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Platinum Replica Electron Microscopy: Imaging the Cytoskeleton Globally and Locally

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Abstract

Structural studies reveal how smaller components of a system work together as a whole. However, combining high resolution of details with full coverage of the whole is challenging. In cell biology, light microscopy can image many cells in their entirety, but at a lower resolution, whereas electron microscopy affords very high resolution, but usually at the expense of the sample size and coverage. Structural analyses of the cytoskeleton are especially demanding, because cytoskeletal networks are unresolvable by light microscopy due to their density and intricacy, whereas their proper preservation is a challenge for electron microscopy. Platinum replica electron microscopy can uniquely bridge the gap between the “comfort zones” of light and electron microscopy by allowing high resolution imaging of the cytoskeleton throughout the entire cell and in many cells in the population. This review describes the principles and applications of platinum replica electron microscopy for studies of the cytoskeleton.

Keywords

Electron microscopy; Platinum replica; Cytoskeleton; Actin filaments; Tomography

INTRODUCTION

Structural studies are essential for understanding functional principles in biology. They reveal how smaller components of a system work together as a whole. An ideal structural approach would combine high resolution of details with full coverage of the whole. However, it is usually difficult to satisfy both requirements. In practice, different trade-offs are needed for each biological question.

In cell biology, light microscopy (LM) and electron microscopy (EM) are main imaging techniques for structural studies. Their “comfort zones” in terms of resolution and coverage barely overlap. Conventional LM can image many cells in their entirety, but at resolution limited by diffraction to ~200 nm. EM can reach ~2 nm resolution, but is rather demanding in terms of time, skills and effort required to produce high quality samples in sufficient amounts. LM is easily compatible with live cell imaging and identification of molecules,

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although with little information about the structural context of labeled components. EM cannot image live cells. Moreover, many EM approaches are poorly compatible correlative light and EM (CLEM) of living cells that can link cell structure and dynamics. Incorporating immunochemistry into EM is also challenging, but can reveal labeled structures together with their surroundings.

Structure of the cytoskeleton, and especially of the actin cytoskeleton, presents particular challenges for high resolution structural analyses. The nanometer size and tight packing of cytoskeletal filaments makes them unresolvable by either diffraction-limited or subdiffraction (super-resolution) LM. This goal so far can be achieved only by EM. However, actin filaments are notoriously difficult to visualize by traditional EM. In plastic-embedded samples, organization of stable actin filament bundles can be revealed, but delicate actin filament networks, such as those in lamellipodia or at the surface of membrane organelles remain uninterpretable even in best examples (Kukulski et al., 2012; Luduena and Wessells, 1973; Tilney et al., 1980; Wu et al., 2010).

CryoEM is praised for its ability to analyze biological samples in their native hydrated state (Lucic et al., 2013). However, because of low contrast and sensitivity to irradiation of cryoEM preparations, cryoEM is mostly used to analyze samples suitable for averaging. In cells, cryoEM can image only very small regions and is best in detecting membranes, but rarely used to image the cytoskeleton (Mahamid et al., 2016; Medalia et al., 2007). The amount of cellular data that can be collected by cryoEM is limited by lengthy image acquisition, sample damage during imaging, sophisticated digital processing of each field, and availability of expensive equipment. CryoEM is also incompatible with specific identification of structural components except for educated guess (Lucic et al., 2013).

Platinum replica electron microscopy (PREM), also known as rotary shadowing EM, can uniquely bridge the gap between LM and EM, especially for structural analyses of the cytoskeleton (Svitkina, 2016; Svitkina and Borisy, 1998). PREM yields many cells per experiment and affords high resolution typical for EM. Various cytoskeletal structures including dynamic actin networks can be well preserved and analyzed by PREM at a broad range of scales up to single filaments, even when they are densely packed (Figure 1). PREM data are suitable for direct visual evaluation, thus allowing immediate screening of many pleiomorphic cells to sort out their general and variable structural features. Sample preparation for PREM, including immunolabeling, is fast and efficient compared with other EM techniques. PREM is compatible with CLEM and requires inexpensive equipment and short training. As any other technique, PREM is not universal. It is limited to relatively thin samples, requires samples attached to glass surfaces and is not ideal for studies of membrane structures. However, the combination of its strengths and weaknesses makes PREM a highly productive and informative approach to study cytoskeleton organization in different cells at various physiological conditions.

PREM PRINCIPLES AND PROCEDURES

In PREM, a transmission electron microscope (TEM) is used to visualize fine 3D topography of the sample surface. The entire procedure developed in our lab for studies of

cultured cells includes: (1) exposure of internal structures of interest by detergent extraction or mechanical rupture; (2) chemical fixation by sequential application of glutaraldehyde, tannic acid, and uranyl acetate solutions; (3) critical point drying; (4) rotary shadowing with platinum; (5) stabilization of the platinum layer by carbon coating; (6) dissolution of the glass coverslip by hydrofluoric acid; and (7) mounting of released samples onto formvar-coated EM grids for TEM observation (Figure 1A).

A key part of the procedure is step 4, when a heavy metal (platinum) is deposited onto the sample forming a replica of the sample surface. Heavy metals reveal 3D topography of coated surfaces by deflecting electrons and thus increasing the sample contrast. Sample coating is conducted in vacuum by heating and evaporating the metal. Oblique shadowing better reveals sample three-dimensionality. In this case, upper structures appear darker, because they receive more metal and deflect more electrons, so that fewer electrons reach the detector. In contrast, lower parts of the sample appear lighter because they are shaded by the upper structures. Because of this inverse contrast relative to light-illuminated objects, PREM images look more intuitive as negatives. Inverted PREM images resemble those obtained by scanning EM (SEM), which relies on recording the scattered electrons. However, PREM typically achieves higher resolution than SEM, because of thinner metal coating and greater resolving power of TEMs, although high-end SEMs can achieve comparable resolution.

Compared with unilateral shadowing, rotary shadowing helps avoid deep shadows and thus yields images of higher quality (Heuser and Kirschner, 1980). PREM resolution depends on the metal grain size and the thickness and the angle of shadowing. Metals with smaller grains give better resolution, but usually have higher melting temperatures, which makes evaporation more difficult. Platinum is a popular metal, because of good compromise between the grain size and ease of evaporation. Thinner coating gives better resolution, but lower contrast. A lower shadowing angle provides higher contrast, but less penetration into the sample depth. As metal coating is not cohesive, it is subsequently stabilized by a layer of carbon, which can keep metal grains together, and is fairly transparent for the electron beam.

For metal coating in the vacuum, the sample must be dry (step 3). Two main approaches, freeze-drying and critical point drying (CPD), help prevent sample damage by surface tension during drying. Optimal freeze-drying can produce higher resolution images than CPD and, for example, resolve protofilaments in microtubules (Heuser and Kirschner, 1980), which are usually not seen after CPD. However, freeze-drying is technically challenging, especially in application to whole cells, which limits its success rate. Accordingly, freeze-drying appears more productive for analyzing single particles (Heuser, 1989), although can be successful for studying cells (Heuser and Kirschner, 1980; Hirokawa, 1982; Morone, 2010). CPD, on the other hand, is highly reliable and yields much high quality material with each run (Svitkina, 2016), while resolution of protofilaments is unnecessary to address the organization of cytoskeletal filaments in the cell.

Because of metal shadowing, PREM reveals only the sample surface. For analyses of the cytoskeleton, it needs to be exposed to shadowing (step 1). This can be achieved by detergent extraction or mechanical rupture of cells. Detergent extraction displays the

cytoskeleton in its entirety (Figure 2), but only if extraction conditions have been carefully adjusted for the sample. Nonionic detergent Triton X-100 is commonly used to dissolve cell membranes, whereas preservation of the cytoskeleton relies on various cytoskeleton-preserving buffers supplemented with stabilizing reagents, such phalloidin for actin filaments, taxol for microtubules, or a nonspecific stabilizer polyethyleneglycol (Svitkina, 2016; Svitkina et al., 1995). Mixing Triton X-100 with glutaraldehyde at different ratios (Small, 1981; Svitkina, 2016) is another way to find conditions that simultaneously expose and preserve structures of interest. While looking for optimal conditions, sample quality should be evaluated both by LM to judge retention of specific proteins and by PREM to assess exposure and preservation of fine details. As a final test, correlative PREM of the cells imaged by LM can definitively show whether any distortions or losses occur during extraction (Svitkina and Borisy, 1998).

A shortcoming of detergent extraction is that it precludes analyses of the membrane-associated cytoskeleton. A partial solution for this problem is mechanical rupture of the cell to expose its interior without dissolving membranes. In two main rupture approaches, freeze-fracture (Heuser, 1989) and unroofing (Collins et al., 2011; Heuser, 2000; Morone, 2010; Ong et al., 2014; Svitkina et al., 1997), the mechanical impact is applied to frozen or buffer-immersed cells, respectively. Mechanical rupture methods are not highly reproducible, but with experimentation and practice, they can yield a desired assortment of structures in the preparations, although some portions of the cell are lost (Figure 2).

Fixation of the sample (step 2) is necessary before CPD, but also beneficial before freeze-drying. Unfortunately, it has lately become customary to make ‘fixation’ almost synonymous with ‘artifact’, usually, to promote alternative procedures, such as cryofixation and live cell imaging. Although the latter methods are powerful in certain aspects, they have their own sources of artifacts and cannot yet replace such rich source of information as properly fixed cells. In application to PREM, we have thoroughly evaluated various fixation protocols using different tests including CLEM to monitor potential artifacts (Korobova and Svitkina, 2010; Svitkina and Borisy, 1998; Svitkina and Borisy, 1999; Svitkina et al., 2003; Svitkina et al., 1995; Yang et al., 2007). These data show that our protocol faithfully preserves the spatial distribution of various cytoskeletal proteins observed by LM in living cells. Our preferred fixation procedure includes sequential fixation with glutaraldehyde, tannic acid and uranyl acetate (Svitkina, 2016). After application of formaldehyde, cytoskeletal structures often appeared fragmented in the EM images even though they appeared properly preserved at LM level (our unpublished data). Deleterious effects of osmium tetroxide on actin filaments have been long known (Maupin-Szamier and Pollard, 1978; Small, 1981).

For PREM, samples should be placed on grids in a form sufficiently transparent for the electron beam. For thick samples, which can be handled by forceps, replicas are released by digesting the underlying organic material using household bleach. Thin samples need to be attached to a glass coverslip, which can be dissolved by hydrofluoric acid (step 6) (Heuser and Kirschner, 1980; Svitkina, 2016). For sufficiently thin samples, there is no need to additionally digest the organic material underneath the replica.

APPLICATIONS OF PREM TO HIGH-RESOLUTION STUDIES OF THE CYTOSKELETON

PREM was initially introduced to study freeze-fractured samples to visualize the interface between leaflets of the plasma membrane, but then used to analyze single particles (Heuser, 1989) and internal structure of cells (Heuser and Kirschner, 1980; Svitkina et al., 1995) and tissues (Hirokawa, 1989). Here, I give examples from our work of PREM applications to study the cytoskeleton of cultured cells.

PREM reveals general architecture of the cytoskeleton and presents it as easily interpretable 3D images with clearly resolved cytoskeletal fibers (Figure 1). Over the years, we have developed a reliable and relatively simple protocol that consistently produces high quality data with excellent yield (Svitkina et al., 1995). This protocol consists of detergent extraction, chemical fixation, ethanol dehydration, CPD, and rotary shadowing with platinum and carbon. Using PREM, we were able to get important insights into organization and functions of the cytoskeleton in different subcellular compartments of different cells (Jones et al., 2014; Korobova and Svitkina, 2008; Korobova and Svitkina, 2010; Ong et al., 2014; Svitkina and Borisy, 1999; Svitkina et al., 2003; Svitkina et al., 1996; Svitkina et al., 1997; Yang et al., 2007). PREM combined with unroofing also gives excellent results in visualizing the membrane-associated the cytoskeleton (Collins et al., 2011; Ong et al., 2014; Svitkina et al., 1997).

Intrinsic three-dimensionality of PREM samples is perceptible even in 2D projections, because of the topography-dependent distribution of shades of the metal coating. However, 3D information can also be specifically extracted using tilt images of PREM samples. For basic TEMs with low-tilt stages (up to $\sim 20^\circ$), stereo pairs taken at the $\pm 6-15^\circ$ tilt can be used to see three-dimensionality. TEMs with high-tilt stages can be used to obtain a tilt series in the range of $\pm 60-70^\circ$ for subsequent tomographic 3D reconstruction (Figure 2). Although electron tomography is used predominantly for plastic-embedded or cryo-preserved samples, high stability of PREM samples under electron beam makes them ideal for tomography (Collins et al., 2011).

PREM can be easily combined with immunolabeling of individual proteins with excellent staining efficiency (Collins et al., 2011; Jones et al., 2014; Korobova and Svitkina, 2010; Shutova et al., 2014; Svitkina and Borisy, 1999; Svitkina et al., 1996; Svitkina et al., 1997), which makes PREM unique relative to other EM approaches. For PREM, secondary antibodies are conjugated with colloidal gold, which is well recognizable on the background of the platinum layer because of nearly perfect spherical shape of gold particles (Figure 1). By combining different sizes of colloidal gold, two epitopes can be labeled simultaneously (Shutova et al., 2014). For PREM, the primary antibody should be compatible with glutaraldehyde fixation, because formaldehyde does not adequately preserve the structure or even damages it. If no glutaraldehyde-compatible antibodies are available, an antibody that works with a native protein can be applied to extracted samples in the cytoskeletal buffer before fixation (Collins et al., 2011; Jones et al., 2014; Shutova et al., 2014).

CLEM helps to partially overcome a major drawback of EM - inability to work with live cells. The high success rate of PREM makes it highly suitable for CLEM of live cells, because even a single imaged cell can be efficiently recovered for PREM analysis. Comparison of the two sets of data provides high resolution structural information about the cellular structures involved in specific activities of the cell (Korobova and Svitkina, 2008; Svitkina and Borisy, 1999; Svitkina et al., 2003; Yang et al., 2007).

CONCLUSIONS

Each structural technique has its optimal productive niche. PREM is highly efficient in revealing cytoskeleton structure at a broad range of scales. PREM is fast (~6–8 h of sample processing time), produces much data per experiment, has a high success rate, is compatible with immunochemistry and CLEM, and technically not demanding. PREM data are suitable for direct visual evaluation, which is still more powerful than computer-based analysis. For example, a recent algorithm for tracking actin filaments in cryoEM tomograms could not faithfully report on short or branched filaments (Jasnin et al., 2013), whereas they can be easily visualized by PREM (Collins et al., 2011; Korobova and Svitkina, 2008; Korobova and Svitkina, 2010; Svitkina and Borisy, 1999; Svitkina et al., 1997). As any other technique, PREM is not universal. It is limited to relatively thin samples, which is also the case for other high resolution EM techniques. PREM requires samples attached to glass surfaces and is not ideal for revealing membrane structures. However, it significantly surpasses other structural methods in cell biology for analyses of fine and global architecture of macromolecular protein assemblies, such as the cytoskeleton.

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HIGHLIGHTS

- Platinum replica electron microscopy combines high resolution with broad coverage of the sample
- Platinum replica electron microscopy is easily compatible with immunochemistry, live cell imaging, and electron tomography
- Current knowledge of the fine cytoskeleton architecture comes largely from data obtained by platinum replica electron microscopy

Figure 1.

PREM of a human umbilical vein endothelial cell stained with anti-VE-cadherin immunogold. (A) Workflow of PREM of cultured cells. (B) Overview of the cell with labeled regions enlarged in panels C-G. (C, D) Actin filament networks in the lamellum (C) and the lamellipodium (D). (E) Enlarged region of the branched actin network from the box in D. (F) Junction between two cells. (G) Enlarged focal adherens junction from the box in F; gold particles labeling VE-cadherin are pseudocolored in yellow.

Figure 2.

Electron tomography of an unroofed Ptk2 cell shows a clathrin-coated pit with the associated branched actin filament network (pseudocolored green in the central panel) abutting the constricted neck of the pit. Brown panels: dual-axis tilt images at the indicated angles. Blue panels: vertical section through the pit (top) and horizontal slices (A-D) along yellow lines with corresponding letters. Neck size and degree of invagination can be seen in D and in upper panels.