

1.2) For plating primary neurons, use a Bunsen burner to flame-sterilize the EM grids and concurrently render them hydrophilic. Use tweezers to pick up the EM grid by its edge, not the central gridded area. CAUTION: Never leave a lit Bunsen burner unattended, and do not use gloves or tweezers with plastic components while performing these steps:

1.2.1) Before lighting the Bunsen burner, set the air and gas adjustments to a minimally open position.

1.2.2) Light the Bunsen burner using a striker flint or butane lighter.

1.2.3) Modify the air and gas adjustments to achieve a small, blue inner flame within a taller, lighter blue/violet flame. The tip of the inner flame is the hottest part of the flame. The knob underneath the burner adjusts the amount of gas entering the burner tube, while the barrel of the burner can be turned to adjust the amount of air entering the burner. Turning the air adjustment clockwise decreases the air (resulting in a purple flame) and counterclockwise increases the air (resulting in a yellow flame).

1.2.4) Using metal tweezers (no plastic parts) to hold the EM grid, pass it quickly through the flame twice, facing carbon-side up. The carbon side will appear more matte (less shiny) and with more of a grayish tint than the other side.

1.2.5) Immediately transfer the grid (carbon-side up) into the center of the glass-bottom dish placed within 10 cm of the Bunsen burner. Use one EM grid per dish (Fig. 1). Only use glass-bottom dishes that are pre-sterilized, i.e. via gamma irradiation.

1.3) Use a light microscope to check the grid integrity (carbon holes intact) whilst still keeping the EM grid inside the glass-bottom dish (to avoid contamination). When applying any substance on the grid or anything that will come in contact with the neurons, use sterile procedure and sterile pipette tips.

1.4) In a tissue culture hood using sterile procedure, apply 250 µl of the appropriate coating substance slowly and carefully to the central glass area of the petri dish. Avoid pipetting directly on top of the EM grid. Make sure the appropriate coating substance covers the entire EM grid.

1.4.1) For hippocampal neurons, use poly-L-lysine (PLL, 1 mg/mL) as the coating substance, prepared as previously described [19]. Note that 250 µl are needed per EM grid, per dish, so scale the batch accordingly. For dorsal root ganglion (DRG) neurons, use a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (see Table of Materials and Reagents) as the coating substance, prepare as follows. Note that 250 µl are needed per EM grid, per dish, so scale the batch accordingly.

1.4.1.1) Thaw a stock bottle of the gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells overnight at 4 °C.

1.4.1.2) Keep cold the gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and all components used to make the solution, i.e. by keeping them on ice.

1.4.1.3) While on ice, dilute this gelatinous protein mixture in Neurobasal medium to yield a 1:20 dilution (i.e., dilute 500 µl of the gelatinous protein mixture into 10 ml of Neurobasal).

213
214 1.4.1.4) Divide diluted gelatinous protein mixture into 1ml aliquots, and immediately store any
215 extra aliquots at -20 °C.
216
217 1.4.1.5) Apply 250 µl per EM grid, per dish, as described in 1.4.
218
219 1.5) Cover the petri dish with its top and incubate (either with PLL for hippocampal specimens,
220 OR with the gelatinous protein mixture for DRG specimens) for 1 hour at room temperature in
221 the tissue culture hood.
222
223 1.6) Aspirate all PLL (for hippocampal specimens) or the gelatinous protein mixture (for DRG
224 specimens) from the dishes. To do this, use the vacuum system in the tissue culture hood. Use a
225 sterile pipette tip attached to the vacuum tube. Avoid direct contact with the EM grid.
226
227 1.7) Use an adjustable air-displacement pipette to carefully apply 250 µl of sterile PBS
228 (phosphate-buffered saline) to the EM grid in the central glass area of each petri dish, such that
229 the EM grid is fully covered by PBS. Then, aspirate the PBS from each dish. Repeat three times.
230
231 1.8) Allow the dishes with EM grids to dry under the tissue culture hood for 15 minutes. Make
232 sure they are completely dry by checking under the light microscope in the tissue culture room.
233 Make sure there are no bubbles of moisture in the grid. If so, carefully aspirate next to the EM
234 grid to eliminate this extra moisture. The coated grid should be used immediately for plating
235 the neurons.
236
237 2. Preparing and Plating Primary Neurons on EM Grids
238
239 2.1) To plate primary neurons, first dissect, trypsinize (using 2.5% trypsin) and triturate to
240 dissociate the hippocampi (or dorsal root ganglion of 18-day old rat embryos) into individual
241 cells, as previously described [19].
242
243 2.1.1) Triturate is a common term used by neurobiologists to describe dissociating clumps of
244 cells into individual cells, particularly by use of a glass pipette with a fire-polished tip. The result
245 is achieved by carefully and slowly drawing and releasing the cells, up and down, multiple times
246 (~30) through the pipette.
247
248 2.2) Follow procedures as previously described for DRG dissection and cell isolation [24], taking
249 note to use 0.25% trypsin (in HBSS) to isolate the rat DRG neurons, rather than use the enzymes
250 suggested (papain, collagenase/dispase) that are more suitable for mouse DRG neurons.
251
252 2.3) Calculate the number of isolated neurons (i.e. using a hemocytometer) and use this value
253 to calculate the appropriate volume of cells to apply to each dish to achieve a concentration of
254 50,000 cells/mL per dish. The maximum volume to apply is 250 µl. Note that this only fills the
255 central glass area, not the entire dish.

256
257 2.4) Incubate the dishes for 30 minutes in a CO_2 incubator at 37 °C. Allow cells to recover and
258 adhere.
259
260 2.5) Slowly add 1.5 ml of warmed media to each dish, taking care not to disturb the EM grid.
261 The type of media depends on the cell type (hippocampal or DRG).
262
263 2.5.1) Prepare the appropriate media for either hippocampal neurons [19] or dorsal root ganglion
264 neurons [24], which is to be warmed in a 37 °C water bath prior to use. Incubate the dishes
265 overnight in the CO_2 incubator.
266
267 2.5.2) For hippocampal neurons, change the media the next day. Change half of the media
268 every two days onward for 14 days. Warm the media in a 37 °C water bath prior to use.
269
270 2.5.3) For DRG neurons, the day following dissection/plating, prepare a fresh stock of media
271 with an anti-mitotic agent (Uridine and 5'-Fluoro-2'-deoxyuridine; make a 10 mM stock solution
272 of each, separately; use a final concentration of 10 µM for each). Remove half of the DRG media
273 (875 µl) and add 875 µl of fresh media with the anti-mitotic agent.
274
275 2.5.3.1) For DRG neurons, every two days for the first week, alternate changing media between
276 anti-mitotic media and standard DRG media. For the second week, change standard DRG media
277 every two days.
278
279 3. Vitrifying Neurons on EM Grids
280
281 3.1) Prepare equipment and all materials for freezing and storing the gold EM grids at cryogenic
282 temperature: a vitrification device with a humidity chamber [17], fine-point specialized tweezers
283 for the vitrification machine, long flat point tweezers, dewar(s) of liquid nitrogen (LN_2), coolant
284 container, EM grid storage box, calcium-free filter paper.
285
286 3.2) Start the vitrification device. Set the humidity to 100% and temperature to 32 °C. In the
287 "Console" section, set the blot time to zero seconds. This allows for manual blotting through
288 the side-window of the vitrification machine's humidity chamber.
289
290 3.3) Handle the calcium-free filter paper with gloves, layering them such that three papers are
291 stacked. Cut the stack into 0.5 cm wide strips that are ~2 cm long. Bend them at a 90° angle
292 such that one side of the paper has a 0.5 cm x 0.5 cm face (Fig. 2). Using tweezers, remove the
293 middle paper and place it on another calcium-free filter paper until use.
294
295 3.4) Put the grid storage button in the button holder within the vitrification chamber. Fill the
296 inner chamber of the coolant container with liquid nitrogen and wait until complete
297 evaporation. Fill the outer chamber of the coolant container with liquid nitrogen until it attains

298 stable temperature for proceeding to the next step. Fill the inner chamber with high-purity
299 gaseous ethane that will condense to a liquid state within the cooled chamber.
300
301 3.5) Move the dish(es) from the incubator to a large 100mm polystyrene dish for transport to
302 the vitrification room, if not within the immediate vicinity. Use the specialized vitrification
303 tweezers to carefully pick the EM grid from the dish. Note which side the neurons are growing
304 on the EM grid; the position will matter for the next step. Use the black sliding lock on the
305 tweezers to securely lock the tweezers on the EM grid.
306
307 3.6) Insert the tweezers into the vitrification machine such that side of the EM grid on which
308 the neurons are adhered faces to the left, away from the side-opening hole of the vitrification
309 machine. Retract the specialized tweezers into the vitrification machine.
310
311 3.7) Place the coolant container in the appropriate holder of the vitrification machine. It should
312 be filled with adequate LN_2 and liquid ethane. Using the appropriate screen command of the
313 vitrification machine, raise the coolant chamber upward until it's flushed with the bottom of
314 the humidity chamber.
315
316 3.8) With the flat point tweezers, grasp one edge of the filter paper such that the shorter side
317 (the 0.5 cm x 0.5 cm face) is perpendicular to the tweezers. This face will come into direct
318 contact with the EM grid for blotting (Fig. 2B). Carefully insert the filter paper into the side-hole
319 of the vitrification machine‘s humidity chamber (Fig. 2A). Stably hold the paper against the EM
320 grid (the side facing away from the specimen) for 10 seconds. Discard the paper afterward and
321 immediately plunge-freeze the specimen in the liquid ethane using the vitrification machine’s
322 automation.
323
324 3.9) Carefully transfer your frozen-hydrated EM grid to one of the slots in one of the grid
325 storage buttons. Repeat the process for additional EM grids in the dishes. The EM grid storage
326 buttons used in these experiments can store multiple frozen-hydrated EM grids.
327
328 4. Image Collection, Processing and Annotation
329
330 4.1) Proceed to collect 2-D electron micrographs and/or 3-D tilt series [5] of the neurites using a
331 cryo-electron microscope under a low-dose condition. The 2-D images were intended to assess
332 the quality of the grid in terms of ice thickness and the possible areas to be useful for 3-D tilt
333 series.
334
335 4.1.1) In this case, all images were collected using a 4k x 4k CCD camera attached to a 200-kV
336 electron microscope equipped with a single tilt liquid nitrogen cryo transfer holder, at 20K
337 magnification at a target underfocus of 7 µm and sampling of 4.4Å/pixel.
338
339 4.1.2) To obtain a 3D tomogram of the sample, take a series of projection images while
340 incrementally tilting the sample along one axis of the transmission electron microscope (TEM).

341 Given the tilt angles and other experimental settings, there are several different softwares
342 available to automatically collect the tilt series [5]. The 3-D tilt series shown here were collected
343 on the same microscope using semi-automated tilt series acquisition software [26] over a range of
344 −60° to 60° at 5° increments with a cumulative dose of ~60 e/Å^2.
345
346 4.2) Reconstruct the tilt series of the neurites using image processing software [21] as previously
347 described for other samples [5, 29].
348
349 4.3) Color-annotate the 3-D features of the neurites by first segmenting the tomogram and
350 creating a surface model using a 3-D image processing software as previously described [5].
351
352
353 **REPRESENTATIVE RESULTS:**
354 Prior to freezing and imaging via cryo-ET, light microscope images should be taken of the EM
355 grid on which the neurons are growing. Neurites should be clearly visible without significant
356 overlap with one another. A colored box in Figure 3A represents an area that is zoomed-in to
357 show a higher magnification in Figure 3B, in which neurites extend across the latticework of the
358 grid. Each grid-square is composed of a holey carbon film that supports the neurons and their
359 neurites. These holes are apparent in a low-dose cryo-EM image taken at 4K magnification,
360 which shows the neuron body as a relatively large, dark, electron-dense object (Fig. 3C), from
361 which neurites project outward.
362
363 A part of the neurite resting above a hole in the carbon is selected in a colored box in Fig. 3C,
364 and zoomed-in at a higher magnification (20K) in Figure 3D. At this magnification, clear internal
365 cellular structures including microtubules, vesicles, and mitochondria should be clearly
366 apparent (Fig. 3D), particularly in the optimally thin ice as generated by following this protocol.
367 The dark black structures in the center of the image (Fig. 3D) are hexagonal ice particles that
368 are considered as contamination and should typically be avoided; however, given that they are
369 very sparse and outside of the neurites themselves, they do not interfere with the
370 reconstruction and color-annotation of the internal structures for analysis and interpretation
371 (Fig. 4). Another low-dose, low magnification image is shown in Figure 5, from which a neurite
372 can be located, after which several 2-D images can be taken and digitally stitched together
373 using image processing software to generate a montage (Fig. 6).
374
375 The initial low plating concentration (50,000 cells/mL per plate) of neurons on the EM grids
376 allows for neurons that are spaced far enough apart to ensure clear visualization of their
377 neurites (Fig. 3C); concentrations higher than recommended (>50,000 cells/mL per plate) can
378 result in suboptimal images of crowded neurites that are difficult to trace or attribute cellular
379 components (Fig. 7B, C).
380
381 **Figure 1. Scheme for growing neurons on EM grids within glass-bottom dishes.** (A) Glass-
382 bottom dishes are shown, partially filled with cell media (pink). (B) Each dish contains one gold
383 EM grid, as a closer view reveals. (C) Coating of the EM grid with poly-lysine is shown as a

384 cartoon side-cutaway. The glass bottom dish is composed of a square glass coverslip that is
385 attached to the bottom of a plastic culture dish, covering a circular opening that was originally
386 cut out of the bottom. These glass bottom dishes are gamma sterilized and commercially
387 bought as pre-made.
388
389 **Figure 2**. **Schematic for manual blotting of EM grids with neurons adhered. (A)** Close-up of the
390 vitrification machine's sliding entry slot (black arrow) to the humidity/blotting chamber, in
391 which tweezers will be inserted to blot the specimen manually. **(B)** Cartoon scheme showing
392 how the calcium-free filter paper (0.5 cm x 0.5 cm face) should be handled using the flat-point
393 tweezers and inserted through the entry slot into the humidity chamber of the vitrification
394 machine for blotting the EM grid on which the neurons are adhered (droplet of pink cell media
395 shown). The EM grid is held by fine-point specialized tweezers inside the humidity chamber.
396
397 **Figure 3. Visualizing frozen, hydrated neurons on gold electron microscopy (EM) grids.** (A)
398 Light microscope image at 10X magnification of the central area of an EM grid on which rat
399 primary neurons have been growing for 2 weeks. (B) Zoomed-in view of the aqua box shown in
400 (A), in which neurons and their neurite projections are visible (pink arrows). (C) Electron
401 micrograph at 4K magnification of a neurite projecting outward from the neuron body (red
402 arrow). Schematically corresponds to an area (i.e., the red box) within one of the grid squares
403 shown in (B). Blue box is viewed close-up in (D), where the neurite's internal features are
404 clearly visible at 20K magnification (green arrow – mitochondria; orange arrow – microtubules;
405 vesicle – blue arrow).
406
407 **Figure 4. 3-D reconstruction and annotation of a rat DRG axon tomogram.** (A) Tomographic
408 slice from a reconstructed stack of images taken at different tilt angles of one DRG axon. (B)
409 Corresponding 3-D annotation of the same axon.
410
411 **Figure 5. 2-D cryo-EM image of a rat DRG neuron (center-left) with axonal projections (red
412 box) at 4K magnification.**
413
414 **Figure 6. Montage of four 2-D cryo-EM images of a single rat DRG axon.** Each image was taken
415 at 20K magnification.
416
417 **Figure 7. 2-D cryo-EM images of neurites. (A) A** rat DRG axon imaged closer in proximity to the
418 cell soma. **(B, C)** Examples of over-crowding of neurites due to high plating concentration of
419 neurons on EM grids. All neurites were flash-frozen two weeks after plating on gold EM grids.
420 All images were taken at 20K magnification.
421
422 **Figure 8. 3-D reconstruction and annotation of a rat hippocampal neurite tomogram.** (A)
423 Tomographic slice from a reconstructed stack of images taken at different tilt angles of one
424 hippocampal neurite. (B) Corresponding 3-D annotation of the same neurite.
425
426

427 **DISCUSSION:**
428 We show that rat embryonic neurons (dorsal root ganglion and hippocampal) can be grown on
429 gold electron microscopy (EM) grids and frozen in vitreous ice thin enough for their neurites to
430 be imaged using 2-D cryo-EM and 3-D cryo-ET. While hippocampal neurites have previously
431 been imaged using cryo-ET [8,10,18,23], a protocol detailed enough for successful replication using
432 commercially available devices has been lacking. Furthermore, while their research has
433 pioneered the use of cryo-ET for visualizing hippocampal neurites, our studies explore the
434 applicability of cryo-ET to more than one type of neuronal specimen.
435
436 Here, we describe a detailed protocol preparing EM grids with either hippocampal and dorsal
437 root ganglion (DRG) neurons, blotting them to achieve optimally thin ice, and plunge-freezing
438 them for imaging by cryo-ET. Optimal blotting time is one which does not result in ice too thick
439 for the electron beam to penetrate, nor too thin that the vitreous ice appears with notably
440 different gradient from edge of the holey carbon to the innermost of the holey carbon.
441 Furthermore, achieving optimally thin ice through using a vitrification machine [17] may be
442 slightly different for each project, and robust freezing using the vitrification machine requires a
443 learning curve.
444
445 While previous research has focused on hippocampal neurons, also explored here in our
446 studies, we have gone further to show images at the nanometer scale of intact, frozen-hydrated
447 whole axons of the dorsal root ganglion (DRG), never imaged before using cryo-EM or cryo-ET.
448 DRG cells were chosen since they (in contrast to hippocampal neurons) exclusively give rise to
449 axons [34]. A protocol for imaging them by cryo-EM/cryo-ET could be useful for those interested
450 to deduce axonal ultrastructure i.e. in sensory neurons. We also present and discuss both
451 optimal and suboptimal results, as well as the potential artifacts that one could encounter using
452 cryo-ET for such specimens.
453
454 At the proper cell concentration (50,000 cells/mL per dish), spacing of neurons is such that
455 neurites allow for excellent access using light and electron microscopy (Fig. 3A and B) with one
456 or two neurons appearing per grid-square (Fig. 3B). Allowing enough separation between the
457 neurons is necessary for clear visualization of the neurites via cryo-EM (Fig. 3C and D). The
458 neurons and their neurites are supported on a holey carbon film of the gold grid, as seen clearly
459 in the cryo-EM images (Fig. 3C and D). The neuron bodies, which can vary from ~15 to 50 µm
460 for hippocampal neurons and dorsal root ganglion (DRG) neurons [30,33] are too thick for imaging
461 using cryo-EM and appear completely black, as is typical for very electron-dense objects and
462 thick specimens, as compared to the relatively thinner projections of neurites (not exceeding 1
463 µm in diameter) radiating from them (Fig. 3C, 5).
464
465 Resin-embedded 3-D brain tissue does not reveal neurites that are completely circular in cross-
466 section [14]; in fact, their morphology is variable. It is thus not known if the non-circular shape of
467 neurites is natural or a specimen preparation artifact. The measured thickness of the DRG axon
468 (Figure 4) grown on the TEM grid, blotted and imaged as shown in this study was 0.32 µm in the
469 z-direction (in the depth of the vitreous ice) and an average width of 0.53 µm in the x-y plane.

470 The measured thickness of the hippocampal neurite (Figure 8) grown on the TEM grid, blotted
471 and imaged as shown in this study was 0.50 µm in the z-direction (in the depth of the vitreous
472 ice) and an average of 0.72 µm in the x-y plane. The blotting process as described herein could
473 flatten the neurites although they remained hydrated in their original buffer during the entire
474 process of blotting and plunge-freezing process.
475
476 The slight flattening of neurites on the grid as observed by cryoET does not appear to affect the
477 structure of its internal constituents such as vesicles as shown herein, which are almost
478 perfectly circular and show no signs of dehydration. By contrast, the chemical processes
479 commonly used in 'traditional' EM preparations, particularly chemical fixation with
480 glutaraldehyde and paraformaldehyde followed by dehydration in ethanol, result in
481 uncontrolled tissue shrinkage. The potential of encountering such a damaging cellular effect is
482 eliminated in the method we present for cryoET in our manuscript.
483
484 Furthermore, our neurites samples were immediately plunge-frozen in liquid ethane, resulting
485 in instant 'embedding' in vitreous ice. Tissues that are frozen in a vitreous state appear much
486 more similar to the physiological state than other techniques. The level of ultrastructural detail
487 is not the only aspect that makes the technique described in our protocol to be valuable to
488 neuroscience researchers. The technique of growing neurons on EM grids, immediately
489 blotting/plunge-freezing them to preserve in a vitreous state for screening/imaging by cryoET is
490 an attractive technique because it opens up the possibility of examining ultrastructure of
491 neurons under different genetic, chemical or environmental stresses without the concern of
492 potential dehydration artifacts.
493
494 The blotting conditions prior to freezing, as detailed in this protocol, are important to generate
495 ice thin enough for visualization by cryo-ET of the ultrastructural features, including internal
496 components such as mitochondria, vesicles and microtubules (Fig. 4, 6, 7A). Mitochondria, for
497 example, could be identified in our preparations since they appear structurally similar to what
498 has been seen in cells (the edges of mouse embryonic fibroblasts) via cryo-electron tomography
499 [20]. They also display cristae, which can be seen in the 3-D color-annotated images present in
500 this manuscript. Suboptimal images can result from thick ice in which the cryo-EM image
501 appears generally opaque, preventing successful identification of such features as mitochondria
502 and microtubules within the neurite. A lack of sufficient details for preparing such specimens,
503 particularly regarding how to produce optimally thin ice, almost certainly contributed to the
504 limited success in defining the ultrastructure of neurites [8,10,17,23].
505
506 Suboptimal images can also result from plating too many neurons on the grid (>50,000 cells/mL
507 per dish), which results in overcrowding of neurites (Fig. 7B, C). Blurry images may be a result of
508 incorrect defocus and must be adjusted; the defocus in the images shown here was taken with
509 a targeted underfocus of 7 µm. Images were collected at that defocus to ensure higher contrast
510 and visibility of ultrastructural features; however, a lower defocus (i.e., 2 to 3 µm) could also be
511 used to obtain higher resolution details of such features.
512

513 Other factors can contribute to suboptimal images. For example, although it may be tempting
514 to use other features of the vitrification machine, particularly the semi-automated blotting
515 feature, rather than manual blotting described herein, this gave undesirable results. Double-
516 sided blotting was initially attempted using this semi-automated feature, in which the
517 vitrification machine (rather than the user) blots the sample directly using such parameters as
518 blotting time and number of blots as specified by the user. However, after imaging such
519 samples, it was found that all neurons had lysed open, spilling out their contents (multi-
520 vesicular bodies, etc.). Hence, the vitrification machine is best used in this case for its
521 temperature-controllable humidity chamber in which the sample is blotted prior to plunge-
522 freezing. Maintaining the sample in such a state (with humidity set close to 100%) minimizes
523 water loss during preparation and helps to ensure optimal vitreous ice post-freezing [3,7].
524
525 Ideally, to minimize exposure of the neurons to external perturbations, they should be grown in
526 a CO_2 incubator (temperature set to 37 °C) in close proximity to the room with the vitrification
527 machine, and the temperature for this apparatus should be set to 37 °C prior to flash-freezing
528 the neurons on the EM grids. One should avoid exposing neurons to large fluctuations in
529 temperature prior to blotting. In this report, temperature was set to 32 °C for blotting
530 purposes; neurons were grown in a different building and a time lapse of ~10 minutes occurred
531 between carrying the plates of neurons from one room to another. Care was taken to protect
532 the samples in a water-resistant, semi-insulated container during the transport process. A
533 similar change in temperature and CO_2 concentration (as experienced by the neurons prior to
534 blotting/plunge-freezing) occur to the neurons every ~2 days when the neurons are taken out
535 of the incubation chamber for their media to be changed under the biosafety hood, as is
536 typically done for neuronal cultures. This is not seen to result in toxic effects to the cell.
537
538 Regarding the presence of what appear to be electron-dense granules within the neurites'
539 mitochondria, several conflicting reports exist. Such granules are found in a variety of different
540 tissues, not specifically neuronal cells. They are perceived as a sink of cations that regulate the
541 internal ionic environment of the mitochondrion. They also appear to create contact sites
542 between inner and outer mitochondrial membranes in which enzymes can function efficiently
543 [16].
544
545 Experimental studies indicate that calcium and other divalent cations accumulate in
546 mitochondria when these ions are present in the fluid bathing either isolated mitochondria or
547 intact cells. It appears likely that the ions are organically bound to the pre-existing intra-
548 mitochondrial granules. It is suggested, therefore, that these granules are linked to the
549 regulation of the internal ionic environment of the mitochondrion [31].
550
551 Furthermore, the neurons in our preparation were cultured on EM grids for 14 days, the timing
552 of which is typical for purposes of neurite outgrowth [15, 36], prior to imaging by cryoEM/ET. It has
553 been reported that there is a dramatic increase in intracellular calcium concentration in aged
554 neurons as compared to young neurons [32]. Therefore, it is plausible that the mitochondria in

555 the neurites of these aged neurons would display mitochondrial granules not necessarily as a
556 sign of stress, but rather because they possess a higher presence of intracellular calcium.
557
558 Electron-dense mitochondrial granules have also been reported in mouse embryonic fibroblasts
559 grown in-culture and imaged by the same technology (cryo-electron tomography) as used in
560 our manuscript [20]. These cells did not show typical patterns of ultrastructural change associated
561 with cellular injury, such as cytoskeletal disruption and vacuolization of the cytoplasm. Likewise,
562 within our neurites as visualized by cryoET, we did not observe a disrupted cytoskeleton that
563 would otherwise be associated with cellular toxicity. Given the above considerations, we
564 conclude that the neurons in our preparation did not display mitochondrial granules as a result
565 of stress or toxicity.
566
567 To sterilize the EM grid surface to prevent contamination to the neurons, and also to generate a
568 favorable, hydrophilic surface on the EM grid to which the poly-L-lysine could adhere, flaming
569 the EM grids prior to plating neurons was found to be advantageous. UV sterilization was not
570 chosen since it requires irradiation of the grid for a longer time (e.g. 20-30 minutes) in the
571 biosafety hood. Then, the grids would need to be re-exposed again to the environment when
572 being glow-discharged for purposes of hydrophilizing the grid's surface. Glow-discharging the
573 grid ensures that the subsequent poly-lysine coating would effectively 'stick' to the grid. The
574 technique of flaming the grids is advantageous since it combines both the hydrophilizing step
575 and sterilization step in one. Primary neurons are known to be more sensitive than tumor-
576 based cell lines and it is crucial to keep their environment (grid, petri dish) as sterile as possible.
577
578 To visualize features that extend along the length of a neurite, it is preferable to first take a
579 lower magnification image at a lower electron dose (Figures 3C and 5), which covers more of
580 the grid area for purposes of selecting which neurite to image. Then, 2-D cryo-EM images can
581 be taken at intervals and digitally stitched together into a montage (Fig. 6). This technique may
582 be useful for ultrastructural features, such as microtubule organization, over a greater area
583 than that of a single cryo-EM image.
584
585 Taking low-dose, low-magnification images (Figs. 3C, 5) is also useful in identifying areas of the
586 neurite that are consistent in diameter across the holey carbon film (both in the holes and
587 across the carbon) and hence more ideal for imaging and subsequent analyses. Enlarging of
588 neurites over holes of the carbon film is typically seen in larger neurites (Fig. 5) with clearly
589 more internal material than thinner neurites (Fig. 3C). This phenomenon is thought to occur
590 due to the lack of physical support in the holes of the carbon film. When the EM grid is
591 removed from the dish in which the neurons were growing, and subsequently blotted from the
592 backside, neurites with more internal mass (Fig. 5) than others (Fig. 3C) tend to expand and/or
593 sag into the holes. Although this appears to be an artifact, it has a benefit as to show more
594 clearly the internal constituents of the neurite. Using a continuous carbon film overlaid on this
595 holey carbon film may help to circumvent this issue in future studies. This setup was initially
596 attempted but resulted in vitreous ice too thick for imaging. Subsequent trials with different
597 thicknesses of continuous carbon overlaid on the EM grid may need further investigations.

An important benefit of the current method is that the spatial organization of cell components within the neurite can be visualized at the nanometer scale by taking several 2-D images of a single neurite at different tilt angles, and reconstructing this stack of images into a tomogram. Individual structural features can then be manually annotated for each 2-D slice of the 3-D stack in different colors using the appropriate software (see Table of Materials and Reagents) as shown for an axon of a rat E18 dorsal root ganglion (DRG) neuron (Fig. 4) and a neurite of a rat E18 hippocampal neuron (Fig. 8).

By choosing to take an image every 5 degrees during the tilt series, rather than 1 or 2 degrees, fewer images are collected but a higher electron dose can be used per image. This results in higher contrast and less noise in the raw images. This is better for purposes of reconstruction (alignment by cross-correlation as one step) and subsequent tomogram annotation, which is done by-hand for each image comprising the tomogram stack in the z-direction. Choosing an increment size also depends on the size of the specimen; between different tilt angles, larger specimens will not vary as much. In this case, using a larger increment size (5 degrees) was thought to be more suitable due to the relatively large specimen size of the neurite.

Furthermore, adding fiducial gold markers to the EM grid would assist for fine image alignment if desired. Fiducial markers were not used because the sample was relatively large compared to other cryo-EM samples (viruses or proteins in isolation) and showed high contrast with a variety of structural features that could be used for proper alignment of each of the 2-D images (comprising the 3-D stack) by cross-correlation. The resulting image stack was well aligned using this approach, and useable for subsequent 3-D color annotation.

For future studies, using a microscope with an energy filter could reduce noise caused by inelastically scattered electrons but such a feature may not be readily available at most electron microscopy facilities. Our procedure reveals what images can result from a standard 200-kV microscope with no energy filter, which may be more commonly available to the neuroscience community at large.

The protocol described here is optimized for preparing and visualizing both rat embryonic DRG axons and hippocampal neurites using commercially available equipment for cryo-EM and cryo-ET. One of the main advantages of this technique is that it yields structural information from whole neurites, not sectioned, milled or treated by various chemical reagents in order to view their ultrastructure. We have demonstrated how cryo-ET is a particularly excellent method for use in future ultrastructural analyses of neurons in a close-to-native state for examining the impact of neurological disease on neurite structure.

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S.H.S. dissected, grew and vitrified DRG and hippocampal cells; collected cryo-EM and cryo-ET data of DRG and hippocampal axons; reconstructed and color-annotated the tilt series. M.R.G. dissected and provided hippocampal cells in M.N.R.'s lab. S.C. assisted in tilt series annotation. S.H.S. was trained by C.W. on how to dissect and grow neurons. S.H.S., W.C.M. and W.C. conceived the experiments. S.H.S. prepared the manuscript with input from other authors.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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