

Electron Microscopy of the Cytoskeleton of Cultured Cells

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Introduction

Light and electron microscopy each have certain advantages and limitations for the investigation of the cytoskeleton. Light microscopy, especially with recent progress in fluorescence imaging, affords the opportunity to analyze the kinetics of dynamic processes in the living cell [2-5](#). However, the spatial resolution of light microscopy is limited to approximately $\lambda/2$, -that is, half the wavelength of the imaging light which, in the green, is about 250 nm. This limitation constrains understanding the supramolecular organization of the cytoskeleton for which information is required below the 10 nm level. Electron microscopy, in contrast, affords high resolution but provides only static images and is not applicable to living cells. Correlative analysis of the same cells by light and electron microscopy,--that is, high temporal resolution analysis of fluorescent features in a living cell followed by high resolution spatial analysis of structural features in the same cell, provides an opportunity to combine the advantages of both techniques and establish functional connections between cytoskeletal dynamics and supramolecular organization. A critical issue for any mode of microscopy is whether the preparative procedures themselves introduce changes and artifacts into the specimen. A telling advantage of correlative microscopy is the potential for evaluating the issue of artifact.

The success of correlative microscopy imposes special demands at both the light and electron microscopic level. For fluorescence light microscopy, a suitable fluorescent probe must be prepared

either by chemical derivatization [6,7](#) or by molecular biological conjugation to green fluorescent protein [8-10](#). The derivitized protein needs to be introduced into the cell either by microinjection [11,12](#) or by expression [8-10](#). Finally, issues of photobleaching, photodamage and phototoxicity need to be attended to, generally through low light level imaging and the use of sensitive cameras [13-15](#). At present, a variety of chemical and molecular biological procedures are available for fluorescently labeling specific components of the cytoskeleton and visualizing them in the living cell. However, among countless electron microscopic techniques, very few were designed for correlative microscopy, particularly for cytoskeletal components which are known to be labile and sensitive to external conditions. The key requirements of a suitable electron microscopic procedure are quality, reproducibility and yield. Yield is essential because detailed observation of individual living cells places a high investment of investigator time and effort in a single cell. If the efficiency of recovering a cell for electron microscopy is low, the investment is lost. Considering different variants of cell preparation for electron microscopy we have chosen detergent extraction - chemical fixation - critical point drying - TEM of platinum replicas as a basic procedure, because each of these steps has been shown to be reliable and capable of producing a higher yield of successful results in comparison with alternative approaches. The overall procedure has been optimized in several respects¹⁶ which significantly improved the quality and consistency of the electron microscopy of the cytoskeleton, thus permitting efficient correlative microscopy.

In this chapter we describe the preparation of cells for correlative electron microscopy after live light microscopic observation of fluorescently labeled cytoskeletal proteins microinjected into the same cells. Since identification of cytoskeletal elements in electron microscopic preparations is an essential part of any correlative study, procedures for immunogold labeling of cytoskeletal components and for myosin S1 decoration of actin filaments are also described.

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Cell Culture and Light Microscopy

Details of cell cultivation, fluorescent probe production, microinjection and light microscopic observation are out of the scope of the present description. However, certain requirements are specific to correlative microscopy and these we discuss.

For high resolution light microscopy, cells need to be grown on glass coverslips. To facilitate the re-localization of the same cells, it is very helpful to have internal reference marks on the coverslip. This is easily obtained by providing coverslips with a finder grating which can be recognized by both light and electron microscopy. We routinely use 22 x 22 mm coverslips which we coat with a thin layer of gold through a locator grid (400 mesh, Ted Pella, Inc., Redding, CA) (see [Fig. 1](#)). After gold coating, coverslips are baked at 160°C overnight to prevent dislocation of gold grains by cultured cells. Cells for correlative microscopy are selected within clear uncoated glass areas, corresponding to the solid parts of the locator grid.

The coated coverslips may be mounted into different types of chambers suitable for cell cultivation, microinjection and observation [17](#). Since correlative microscopy requires the fast cessation of dynamic cellular processes at the end of the light microscopic observation, the chamber design should allow for

the fast exchange of media. In our laboratory, we typically use 35-mm tissue culture dishes with a circular 18 mm hole in the bottom. Coverslips are mounted with gold-coated side up onto these holes using silicon vacuum grease. The minimum amount of grease required to prevent leakage should be used in order to avoid complications during subsequent excision of the central area of the coverslip with the desired cells (see below). Dishes are sterilized with UV irradiation before plating cells. To prevent pH shift in the medium during observation, we use either of two procedures. If the culture medium is bicarbonate-buffered, the medium is overlaid with mineral oil ¹⁷ which prevents evaporation and resulting pH change. Alternatively, HEPES-buffered media are used instead of the bicarbonate-buffered ones. For correlative microscopy, the latter approach allows higher temporal resolution because elimination of oil before processing for electron microscopy takes time and may result in a lapse between light and electron microscopic observations.

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Electron Microscopy

Extraction

For platinum replica electron microscopy the cytoskeleton first has to be uncovered and made available to metal coating. Detergent lysis is the most usual way to remove the cell membrane, thus exposing the cytoskeleton ¹⁸⁻²³. However, immediately upon removal of the cell membrane, the potential for extraction and artifact arises. Consequently, the composition of the extraction solution is extremely important and many extraction procedures have been suggested ²⁴. In our experience, a relatively high concentration of a non-ionic detergent, e.g. 1% Triton X-100, is critical for rapid solubilization of the cell membrane and cessation of cell activity. The detergent is made up in a buffer containing stabilizing supplements. We routinely add high molecular weight polyethelene glycol (PEG) for general preservation of the cytoskeleton ²⁵. Taxol and/or phalloidin are added for the specific preservation of microtubules and actin filaments, respectively. Extraction at room temperature is superior to extraction at lower (4°C) or higher (37°C) temperature. At 4°C, the plasma membrane tends to be retained and is visible as patches overlying the cytoskeleton. At 37°C, a substantial loss of material from the cytoskeleton is evident, especially in the lamellipodia region.

Solutions

1. In our practice we use one of the following cytoskeleton buffers as a base for the extraction solution: buffer M (50 mM imidazole, pH 6.8, 50 mM KCl; 0.5 mM MgCl₂; 0.1 mM EDTA; 1 mM EGTA) or buffer PEM (100 mM PIPES, pH 6.9; 1 mM MgCl₂; 1 mM EGTA). 10x stocks can be prepared for both buffers.
2. Extraction solution: 1% Triton X-100, 4% PEG (MW 40,000) (Serva, Heidelberg / New York) in buffer M or PEM supplemented (optionally) with 10 µg/ml taxol and/or 10 µM phalloidin.
3. Phosphate buffered saline (PBS): 10 mM phosphate buffer, pH 7.4, 150 mM NaCl.

Procedure

1. Remove culture medium from a dish and briefly rinse cells with PBS or other serum-free physiological solution. Remove rinse solution.
2. Immediately add the extraction solution. Exchange of media should be fast to avoid cell damage by drying.
3. Incubate cells for 3 to 5 min at room temperature.
4. Rinse cells with the cytoskeleton buffer 2 or 3 times.

Direct comparison of live and extracted cells demonstrated that the described extraction procedure does not introduce alterations in the distribution of myosin II [26,27](#) and microtubules ([Fig. 2](#)). After extraction, the cytoskeleton remains stable for an extended period of time in the cytoskeleton buffer supplemented with taxol and phalloidin. This allows one to perform certain operations, which are possible only with the unfixed cytoskeleton. Some of them are described below.

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Actin Depletion

One of the major problems for electron microscopic investigations of the cytoskeleton arises from the abundance of actin filaments interfering with the visualization of other cytoskeletal components that may be present in minor amounts. As a solution to this problem, we depleted actin from detergent-extracted cells with the actin-severing protein, gelsolin [26-31](#). Gelsolin treatment did not perturb other cytoskeletal components [26,27](#) ([Fig. 2](#)), but exposed many other cytoskeletal elements that become available for examination ([Fig. 3, 4](#)). This approach was successfully used for the visualization in cultured cells of nonmuscle myosin II [26,27](#), microtubules [30](#), and intermediate filaments [31](#).

Solutions

1. Buffer G: 50 mM MES-KOH, pH 6.3, 0.1 mM CaCl₂, 2 mM MgCl₂, and 0.5 mM DTT.
2. 0.1-0.2 mg/ml of gelsolin dialyzed against buffer G. Both native protein isolated from bovine brain²⁸ and recombinant Ca²⁺-independent gelsolin fragment [32](#) were successfully used for actin depletion [26-31](#).
3. Buffer M or PEM (see section [Extraction](#))

Note: 10 μ g/ml taxol should be added to all solutions for preservation of microtubules, because otherwise all microtubules will be destroyed by the Ca²⁺-containing gelsolin buffer.

Procedure

1. After rinse with a cytoskeleton buffer (see section [Extraction](#)), rinse coverslips once with buffer G.
2. Remove the buffer from the dish. Using cotton swabs, wipe the buffer from the dish and coverslip leaving wet only a small (approximately 5-7 mm) central area containing the finder grid.
3. Add 10-15 μ l of gelsolin solution to the wet central area. The drop of gelsolin solution will remain

within the wet circle and will not spread into the dry glass area. This approach allows the use of very small amounts of gelsolin. Incubate 1 h at room temperature in moist conditions.

4. Rinse 3 times with M or PEM buffer and fix (see section [Fixation](#))

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S1 Decoration

Molecular identification of cytoskeletal components is a necessary part of the investigation of the cytoskeleton. Although immunochemical labeling is the most common solution of this problem (see below), there is an alternative approach for identification of actin filaments, -- that is, decoration by skeletal myosin subfragment 1 (S1) [33,34](#). S1 binding to actin filaments results in the formation of characteristic polar complexes, which can be easily recognized with electron microscopy and thus permit distinguishing actin filaments from other cytoskeletal components.

Besides identification, S1 decoration of actin filaments allows determination of the polarity of actin filaments, which is a key structural feature of their function. Models for various actin activities, such as lamellipodia protrusion [35](#), stress-fiber contraction [36,37](#), cell translocation [38](#), and formation of organized actin networks and bundles [26,27](#) explicitly take into account the polarity of actin filaments in different cellular domains. When studied with negative staining [33,39-43](#) or thin sectioning electron microscopy [34,44-46](#), S1-decorated actin filaments have a classic arrowhead pattern. Barbed or pointed ends of decorated filaments correspond to fast and slow growing ends, respectively [47](#). In platinum replicas, S1-decorated filaments express a helical rope-like appearance rather than an arrowhead pattern [48](#), probably because only the top surface of filaments contributes to the formation of the image, unlike negatively or positively stained filaments, where contrast is generated by top and bottom surfaces of the specimen. The individual turns of the "rope", however, have an intrinsic asymmetry correlated with the polarity of the actin filaments [48](#) ([Fig. 5a](#)). With our approach, we are usually able to visualize the polarity of a large fraction of actin filaments even in areas of dense filament arrays, like stress-fibers of fibroblasts ([Fig. 5b](#)).

Solutions

1. Cytoskeleton buffer M or PEM with 10 μ g/ml taxol and 10 μ M phalloidin (both are optional)
2. 0.25 mg/ml S1 in buffer M or PEM with taxol and phalloidin (optional). We purchase S1 from Sigma and centrifuge the solution at 100,000g for 20 min before use to remove residual myosin filaments.

Procedure

1. After rinse with a cytoskeleton buffer (see section [Extraction](#)), wipe coverslips with cotton swabs around the central finder grid-containing area, as for gelsolin treatment (see above).
2. Add 10-15 μ l of S1 solution to the wet central area and incubate 30 min at room temperature in

moist conditions.

3. Rinse with buffer M or PEM 3 times.

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Fixation

Fixation provides cell structures with a physical resistance against subsequent harsh procedures. Chemical fixation is generally used for cytoskeleton preservation. Cryofixation, which has been used as an alternative, is only sufficient if the water layer is not lost in subsequent procedural steps. Because cytoplasmic structures lose their support and collapse during subsequent water sublimation [49](#), chemical fixation is frequently used even in conjunction with cryofixation [22,50,51](#).

The most commonly employed fixative for electron microscopy is glutaraldehyde. However, glutaraldehyde alone does not provide proper preservation of all cellular structures. Consequently, various supplemental treatments have been introduced and, among them, tannic acid followed by heavy metal treatment is particularly beneficial [48,52-58](#). The mechanism of protective action of tannic acid followed by heavy metals seems to be related to the ability of tannic acid to bind both to proteins and to heavy metals [52](#). When applied in the correct order, these compounds apparently form an extensive chemical complex bound to protein structures that can work as a protective shell resisting shrinkage caused by dehydration and drying. According to these considerations, our procedure includes consecutive fixations with glutaraldehyde, tannic acid and uranyl acetate.

Solutions

1. 2% glutaraldehyde (EM grade) in 0.1 M sodium cacodylate, pH 7.3
2. 0.1% aqueous tannic acid (Mallinckrodt, Inc., Paris, Kentucky; Cat. # 1764). This particular formulation of tannic acid with low molecular weight has been shown to give the best results [59](#)
3. 0.1-0.2% uranyl acetate in distilled water

Procedure

1. After final rinse with a cytoskeleton buffer, add glutaraldehyde solution and incubate for at least 20 min at room temperature. If necessary, specimens can be refrigerated at this stage and stored for several days in sealed dishes to prevent evaporation. Before further processing, specimens should be brought back to the room temperature.
2. Without washing, remove glutaraldehyde and add tannic acid solution; incubate 20 min at room temperature.
3. Rinse specimens in 3 changes of distilled water and incubate 5 min in the last change of water.
4. Add uranyl acetate solution and incubate 20 min at room temperature.

5. Rinse with distilled water.

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Immunostaining

Morphological features of cytoskeletal components, although often suggestive, not always allows unambiguous identification of the structure. The usual way for determination of the molecular identity is immunochemistry. Various techniques have been employed for electron immunostaining. Each technique, however, usually requires certain adjustment for a specific object and/or antigen. The most commonly used approach of immunostaining is the indirect method with an unlabeled primary antibody and a species-specific secondary antibody conjugated with a marker for detection of the antibody. Colloidal gold is the probe of choice for antibody visualization by electron microscopy, because of its perfect circular shape, high electron density and availability in a range of sizes.

We found that our procedures of cytoskeleton preparation for platinum replica electron microscopy are completely compatible with immunoelectron cytochemistry and with the use of colloidal gold as an electron dense marker. The difference in electron density between colloidal gold particles and the platinum layer is sufficient for detection of the immune reaction in coated specimens.

The protocol for immunolabeling for electron microscopy depends mostly on the primary antibody and its ability to recognize antigen under particular conditions. Initial evaluation of the quality of staining at the light microscopic level is always recommended. For most antibodies we use immunostaining after glutaraldehyde fixation, because it provides the least deviation from the basic procedure. However, some primary antibodies do not work with glutaraldehyde-fixed antigens. In this case, we do not recommend to use formaldehyde or methanol fixation, since they often interfere with the preservation of the structure. Instead, we found that in most cases application of the antibody to unfixed cytoskeletons in a cytoskeleton buffer is safe for the structure and may even increase the sensitivity of immunoreaction.

Solutions

1. Phosphate buffered saline (PBS): 10 mM phosphate buffer, pH 7.4, 150 mM NaCl.
2. 2 mg/ml NaBH₄ in PBS (should be prepared immediately before use).
3. Primary antibody diluted in PBS (for post-fixation staining) or in a cytoskeleton buffer with 10 μ g/ml taxol and 10 μ M phalloidin (for pre-fixation staining). The required antibody concentration should be estimated in preliminary light microscopic experiments. We use for electron microscopy the concentration of antibody that produces good immunofluorescence staining.
4. Buffer A: 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05% Tween 20.
5. Buffer A with 0.1% bovine serum albumin (BSA).
6. Buffer A with 1% BSA.
7. Colloidal gold-conjugated secondary antibody diluted in buffer A with 1% BSA. The exact dilution

should be estimated experimentally. In our experience, 1:5 to 1:10 dilutions are most useful for a number of commercially available gold-conjugated antibodies.

8. 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3

Procedure for post-fixation staining

1. After glutaraldehyde fixation (step 1 of the [Fixation](#) procedure), wash specimens with PBS (two brief rinses and 5 min in the third change of PBS).
2. Quench specimens by NaBH₄ (2 changes, 10 min each) at room temperature.
3. Rinse in PBS (3 changes, 5 min in the last change).
4. Remove PBS and wipe coverslips around the central finder grid as for gelsolin treatment.
5. Apply primary antibody in PBS and incubate 30-45 min at room temperature.
6. Rinse in PBS (3 changes, 5 min in the last change).
7. Rinse once in buffer A with 0.1% BSA.
8. Wipe coverslips as before and apply colloidal gold-conjugated antibody.
9. Incubate overnight at room temperature in a sealed dish in moist conditions.
10. Rinse in buffer A containing 0.1% BSA (3 changes, 5 min in the last change) and fix including glutaraldehyde fixation (see section [Fixation](#)).

Procedure for pre-fixation staining

1. After detergent extraction and washing (see section [Extraction](#)), wipe coverslips around the central area as for gelsolin treatment.
2. Apply primary antibody in a cytoskeleton buffer and incubate 15-30 min at room temperature.
3. Rinse in a cytoskeleton buffer (3 changes, 5 min in the last change)
4. Fix in glutaraldehyde 20 min at room temperature
5. Quench in NaBH₄ (2 changes, 10 min each) at room temperature.
6. Follow steps 6-10 of the post-fixation procedure.

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Critical Point Drying

Cytoskeletons before metal coating have first to be dried, partially or completely. Techniques that do

not use drying are not applicable for the study of the cytoskeleton with its complicated 3D structure. Critical point drying and freeze drying (freeze etching) are the two methods most commonly used to prepare cytoskeletons for metal coating. Each of these techniques has certain advantages and disadvantages. Critical point drying is a simple and reliable technique, but requires a preceding dehydration with organic solvents, a procedure that causes significant shrinkage of protein structures [60](#). In addition, water or organic solvent contamination during critical point drying may cause serious artifacts [58](#). Freeze drying induces less shrinkage [24,60,61](#) and provides better preservation of fine biological structures [62](#). Freeze etching after quick freezing has been shown to produce an excellent quality of electron microscopic images [22](#). However, conditions providing successful results are hard to control and the yield of this technique is usually fairly low. The most common problem is the formation of ice crystals during freezing or sublimation, causing displacement of structures and limiting useful area for investigation [51,63](#). Another problem is difficulty in obtaining the correct thickness of the water layer above the cells, which must be thin enough to ensure vitreous ice throughout the cells, but not too thin to avoid cell damage by surface tension [51](#). These limitations make the freeze drying process demanding in terms of time and experience to be routinely used for correlative light and electron microscopy.

We found that critical point drying in combination with the necessary precautions produce images of excellent quality. Thus, tannic acid-uranyl acetate fixation significantly reduces cell shrinkage during dehydration [54,57](#). Special measures to avoid water or intermediate liquid (alcohol or acetone) contamination during dehydration and critical point drying prevent the artifact of apparently "fuse" filaments [58](#). In addition, precautions directed at retaining a layer of liquid above cells help to eliminate serious distortions which may be caused by the gas-liquid interface passing through the specimen. For example, we suggest placing specimens into holders for critical point drying in a horizontal position with lens tissue between them ([Fig. 7](#)). The lens tissue helps to retain a layer of liquid over the specimens thus protecting them from damage by surface tension when specimens are transferred from one dehydrating solution to another.

Solutions

1. Graded ethanols (10%, 20%, 40%, 60%, 80%, and 100%)
2. 0.1-0.2% uranyl acetate in 100% ethanol
3. 100% ethanol dried over molecular sieves. Molecular sieves (4 Å, 8-12 mesh) (Aldrich Chemical Company) were washed out of dust with multiple changes of water, baked overnight at 160°C, and, after cooling, added to 100% ethanol. Ethanol was kept for a few days with molecular sieve before use.

Procedure

1. If oil objectives are used for light microscopy, remove the immersion oil from the bottom of the coverslip with a cotton swab soaked in an appropriate solvent (alcohol or ether).
2. Detach the gold-coated coverslip from the bottom of a dish and quickly transfer it into a wide Petri

dish filled with water. Some silicone grease will remain on the lower side of the coverslip. Lightly press the coverslip down to the Petri dish bottom making sure that the grease does not contaminate the central area of the coverslip containing the finder grid.

3. Using a diamond pencil, cut off the greased edges of the coverslip to obtain a clean central part of the coverslip with the finder grid ([Fig. 7](#), top). It is helpful to use a razor blade as a guide for making cuts and for keeping the coverslip in place. Use sharp diamond pencil and avoid glass crumbs around the cutting area to prevent coverslips from shattering. The optimal size of the central piece of the coverslip containing cells of interest is 6-8 mm.
4. Place a specimen holder for critical point drying into a beaker filled with water. We use a handmade holder, which represents a wire basket ([Fig. 7](#), bottom), fitting the size of the critical point dryer's chamber.
5. Put a sheet of lens tissue on the bottom of the holder and load coverslips one after another (cell side up) using additional lens tissue sheets as spacers. It is not desirable to overload a holder, since it makes the exchange of liquid between coverslips difficult. For the 10x15x10 mm holder and 6-8 mm coverslips, 12 specimens is a recommended limit. Close holder with a wire lid to prevent the last sheet of lens tissue from flowing away ([Fig. 7](#), bottom).
6. Dehydrate specimens by transferring the holder through graded ethanols (10%, 20%, 40%, 60%, 80%, and two times 100%, 5 min in each). To improve exchange of ethanols, use a magnetic stirrer. For this purpose, we use a wire scaffold on the bottom of the ethanol containing beaker and place the specimen basket on the top and the stirrer bar underneath ([Fig. 7](#), bottom).
7. Place holder into 0.1-0.2% uranyl acetate in 100% ethanol and incubate for 20 min
8. Wash two times in 100% ethanol and two times in 100% ethanol dried over molecular sieves, 5 min in each.
9. Place holder into the chamber of the critical point dryer filled with 100% ethanol dried over molecular sieves. Extensively wash the chamber with liquid CO₂ at 5-10°C to remove all traces of ethanol. Keep the level of CO₂ always above the upper edge of the holder. We use 10 changes of CO₂ with 5 min incubation in each change. The CO₂ cylinder should be equipped with a water-absorbing filter. When washing is finished, follow the instructions for critical point dryer.

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Metal Coating

Metal coating provides a contrast to biological specimens. The choice of a metal depends on the technical ease to produce coating (usually, on the melting temperature of a metal) and on the quality of the shadowing (primarily, on the size of metal grains). These two parameters are basically in an inverse relation with each other. Platinum is the most popular metal for coating of biological objects because it represents a reasonable compromise between melting temperature and grain size.

Platinum grains settled down onto the specimen surface are not cohesive and can be easily distorted

during subsequent manipulations or under the electron beam. To prevent this distortion, carbon coating is usually applied on the top of the platinum layer. Unlike platinum, carbon can form a firm cohesive film and thus keep platinum grains together. Carbon is practically transparent for electrons and therefore does not interfere with the formation of image.

The important parameters affecting the quality of the image are the angle and the thickness of metal shadowing. Higher angles result in less contrast but allow for better visualization of the cellular organization in the third dimension because of increased penetration of metal grains into deep hollows. Rotary shadowing has proved to be better compared to one-dimensional coating. Thicker coating reduces resolution but increases contrast and 3D range.

In our experiments, we use platinum evaporation for coating of cytoskeletons. A platinum layer thickness of 2.5-2.8 nm and an angle of 45° with the rotation of a specimen stage produce a fair balance between contrast and depth of the coating. Platinum shadowing was followed by carbon evaporation (2-3 nm thick) at 75°-90° with rotation. An Edwards 12E1 (UK) vacuum evaporator was used for platinum and carbon coating. The average thickness of platinum and carbon layers were estimated using a film thickness monitor QM-300 (Kronos, Inc.).

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Preparation of Replicas

After coating, the platinum-carbon replica of the cytoskeleton has to be released from the coverslip and mounted on electron microscopic grids for examination. If cell areas that are going to be studied are thin and have low electron density, like lamella regions in most cultured cells, removal of glass with hydrofluoric acid is sufficient for replica release. For thick and electron dense cell regions it is desirable first to deplete organic components with a strong oxidative agent, e.g. household bleach. This allows, for example, visualization of cytoskeletal elements associated with the surface of pigment granules of fish melanophores, which are characterized by high electron opacity ([Fig.8](#)). However, it is important to note that depletion of organic material is not compatible with immunogold labelling, since it causes the degradation of antibody molecules associated with colloidal gold particles and consequent elimination of gold label from replicas.

For correlative microscopy, special attention should be paid to the selection of the area of interest. It can be done while the replica is still attached to the coverslip. After drying and metal coating, cells have good contrast and are visible even under the dissection microscope. The pattern of gold shadowing also helps to localize cells.

Solutions

1. 10% hydrofluoric acid (HF)
2. 10-3 % liquid household detergent (e.g. "Ivory")
3. Clorox bleach diluted in distilled water 1:2 to 1:10 depending on the strength of bleach

Procedure

1. Using double-sided tape, immobilize a platinum-carbon coated coverslip on the bottom of a wide Petri dish with cell side up. Attach only very corners of the coverslip to make the detachment easy and safe.
2. Under dissection microscope, make cuts in the platinum-carbon layer around cells of interest using any sharp tool (razor blade or needle). Continue the cuts up to the edges of the coverslip to facilitate the release of the selected area from the rest of the replica.
3. Fill a well of 12-well tissue culture multidish (diam. 25 mm) with HF (approximately 5 ml per well). Place a coverslip onto the surface of the HF with cell side up. In minutes the coverslip falls down leaving the replica floating. After separation of the coverslip, replica falls apart along the introduced cuts. Pattern of the gold shadowing on the resulting pieces helps to identify the desired replica fragments.
4. Using a platinum loop, transfer replica pieces onto the surface of diluted household detergent. This procedure prevents the replica from breaking apart, which usually happens because of large difference in surface tension between HF and water. Overdose of detergent, however, can result in rolling up and drowning of replicas.
5. Transfer replica pieces onto the surface of pure distilled water. Go to step 8 for thin or gold labeled samples or to step 6 for unlabeled electron dense specimens.
6. Transfer replica pieces onto the surface of a diluted household bleach. Time of treatment may vary from 2 to 20 min depending on the cell type and the strength of the bleach.
7. Repeat steps 4 and 5.
8. Mount replica pieces onto formvar-coated electron microscopic grids. Use low mesh or single slot grids to reduce chance of getting the region of interest onto a grid bar. Control under the dissection microscope is helpful for the targeted mounting of replicas on grids.
9. Examine samples in electron microscope. Present images in inverse contrast (as negatives), because it gives a more natural view of the structure, as if illuminated with scattered light. We use a Phillips EM-300 transmission electron microscope at 80 kV for examination of specimens. Negatives are converted to digital files using a Nikon (Melville, NY) Scantouch digital scanner and processed for presentation using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) software.

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Conclusions

We have described an electron microscopic procedure designed for a specific purpose: to correlate dynamic features in living cells with their structural details at the supramolecular level. The overall procedure consists of modifications and refinements of well-known techniques of electron microscopy which, in combination, provide for high sample yield, consistency and quality of results while retaining high sample throughput. This simple way to compare dynamics and structure at high resolution levels should facilitate studies of the cytoskeleton.

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