Correlative Video-Light-Electron Microscopy

A Detailed Protocol with Useful Tricks

Microscopical studies in biology have relied on two complementary microscope technologies - light (fluorescence) microscopy and electron microscopy. Light microscopy is used to study phenomena at a global scale and to look for unique or rare events, and it also provides an opportunity for live imaging, while the forte of electron microscopy is the high resolution. Observation of living cells under EM is still impossible. Traditionally light and electron microscopy (EM) observations are carried out in different populations of cells/tissues. The advent of true correlative light-electron microscopy has allowed high resolution imaging by EM of the same structure observed by light microscopy. Thus a rare event captured by low resolution imaging of a population or transient events captured by live imaging can now also be studied at high resolution by electron microscopy. This chapter describes correlative light-electron microscopy with details and useful tricks, including the way to localize the same cell after its transfection with a protein fused with a fluorescent tag, examination under the microscope in living condition, fixation, immunolabelling, embedding, and observation under EM. We also illustrate here the kinds of questions that the correlative video-light-EM (CVLEM) approach was designed to address, as well as the particular know-how that is important for the successful application of this technique. The potential and difficulties of this approach, along with the most impressive breakthroughs obtained by these methods, are discussed.

1. Introduction

Although advanced optical microscopy techniques can push resolution to 50-100 nm and even below, it is still much less than the resolution of EM and far from the resolution needed for the study of, for instance, the organization of assemblies of proteins and lipids in biological specimens. Often, the analysis of immunofluorescently-labeled structures needs a better-than-light-microscopy resolution. On the other hand, although the spatial resolution of EM is superior, its field of view is limited: a resolution of 1 nm can only be realized when small (2x2 µm²) areas are
Consequently, the study of rarely occurring events in cells or tissues is extremely tedious and time consuming. This limitation has motivated researchers to embark on the development of imaging methods that combine, for example, LM and EM - correlative microscopy (CM). CM uses a combination of microscopy methods for the study of rare cellular events of unique samples.

Now CVLEM is a rather complex procedure with the possibility to use several techniques for the identification of the organelle of interest subsequently under LM and then under EM. The CLEM procedure includes several stages: 1) observation of the structures labeled with fluorescent protein (FP, i.e., green FP) or other fluorescent markers in living cells; 2) immobilization (fixation or freezing); 3) immuno- or other type of labeling with gold or other markers suitable for EM; 4) embedding; 5) identification of the just examined cell in the resin block or within the frozen sample; 6) sectioning of thin or thick serial sections and identification of the cell on the resin block and cutting of thin or thick serial sections; 7) EM analysis and structure identification [1]. Each of these steps could be performed by different ways and all techniques have their own advantages and disadvantages (reviewed in [2]). Here, we are presenting an example of only one from many existing methods of CVLEM. We describe only those protocols that are indispensable for the presented type of CVLEM. Protocols of transfection, observation under a confocal microscope, EM tomography, the reader can find in corresponding protocol books.

2. Procedure

2.1. Observation of living cells and fixation.

2.1.1. Grow i.e. HeLa cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37°C and 5% CO₂.

2.1.2. Suspend HeLa cells using standard procedures and plate cells for CVLEM on a MatTek Petri dish with the CELLocate cover slip attached to its bottom. The
CELLocate cover slip contains an etched grid with coordinates that allow the localization of the cell of interest at any step in the preparation.

2.1.3. Transfect HeLa cells with cDNA of the GFP fusion protein using any standard method of transfection or microinjection of cDNA into the nucleus.

2.1.4. By 6 h to 48 h (depending of the fusion protein and method of transfection used) after transfection place the dish under an inverted fluorescence microscope or laser scanning confocal microscope.

2.1.5. Select the transfected cell of interest, and identify its position related to the coordinates of the CELLocate grid.

2.1.6. Draw (or photograph) the position of the cell on the map of the CELLocate grid. For instance, the cell could be near the cross of horizontal line A and vertical line 3 (see arrow in Fig. 1A, 2A, see Note 2). Another way to map the cell of interest is to scrap cells around the cell of interest using wood stick and then cells remaining in the centre of ring could be visible (Fig. 1C, D), Finally, the pattern of cell culture could be used for the labeling of the cell position (Fig. 1B)

2.1.7. Observe the dynamics of the GFP-labeled structures in the selected living cell using a multiphoton-, a laser scanning confocal-, or a digitalized fluorescent-inverted microscope, which allows the grabbing of a time-lapse series of images by a computer.

2.1.8. At the moment of interest, add fixative A to the cell culture medium while still grabbing images (fixative A: medium volume ratio is 1:1). Fixation usually induces the fast fading of GFP fluorescence and blocks the motion of labeled structures in the cell.

2.1.9. Stop grabbing time-lapse images and keep the cells in fixative for 5-10 min (during this time it is useful to grab a Z-series of images of the cell).

2.1.10. Replace the mixture with the fixative 1 and keep the cells in the fixative 1 for 5 min.

2.1.11. Replace fixative 1 with fixative 2 and keep cells there for 30 min.

2.1.12. Wash with 0.2 M HEPES (pH 7.2-7.3) for 10 min (see Note 3).

2.2. Immunolabelling for EM with Nanogold

2.2.1. Wash the fixed cells for 3 x 5 min with 0.2 M HEPES (pH 7.2-7.3).

2.2.2. Incubate the cells with the blocking solution for 30 min, and then with the primary antibodies diluted in blocking solution, overnight.

2.2.3. Wash the cells for 6 x 2 min with 0.2 M HEPES (PH 7.2-7.3).

2.2.4. Dilute the Nanogold-conjugated Fab fragments of the secondary antibodies x50 times in the blocking solution and add this to the cells; incubate for 2 h.

2.2.5. Wash the cells again for 6 x 2 min with 0.2 M HEPES (PH 7.2-7.3).

2.2.6. Fix the cells with 1% glutaraldehyde in 0.2 M HEPES buffer (pH 7.2) for 5
2.2.7. Wash the cells for 3 x 5 min with 0.2 M HEPES (pH 7.2-7.3), and then for 3 x 5 min in distilled water.

2.2.8. Incubate the cells with the gold-enhancement mixture for 6-10 min according to instruction of the manufacturer. The cells will become violet-grey in color if the gold enhancement is successful.

2.2.9. Wash cells for 3 x 5 min with distilled water.

2.3. Enhancement of Sample Contrast, Sample Locating, and Embedding (see Note 4)

2.3.1. Wash cells 3 times with 0.2M Cacodylate Buffer (pH 6.9).

2.3.2. Treat cells with the reduced osmium for 1 h on ice in the darkness.

2.3.3. Wash cells with 0.2 M Cacodylate Buffer (pH 6.9) 3 x 5 min.

2.3.4. Treat samples with 0.3% Thiocarbohydrazide in 0.2 M Cacodylate Buffer (pH 6.9) for 10 min.

2.3.5. Wash cells 3 times with 0.2 M Cacodylate Buffer (pH 6.9).

2.3.6. Treat cells with 1% OsO4 in 0.2 M Cacodylate buffer (pH 6.9) for 30 min. (To further enhance contrast, one can add three additional steps between steps number 6 and 2.3.7. Rinse samples with 0.1 M sodium cacodylate (pH 6.9) buffer until all traces of the yellow osmium fixative have been removed.

2.3.8. Stain sample with a 1% solution of tannic acid in 0.05 M sodium cacodylate buffer for 45 min.

2.3.9. Replace the tannic acid with a 1% solution of anhydrous sodium sulfate in 0.05 M sodium cacodylate buffer and leave for 5 min.)

2.3.10. Rinse sample in de-ionized water and then dehydrate stepwise with 70%, 90%, and 100% ethanol (3 x 5 min for each) for the dehydration of the specimens.

2.3.11. Prepare EPON solution as described in materials.

2.3.12. Prepare a 1:1 EPON/100% ethanol mixture (acetone and propylene oxide could dissolve plastic Petri dishes). Vortex until thoroughly mixed and stores all solutions in the fume hood.

2.3.11. Treat sample with a 1:1 EPON/100% ethanol mixture for 1 hr.

2.3.12. Replace the 1:1 EPON/100% ethanol mixture with EPON alone for 4 h with one exchange of EPON after 2 h.

2.3.13. Keep the cells in EPON for 1-2 h at room temperature, and then leave them in an oven at 60 °C for 12 h.

2.3.14. After 12 h of polymerization of the EPON, place a small droplet of a fresh resin on the site where the examined cell is located, and insert a resin cylinder (prepared before by polymerization of the resin in a cylindrical mold) with a flat lower surface; leave the samples for an additional 18 h in the oven at 60 °C. The
resin cylinder should be placed on the semi polymerized EPON just over the cell of interest (Note 5).

2.3.15. Polymerize for 24 h hours at 60 °C.

2.3.16. Carefully pick up the resin from the Petri dish and glass; this is easy to do by gentle bending of the resin cylinder to and fro. The resin block and the empty MatTek Petri dish after block detachment could be separated easily. If the cover glass with a coordinated grid cannot be detached from the cells included into the resin, the latter should be placed into commercially available hydrofluoric acid (do not use glassware for this) for 30-60 min.

2.3.17. Removed the glass bottom of tissue culture dishes by hydrofluoric acid for 30-60 min.

2.3.18. Control the completeness of the glass dissolution under a stereomicroscope.

2.3.19. Wash the samples in water after the complete removal of the glass.

2.3.20. Leave the samples in 0.2 M HEPES buffer (pH 7.3) for 60 min to neutralize the hydrofluoric acid.

2.3.21. Wash the samples in water and allow them to dry. The round basement of the sample with diameter of about 1 cm or more should not be cut.

2.4. Identification of the cell of interest on Epon blocks (Fig. 1).

2.4.1. Place the embedded sample under a stereomicroscope using a fiber optic illuminator.

2.4.2. If the gridded cover slip was used the furrows on the cover slips are filled with Epon and after polymerization and dissolution of the glass or its detachment the furrows appeared as combs because Epon forms a replica from the gridded glass. The relief of grid is visible under a stereomicroscope. Using numbers and letters on the grid it is easy to find the position on the surface of the sample where the cells are situated.

2.4.3. Use the composite of bright field DIC images or manual drawings of the region of interest to locate the region of interest on the Epon block.

2.5. Sample Orientation and EM Sectioning from the Very First Section (see Note 6)

2.5.1. Place the sample under the transmission light microscope.

2.5.2. Find the cell of interest among the cells within the sample according to the coordinated grid (Fig. 1A) or pattern of the cell layer (Fig. 1B).

2.5.3. Put the resin block into the holder of an ultratome and examine it under a stereomicroscope.

2.5.4. Place the holder with the sample under the stereomicroscope and using a
steel needle and rotating the sample in the holder, make two small cavities in such a way that they form a horizontal line (broken line in Fig. 2A and C) with our cell appearing in the center of the sample.

2.5.5. Introduce the holder into the ultratome in such a way that the segment arc of the ultratome is in the vertical position and the two cavities form a horizontal line.

2.5.6. By rotating the glass knife stage align the bottom edge of the pyramid parallel to the knife-edge. Using the segment arc, orient the plane of the sample vertically.

2.5.7. Bring the sample as close as possible towards the glass knife.

2.5.8. Adjust the gap (which is visible as a bright band if all three of the lamps of an ultratome are switched on) between the knife-edge and the surface of the sample. The gap has to be identical in width between the most upper and lower edges of the sample during the up and down movement of the resin block. This ascertains that every point of the sample surface containing the cell of interest is at the same distance from the knife-edge.

2.5.9. Slowly moving the sample up and down, continue its approach until the knife begins to cut one of the edges of the sample. The sectioning begins from either the upper or the lower part of the sample, the middle part of the sample where the cell of interest is situated will be unaffected because the length of the radius passing through the cell is shorter than the radii passing through the upper and the lower edges of the sample.

2.5.10. If the sectioning is to begin from the upper part of the sample, tilt the segment arc to approach the lower edge towards the knife. If the sectioning is to begin from the lower edge of the sample, tilt the segment arc and approach the upper edge of the sample towards the knife. A vertically oriented sample should produce equal sections from both the upper and lower edges of the samples (Fig. 2C).

2.5.11. Note down precisely all of parameters relating to the position of the sample in the ultratome, i.e. the degree of rotation of the sample in the holder, the degree of tilting of the segment arc, and the degree of rotation of the knife in its stage. Do not take the sample from the holder and do not rotate the sample inside the holder.

2.5.12. Take the sample and trim it to provide a narrow horizontal pyramid. The pyramid should be as narrow as possible, and the cell of interest should be at the centre of the pyramid. The length of the pyramid should be smaller than 0.9 mm and its height smaller than 0.1 mm. In this case you could place on the slot 18 serial sections. An experienced person can trim a pyramid directly with a razor blade.

2.5.13. Introduce the sample back into the ultratome, and lock it in exactly the same position as before (preserving all of the parameters of sample positioning; this is very important).

2.5.14.
Replace the glass knife with the diamond one, and position the latter towards the pyramid. If the sample is not parallel to the knife, adjust the angle of the diamond knife by rotating the knife stage to make its edge parallel to the plane of the pyramid. Do not change any other parameters of the sample position.

2.5.15. Approach the sample towards the edge of the knife until the gap is extremely narrow. Using a 200-nm approaching step, begin the sectioning. Take serial sections according to the instructions with the ultratome. It is enough to take only 10 sections to pass 2 µm from the bottom of the cell.

2.5.16. Identify the position of the organelle of interest within the EM sample according to the Z-stacking, and select those thick sections that should correspond to this position. For instance, if the organelle of interest is situated at 500 nm from the surface of the sample (bottom of the cell), it is enough to collect only the first three 200-nm serial sections (or ten 50-nm sections). If the position is at 1 µm in height, it will be necessary to collect from the fourth to the eighth serial 200-nm sections (or twenty 50-nm sections).

2.6. Picking up the Serial Sections with the Empty Slot Grid (Fig. 3, see Note 7)

2.6.1. Clean the empty (not covered) slot grid (the donor transfer grid) and one slot grid covered with formvar-carbon film (the acceptor grid).

2.6.2. When the normal (not bent) slot grid is taken by the tweezer it is not convenient to take sections from the water, because the axes of grid cannot be oriented paralleling to the plane of water surface. One needs a holder on the grid. The holder on the slot grid could be done by bending just the small part of the grid from horizontal position. Using a tweezer bend just the small part of the edge of the empty slot grid from horizontal position. This bent rim will serve as a holder for the tweezer.

2.6.3. Take the slot grid with supporting film from a container by the self-closing tweezer and place it film-down on parallel holders covered with scotch tape (Fig. 3C, below).

2.6.4. Take the empty slot grid using the just made holder and place it over the band of serial sections not touching the water and in such a way that the slot is projected on the band of serial section.

2.6.5. Touch water with the donor grid in such a way that sections should be inside the slot and move the slot grid away. The droplet of water and sections will be inside the slot (Fig. 3A-C).

2.6.6. To avoid dirt on the sections it is better to place a small droplet of glass handled distilled water on the acceptor slot grid. Using stereomicroscope put a small droplet of water on the supporting film of the acceptor grid (Fig. 3D).

2.6.7.
Put the donor grid with serial sections on acceptor grid as it is shown in Fig. 3E, F.

2.6.8. Using sharp and very narrow filter paper orient slot in parallel to each other and eliminate the excess of water from the space between slot grids (Fig. 3G).

2.6.9. Dry grids during at least 20 min.

2.6.10. Carefully eliminate the donor grid (Fig. 3H).

2.6.11. Take the dried acceptor grid and check whether sections are in correct position (Fig. 3I).

2.7. **EM Analysis**

2.7.1. Using stereomicroscope (to avoid mistakes) place the slot grid into the holder of EM and introduce it into EM.

2.7.2. Find the very first serial EM sections using the traces (usually letters) of the coordinated grid filled with the resin. These are visible mostly on the first two sections. If the central position of the cell of interest within the pyramid is used as a marker, identify the central cell within the sections.

2.7.3. Take consecutive photographs (or grab the images with a computer using a video camera) of the serial sections beginning from the very first section since the organelle of interest (just observed under the LSCM) appears and until is no longer seen. Having images from FL is useful to identify the cell of interest.

2.7.4. If EM and FM images are reflective it is necessary to change the reflection position of FM images (see Note 8)

3. **Materials**

3.1. **Observation of Living Cells and Fixation**

3.1.1. Cells of interest, i.e. HeLa cells (No. CCL 185; American Type Culture Collection, Rockville, MD), cDNA and transfection reagents;

3.1.2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine (GIBCO BRL, Life technologies)

3.1.3. MatTek Petri dish with CELLocate (MatTek, Ashland, MA).

3.1.4. A digitalized fluorescent-inverted microscope or laser scanning confocal microscope (i.e. from Leica Microsystems Spa, Milan, Italy, www.leica-microsystems.com).

3.1.5. HEPES buffer (0.2 M). Dissolve 4.77 g HEPES in 100 ml distilled water and add of 1 N HCl droplet by droplet to provide a pH of about 7.2-7.3

3.1.6. Fixative 1. 0.05% glutaraldehyde plus 4% formaldehyde in 0.15 M HEPES (pH 7.2-7.3). Dissolve 8 g paraformaldehyde powder in 50 ml 0.2 M HEPES buffer, stirring and heating the solution to 60 °C. Add drops of 1 N NaOH to clarify the solution. Add 1.25 ml 8% glutaraldehyde and 50 ml 0.2 M HEPES buffer. Dilute
twice before use.

3.1.7. Fixative 2. 4% formaldehyde in 0.15 M HEPES (pH 7.2-7.3). Dissolve 4 g paraformaldehyde powder in 100 ml HEPES buffer, stirring and heating the solution to 60 °C. Add drops of 1 N NaOH to clarify the solution.

3.2. Immunolabelling for EM with Nanogold

3.2.1. See item 3.1.4.
3.2.2. Blocking solution. Dissolve 0.50 g BSA, 0.10 g saponin, 0.27 g NH4Cl in 100 ml of 0.2 M HEPES (PH 7.2-7.3).
3.2.3. Nanogold-conjugated monovalent Fab fragments (Nanoprobes, Incorporated: 95 Horseblock Road, Unit 1, Yaphank, NY, USA).
3.2.3. Gold-enhance mixture. Use a gold-enhance kit (Nanoprobes, Incorporated: 95 Horseblock Road, Unit 1, Yaphank, NY, USA). Using equal amounts of the four components (Solutions A, B, C and D), prepare about 200 µl of reagent per Petri dish (a convenient method is to use an equal number of drops from each bottle). First mix Solution A (enhancer; green cap) and Solution B (activator; yellow cap). Wait for 5 min, and then add Solution C (initiator; purple cap), and finally Solution D (buffer; white cap). Mix well.
3.2.4. 1% glutaraldehyde in 0.2 M HEPES buffer (pH 7.2-7.3). Mix 1 ml of 50% glutaraldehyde (EM grade, Electron Microscopy Sciences, Hatfield PA) with 49 ml of 0.2 M HEPES (pH 7.2-7.3). Store at 0-4 °C.

3.3. Enhancement of Sample Contrast, Sample Locating, and Embedding

3.3.1. 0.2 M Cacodylate buffer. Dissolve 2.12 g sodium cacodylate (Electron Microscopy Sciences, Hatfield PA) in 100 ml of distilled water. Add 1 N HCl to provide a pH of about 6.9.
3.3.2. Reduced OsO4. Mix 2% OsO4 in water 3% Potassium Ferrocyanide in 0.2 M cacodylate buffer (pH 7.2) 1:1.
3.3.3. 0.3% Thiocarbohydrazide. Dissolve 0.1 g of Thiocarbohydrazide in 33.3 ml 0.2 M Cacodylate Buffer (pH 6.9). Use immediately after preparation.
3.3.4. 1% OsO4. Mix 2 ml of 4% OsO4 ((Electron Microscopy Sciences, Hatfield PA) with 6 ml of 0.2 M Cacodylate buffer (pH 6.9). Use immediately after preparation.
3.3.5. 1% Tannic Acid. Dissolve 0.1 g Tannic Acid (TAAB Laboratories Equipment Ltd) in 0.05 M Sodium Cacodylate buffer (pH 6.9) prepared by dilution of one volume of 0.2 M Cacodylate buffer (pH 6.9) in three volumes of distilled or de-ionized water.
3.3.6. Ethanol. To prepare N% Ethanol mix N ml of 100% Ethanol (Electron Microscopy Sciences, Hatfield PA) with 100-N ml of de-ionized water.
3.3.7. EPON mixture (TAAB 812, DDSA, MNA, DMP30 in a ratio of 24:9.5:16.5:1,
respectively). Put 20.0 g EPON, 13.0 g Dodecenyl succinic anhydride (DDSA) and 11.5 g Methyl nadi anhydride (MNA) into the same test tube. Heat the tube in the oven for 2-3 min at 60 °C and then vortex it well. Add 0.9 g tri-Dimethylaminomethyl phenol (DMP-30, all from Electron Microscopy Sciences, Fort Washington, PA) and immediately vortex the tube again. It is possible to freeze the EPON in aliquots and to store it for a long time at 20 °C before use.

3.3.9. 1:1 EPON/100% ethanol mixture. Mix 10 ml of 100% ethanol with 10 ml of EPON mixture.

3.4. Identification of the cell of interest on Epon blocks


3.5. Sample Orientation and EM Sectioning from the Very First Section


3.6. Picking up the Serial Sections with the Empty Slot Grid

3.6.2. Diamond knife (Electron Microscopy Sciences, Hatfield PA).
3.6.3. Eyelash with handle (Ted Pella Inc., Redding, CA).
3.6.4. Chloroform
3.6.5. 1x2 mm slot-grids (Electron Microscopy Sciences, Hatfield PA).
3.6.6. 1x2 mm slot-grids covered with formvar and carbon (Electron Microscopy Sciences, Hatfield PA).
3.6.7. Parallel holders covered with scotch tape is not commercially available (Note 1).

3.7. EM analysis

3.7.1. A stereomicroscope (see item 2.4.1.).
3.7.1. An electron microscope with a goniometer (i.e. FEI, Eindhoven, The Netherlands).

4. Notes

4.1. Take a list of thick paper and covered it from both side with a double covered scotch tape. Then cut with scissor two narrow (3 mm in width) peaces and attach them to the glass with the distance of 2 mm between them.
4.2.
Then the figure will be visible after polymerization of Epon as the replica of the roof on the cover slip.

4.3. For subsequent immuno labelling, cells should be treated in the culture dish without removing the cover glass.

4.4. If you plan to use EM tomography is could be helpful to avoid the use of lead citrate staining (see corresponding Chapter in this book) that often gives not equal staining of the thick sections. For this purpose one could use Thiocarbohydrazide staining after fixation with aldehyde.

4.5. The central position of the cylinder is important to ensure the possibility of use the transmission light for the examination of EPON samples. If the wall of the cylinder is situated near the cell of interest, the light will be scattered.

4.6. This method is applicable for CLEM even if cells were looked at as CVLEM. If you use EM tomography, the thickness of EM sections should be 200-250 nm. If you use routine serial sections the thickness could be from 40 to 80 nm. Thinner sections demand more grids necessary for the picking up of serial section. Thus, one should find a compromise between the difficulty, cost, and demands.

4.7. Standard method to pick up the serial sections for routine EM is not sufficient for CVLEM due to significant rate of possible mistakes during manipulations with sections. CVLEM the demands for the reproducibility of this step are much higher. The method should give almost 100% of success. Here, we described a specific method that after some training gives such a result.

4.8. To achieve success the researcher should make all procedures with the reproducibility of about 99%. Therefore we recommend performing all manipulations with grids under stereomicroscope. During manipulations with sections and samples it is better to use self-closing tweezers and slot grids that are previously tested under the stereomicroscope.

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