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An efficient and reproducible process for transmission electron microscopy (TEM) of rare cell populations

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Abstract

Transmission electron microscopy (TEM) provides ultra-structural details of cells at the sub-organellar level. However, details of the cellular ultrastructure, and the cellular organization and content of various organelles in rare populations, particularly in the suspension, like hematopoietic stem cells (HSCs) remained elusive. This is mainly due to the requirement of millions of cells for TEM studies. Thus, there is a vital requirement of a method that will allow TEM studies with low cell numbers of such rare populations. We describe an alternative and novel approach for TEM studies for rare cell populations. Here we performed TEM study from 10,000 HSC cells with quite ease. In particular, tiny cell pellets were identified by Evans blue staining after PFA-GA fixation. The cell pellet was pre-embedded in agarose in a small microcentrifuge tube and processed for dehydration, infiltration and embedding. Semi-thin and ultra-thin sections identified clusters of numerous cells per sections with well preserved morphology and ultrastructural details of golgi complex and mitochondria. Together, this method provides an efficient, easy and reproducible process to perform qualitative and quantitative TEM analysis from limited biological samples including cells in suspension.

1. Introduction

Transmission electron microscopy (TEM) provides ultra-structural details of cells at the sub-organellar level, most generally by using tissue samples. However, ultrastructures of several rare populations in suspension such as hematopoietic stem cells (HSCs) are under explored, due to requirement of millions of these cells for TEM studies (Bozzola, 2007; Leapman, 2004). It is easy to process solid pieces of tissues for electron microscopy, while processing of cells in suspension remains challenging (Bozzola, 2007). Many methods have been adapted for cell pellets, but generally they require millions of cells. For this reason, details of cellular ultra-structure and organelle content of rare populations remain elusive. In this

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article we describe a different and novel approach for TEM studies for rare cell populations i.e. stem cells. The essential details involved in specimen preparation are covered.

Cellular analysis of rare cells or biological samples in suspension mostly relied on scanning electron microscopy (SEM), or other alternative techniques, with information limited to the cell surface (Tanaka and Yamagata, 1992). The main limitation to perform TEM studies from few cells in suspension is to localize them for TEM processing. To overcome this, several alternative approaches have been adopted including BSA/ bis-acrylamide (BSA-BA) mediated polymerization of cell suspension or TEM analysis from cytospin preparations (Mather et al., 1981; Taupin, 2008). These methods have inherent drawbacks, as very few cells were found over the section. In addition, it is difficult to identify the samples in the solidified gel. It may also cause alterations in cellular organization due to cell spreading and the fragility of the cell structure during separation of cells from cytospin slides.

Here, we report an efficient protocol with consistent results from scarce biological samples for TEM. To counter the difficulty in identifying the small cell pellets in the solidified agarose gel, we utilized the Evans blue staining after fixation to localize a tiny cell pellet of 10,000 cells in a small microcentrifuge tube as well as in the agarose gel. The small agarose gels with tiny cell pellet were then processed for osmification, serial dehydration and embedding in LX112 before sectioning. Interestingly, this method enabled us to characterize ultrastructure and subcellular organization of hematopoietic stem cells (identified as Lin⁻ Sca-1⁺ c-Kit⁺; LSK), a rare population in the bone marrow (0.2-0.5%). Here we focus to provide the experimental details that would be helpful to perform ultrastructural investigations of many rare but highly important populations. Together, this method provides an opportunity to efficiently and reproducibly process for TEM analysis limited biological samples including cells in suspension, in a much easier and more consistent manner to get both qualitative and quantitative data than previously described methods.

2. Methods

2.1. Animal Strains and Chemicals

C57Bl/6 inbred mice of 2-4 month age were used in study for the stem cells (LSK) isolation. All experimental procedures were approved by the institutional animal committee at the Cincinnati Children's Research Foundation. Paraformaldehyde, Gluteraldehyde, cacodylate buffer, 1% aqueous osmium tetroxide (OsO₄), uranyl acetate, lead acetate, 200 mesh grids and resin LX112 were from Electron microscopy sciences (Hatfield, PA). Evans Blue and low melting agarose were procured from Sigma-Aldrich (St. Louis, MO).

2.2. Cells, fixation, staining and pre-embedding

Bone marrow from 2-3 mice were stained with different makers including Lineage, c-Kit, Sca-1 as standard protocols described before (Chen et al., 2008). Hematopoietic stem cells (Lin⁻ Sca-1⁺ c-Kit⁺; LSK population) were sorted using FACS Aria (BD instrument). Sorted 10-20K LSK cells were spun down at 3200 RPM (1000g) for 10 min, medium was gently taken out with ~ 200ul medium leaving in tubes at each step (at this stage, the cell pellet was not visible in the tube). Cells were fixed by adding 0.2 ml of 2× fixative (1× concentration- 3% Paraformaldehyde and 2.5% Gluteraldehyde in 0.1M cacodylate buffer) at RT for 1 h (Bozzola, 2007; Hopwood, 1970). After fixation, cells were centrifuged at 3200 RPM for 10 min at 30° C, washed with 0.1M cacodylate buffer with leaving ~ 200ul buffer in the tube. Cells were stained by adding 200ul of 2mg/ml solution of Evans Blue to tube and incubated for 20 min following 3 washes with buffer to stain cells with blue color, this step was really helpful to localize cell pellet in Microcentrifuge tube and to follow the next steps. The cell suspension was transferred to 0.5ml tubes and centrifuged at 3200

RPM for 10 min. Now a tiny cell pellet was visible in tube and buffer was removed gently with leaving ~ 50 µl buffer in tube. After that, 200 µl of 4% low melting agarose was added to the tube and immediately centrifuged at 3200 RPM for 10 min at 30° C. The cell pellet was localized and tubes were transferred at 4°C/ice for 20 min to solidify agarose. Cell pellet in solidified agarose was carefully taken out of the tube with the help of a 27 G needle and buffer (Anderson, 1965; Saini et al., 2006) and trimmed into small piece with a scalpel. Agarose piece with cell pellet was transferred to a new 1.5 ml tube and washed 2-3 times using buffer and processed for TEM.

2.3. Transmission electron microscopy

The cell pellet in agarose piece was treated with 1% OsO₄ solution for 1 h at 4°C, that helps to provide an enhanced contrast to TEM images. Samples were further processed for dehydration, infiltration and embedding into LX-112 resin with serial changes into following solutions: 25% ethanol at RT for 15 min, 50% ethanol at RT for 15 min, 75% ethanol at RT for 15 min, 95% ethanol at RT for 15 min, 100% ethanol at RT for 15 min, twice; Ethanol:LX-112 (3:1) at RT for 30 min, Ethanol:LX-112 (1:1) at RT for 30 min, Ethanol:LX-112 (1:3) at RT for 30 min; pure LX-112 at RT for 60 min, twice. Finally, the samples were transferred in pyramid tip mold (Ted Pella; 10585) and polymerized at 60°C for 72 h. Semi-thin sections (1 µm) were cut using an ultra-microtome (Leica EM UC7) after attaching pyramid on mounting cylinders (Ted Pella; 10580) and stained with toluidine blue to identify the position of cells. Ultra-thin sections (70-100 nm) were cut and collected on 200 mesh grids. The grids were stained with 1% uranyl acetate, at RT for 10 min, followed by Reynolds lead citrate, at RT for 5 min. Sections were examined with a Hitachi model H-7650 electron microscope, at 80 KV, using the AMT-600 image capture engine software. Images were transferred to photoshop software for final processing.

3. Results and Discussion

A conclusive method to localize a tiny pellet containing minimum number of cells followed by TEM analysis is described. We used 10,000 HSC (LSK) cells for experiments, which forms an almost invisible cell pellet in 1.5 ml tube. Staining with Evans blue post-fixation and transferring the cells to a 0.5 ml microcentrifuge tube led to the visualization of a tiny cell pellet of 10K cells, that was directly embedded in agarose (Figure 1Ai-ii). This cell pellet was easily identifiable after osmification with osmium tetroxide treatment in agarose and during the dehydration process and after embedding into LX-112 (Figure 1Bi-ii). We observed darkening of the cell pellet after osmification with 1% OsO₄, while minimal change in color of agarose piece was observed (Figure 1Bi-ii). Blocks were trimmed to the cell pellet, sections were cut from plastic to agarose containing pellet (Figure 1Biii). Semi-thin sections from blocks containing a tiny pellet showed numerous cells clustered together (Figure 1C). The obtained ultra-thin sections were viewed under electron microscope and clusters of numerous individual cells were identifiable. The morphology and ultra-structures of these cells was well preserved (Figure 1D). It is pertinent to mention that the cell clusters were quite easier to localize under electron microscope using this method than it would have been if processed as suspension. The electron micrograph of individual cells demonstrated good cell morphology with intracellular details (Figure 2A). Furthermore, structures and integrity of sub-cellular organelles including the Golgi network (Figure 2B) and mitochondria (Figure 2C) were well preserved, as observed at higher magnification of 50,000×. The focus of this article is to provide enough details to execute TEM studies with fewer cells. The biological findings relevant to this analysis will be described elsewhere. This method enables maintaining cell clusters together and preserving cell ultra-structure, which is desirable to analyze multiple cells for quantification of data. This represents a significant advantage to previous published methods (Mather et al., 1981; Taupin, 2008). Together, our

method describes a novel way to localize the cell pellets for TEM using Evans blue, which makes the sample processing for TEM easy to perform and enables qualitative and quantitative data analysis. Typically, sample preparation for TEM requires many times more numbers of cells than what is needed for light microscope, because of the multiple steps in processing the samples for TEM and localizing the cell block to be able to cut them at the right position (Bozzola, 2007; Saini et al., 2006). Thus, scarce biological samples as cell pellet is less tedious to target under the microscope than those processed as suspension, particularly for TEM (Bozzola, 2007). Most often, cell pellets from about ten millions of cells are embedded into agarose/gelatin matrix and processed as 2×2 mm pieces enriched with cell pellet to avoid samples to be too scarce for handling through all the standard washes, dehydration, infiltration and fluid changes, involved in TEM processing (Bozzola, 2007; Saini et al., 2006). Although researchers have adopted immobilization of cell suspension in BSA-bisacrylamide polymerized resin or process of cells from cytopins of several cells in order to perform TEM from scarce sample (Mather et al., 1981; Taupin, 2008), However are not methods of choice due to – 1) tedious job to cut and find cells under TEM due to sparsely distributed cells within BSA-gelatin matrix, 2) cytopsin cell preparation can alter cell morphology due to spreading, cleavage between the cells and slide – hence, often requires re-embedding. Furthermore, monolayers are not easy to localize for sectioning. Osmium post-fixation after PFA-GA fixation is a usual procedure for conventional ultrastructural morphology that helps to provide better contrast to images under electron microscope (Hopwood, 1970). This step cannot be performed for scarce biological samples in suspension before pre-embedding in BSA and BA due to loss of the samples during the subsequent washes required to wash away the osmium or in pre-embedded sample in BSA and BA, as matrix becomes dark due to the staining with osmium (Hopwood, 1970; Taupin, 2008). Thus, the qualities of the electron micrographs were compromised due to no use of osmium. In the present protocol, we used osmification after pre-embedding in agarose in order to provide contrast to TEM images. We observed darkening of cell pellet with little change in color of agarose (Figure 1B) and high quality micrograph were obtained (Figure 2). As expected, the pre-staining with Evans blue allowed us to localize tiny cell pellets without affecting the sub-organelle details. The present method offers improvement for localizing the rare cell population in biological system. The cell type and cell size matter a lot during the sample processing for TEM. Our method setups an improvement to find the smaller cell easily for TEM processing. Additionally, we strongly believe that this method can easily be adopted with even lower cell number (~ 1000 cells) of larger cells such as endothelial and fibroblasts.

4. Conclusion

Together, here we provided a method of choice to perform TEM studies with lower amount of cells as starting material for TEM. This is highly desirable as critical information on morphology and ultrastructures of many scarce cell populations are still elusive. We describe an alternative and novel approach to localize small cell pellet that will be helpful to perform TEM studies of rare cell populations with ease. Furthermore, analysis of cell clusters will provide both qualitative and quantitative data. We believe that this method will be quite useful to understand many new biological information for key but rare cell populations as electron microscopy offers unsurpassed details of cell ultra-structure and organization.

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Figure 1.

Experimental overview of TEM analysis from low number of cells (A) Image of cell pellet from 10,000 LSK cells pre-embedded in agarose inside 0.5 ml Microcentrifuge tube (i) and pellet in agarose after taking out of the tube (ii) with the insight of pellet at the right corner (B) Cell pellet after osmification with 1% OsO₄ and embedding into LX112 resin (i), block after trimming during cutting of sections (ii) and sections ribbon above diamond knife (iii) (C) Low magnification under light microscope of a representative field of a toluidine blue stained section of 1 μm thickness (D) An overview field of the cell cluster observed under electron microscope after staining with uranyl acetate and lead citrate. Scale bars are indicated in each figure.

Figure 2.

Image analysis under electron microscope (A) Electron micrograph of single HSC (LSK) cells after uranyl acetate and lead citrate staining. (B) Higher magnification image of HSC showing Golgi complex (G) and (C) mitochondria (M). The nucleus is labeled as N. Scale bar: a, 2 μ m; b and c, 500 nm.