

Video Article

Sample Preparation and Imaging of Exosomes by Transmission Electron Microscopy

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Abstract

Exosomes are nano-sized extracellular vesicles secreted by body fluids and are known to represent the characteristics of cells that secrete them. The contents and morphology of the secreted vesicles reflect cell behavior or physiological status, for example cell growth, migration, cleavage, and death. The exosomes' role may depend highly on size, and the size of exosomes varies from 30 to 300 nm. The most widely used method for exosome imaging is negative staining, while other results are based on Cryo-Transmission Electron Microscopy, Scanning Electron Microscopy, and Atomic Force Microscopy. The typical exosome's morphology assessed through negative staining is a cup-shape, but further details are not yet clear. An exosome well-characterized through structural study is necessary particular in medical and pharmaceutical fields. Therefore, function-dependent morphology should be verified by electron microscopy techniques such as labeling a specific protein in the detailed structure of exosome. To observe detailed structure, ultrathin sectioned images and negative stained images of exosomes were compared. In this protocol, we suggest transmission electron microscopy for the imaging of exosomes including negative staining, whole mount immuno-staining, block preparation, thin section, and immuno-gold labelling.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56482/>

Introduction

Extracellular vesicles (EVs) are lipid bilayer vesicles secreted by cells, and their size ranges between 30 and 300 nm. EVs were first reported in 1978, with evidence from vesicles of patients with Hodgkin's disease¹. These vesicles were reported to be 40 to 120 nanometers in size. In 1980, it was reported that EVs are involved in the coagulation system and thrombosis, formation of a blood clot inside a blood vessel in cancer patients². After 30 years, EVs have been reported to be an important factor to promote tumor invasion, immune escape, and angiogenesis³. In addition, the functions of EVs have been studied as regulators in cellular interactions in the areas of inflammation, immune disorder, neurological disease, and cancer⁴. Since EVs contain specific biomolecules such as protein, mRNA, and microRNA⁵, their potential application in diagnostics and therapeutics has been analyzed^{6,7}. EVs are a category composed of subgroups including exosomes, prostasomes, oncosomes, dexosomes, microparticles, promininosomes, argosomes, and exosome-like vesicles, depending on their cellular origin and biological function³. In addition, based on their biogenesis, these EVs can be divided into microvesicles (shed microvesicles, 100 - 1000 nm) and exosomes (30 - 300 nm)^{8,9}. Among these, the exosome has been reported as a cell communicator for immune response¹⁰, cancer^{11,12}, and infectious disease¹³.

Interest in the exosomes as a biomarker for early diagnosis is increasing rapidly, and the purification and characterization of exosomes must be accompanied with molecular imaging techniques. The varying sizes and morphologies of exosomes, depending on their origin and function¹⁴, can be distinguished by microscopy techniques with high resolution, such as electron microscopy. Most exosomes were visualized by negative stained Transmission Electron Microscopy (TEM)^{15,16,17}, and these results were confirmed by immunolabeling of a specific protein in whole mount vesicle¹⁸. Several research groups have reported the structure through Scanning Electron Microscopy, Atomic Force Microscopy^{19,20}, and Cryo-TEM^{21,22}. However, while these techniques are useful for studying the exosome structure, they are insufficient for observing the position of specific proteins located inside the exosome. Therefore, we introduced a protocol for imaging exosomes with a specific protein's labelling. We applied block preparation, ultrathin sectioning, and immunostaining for ascertaining the protein's detailed location in the exosome. This was compared with negative staining and whole mount immunostaining, which is traditionally used for characterization of the exosome.

Protocol

1. Block Preparation, Sectioning, Staining and Imaging of the Exosome

1. Pellet the exosomes from culture supernatant of HCT116 cells by centrifugation at 100,000 x g for 1.5 h²³. Remove the culture supernatant and carefully fix the purified exosome pellet with 1 mL of 2.5% glutaraldehyde in 0.1 M sodium cacodylate solution (pH 7.0) for 1 h at 4 °C. For 0.1 M sodium cacodylate, dissolve 4.28 g cacodylic acid in 160 mL of distilled water (DW). Adjust pH to 7.4 with 0.1 M HCl then make up to 200 mL with distilled water.
2. Remove the fixative and rinse the pellets with 1 mL of 0.1 M sodium cacodylate buffer at room temperature. Repeat three times with each change lasting 10 min.
3. Post-fix the samples with 1 mL of 2% Osmium tetroxide for 1 h at 4 °C.
4. Remove the fixative and rinse three times with 0.1 M sodium cacodylate buffer every 10 min.
5. Incubate for 10 min with a graded acetone series (50%, 60%, 70%, 80%, 90%, 95%, 100%, respectively) on the shaker.
6. Remove the acetone and incubate the solution of 3:1 acetone:low viscosity embedding mixture for 30 min. The exosome pellet is in the tube.
7. Remove the medium and add 1:1 acetone:low viscosity embedding mixture medium, then incubate for 30 min.
8. Remove the medium and add 1:3 acetone:low viscosity embedding mixture medium, then incubate for 30 min.
9. Remove the medium and add 100% low viscosity embedding mixture and incubate overnight at room temperature.
10. Embed the sample in pure low viscosity embedding mixture using the embedding mold and bake for 24 h at 65 °C.
11. Prepare sections with 60 nm thickness through an ultra-microtome.
12. Double-stain with 2% uranyl acetate for 20 min and lead citrate for 10 min.
For Reynolds lead solution, add 1.33 g of lead nitrate and 1.76 g of sodium citrate to a total of 50 mL distilled water.
13. Observe the grid under transmission electron microscopy at 80 kV.
14. Click "Acquire" and then click "File" and "Save as" in CCD camera system under the electron microscope at 80 kV. Follow automatic settings for the exposure time.

2. Immuno-staining of Sections and Imaging (Figure 1)

1. Cut 60 nm ultrathin sections using an ultra-microtome, and collect on the nickel grid.
2. Incubate grids in 50 µL drops of 0.02 M glycine for 10 min to quench free aldehyde groups.
3. Rinse in 100 µL of DW three times each for 10 min. Incubate at room temperature for 1 h in PBS containing 1% BSA.
4. Incubate grids in 50 - 100 µL drops of anti KRS antibody²⁴ (1:100 in PBS containing 0.1% BSA) for 1 h (If necessary, this step should be carried out at 4 °C overnight.)
5. Wash grids with five separate drops (50 µL) of PBS containing 0.1% BSA for 10 min each.
6. Transfer grids to drops of 2nd antibody for 1 h (anti-rabbit IgG conjugated to 10 nm gold particle (1:100) in PBS containing 0.1% BSA).
7. Wash grids with five separate drops (50 µL) of PBS containing 0.1% BSA each 10 min.
8. Double-stain with 2% uranyl acetate for 20 min under dark conditions and with Reynold's lead citrate for 10 min, respectively.
9. Click "Acquire" and then click "File" and "Save as" in CCD camera system under a TEM at 80 kV. Exposure time followed automatic settings.

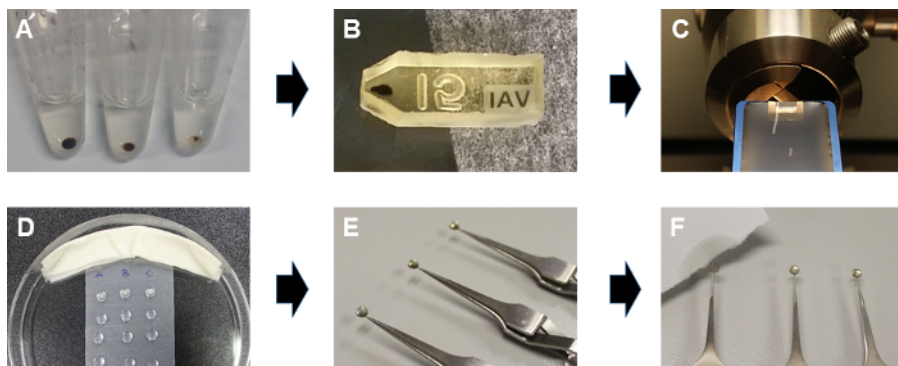


Figure 1: Process of sample preparation and immunostaining. (A) Double fixed exosome is dehydrated and infiltrated with low viscosity embedding mixture resin. (B) Resin embedding. (C) Ultra-thin section with diamond knife. (D) Setup for Immuno-gold labelling. The grids with the ultrathin sections are put on the liquid droplets on a parafilm, which is placed with the wet paper towel. Each section is incubated with droplets of 50 - 100 µL antibody, then washed with buffer. (E) Double staining with uranyl acetate and lead citrate. The lid is covered with aluminum foil for heavy metal staining. (F) Staining solution is removed by filter paper. [Please click here to view a larger version of this figure.](#)

3. Negative Staining

1. Glow-discharge the thin formvar/carbon film coated 200 mesh copper EM grids for 1 min by glow discharger.
2. Fix purified exosomes with 1 mL of 2% Paraformaldehyde (PFA) for 5 min.
Caution: Paraformaldehyde fumes are toxic. All work should be done in a ventilated fume hood.
3. Load 5 - 7 µL exosome suspension solution on the grid and incubate for 1 min. If the concentration of exosome is too high, dilute the concentration to 1/2 - 1/5.
4. Immediately stain with the ~20 drops of filtered 1% uranyl acetate (UA) solution on the surface of the EM grid by syringe.

5. Remove the excess UA solution on the grid by contacting the grid edge with filter paper.
6. Quickly rinse the grid with a drop of water. This step will remove the excess staining solution.
7. Place the grid on the table by holding with tweezers, and cover the grid partially with a culture dish to dry for 10 min under room temperature.
8. Store the grid in an EM grid box for future observation by a TEM at 80 kV.

4. Whole Mount for Immunostaining

1. Glow-discharge the thin formvar/carbon film coated 200 mesh copper EM grids for 30 s.
2. Fix purified exosomes with 1 mL of 2% Paraformaldehyde (PFA) for 5 min.
Caution: Paraformaldehyde fumes are toxic. All work should be done in a ventilated fume hood.
3. Load 5 - 7 μ L fixed exosome solution on the grid and incubate for 5 min. Rinse with 100 μ L of PBS three times each for 10 min.
4. Treat grids with 50 μ L of 0.05 M glycine for 10 min to quench free aldehyde groups.
5. Transfer grids to a drop of blocking buffer (PBS containing 1% BSA) for 30 min.
6. Incubate grids with 50 - 100 μ L anti PD-L1 antibody (1:100 in PBS containing 0.1% BSA) for 1 h (if necessary, this step should be carried out at 4 °C overnight).
7. Wash grid with five separate drops (50 μ L) of PBS containing 0.1% BSA for 10 min each.
8. Transfer grid to a drop of 2nd antibody for 1 h. Anti-mouse IgG conjugated to 9 - 11 nm gold particle diluted at 1:100 in PBS containing 0.1% BSA.
9. Wash grid with five separate drops (50 μ L) of PBS containing 0.1% BSA for 10 min each. Wash grid with two separate drops (50 μ L) of DW. Perform negative staining with 2% uranyl acetate as described from 3.4 to 3.8.
10. Store the grid in an EM grid box for future observation by a TEM at 80 kV.

Representative Results

Currently, exosomes are classified into size and shape categories by transmission electron microscopy. **Figure 2** shows negative stained exosome and immune-labelled exosome in whole mount status. **Figure 3** shows sectioned exosome and immuno-labelled exosomes after thin sectioning. Immuno-gold staining using antibodies of specific proteins is used to positively identify an exosome and classify the types of proteins in the exosome. The protocol in this paper uses whole mount immunostaining and immunostaining with plastic sectioned exosome.

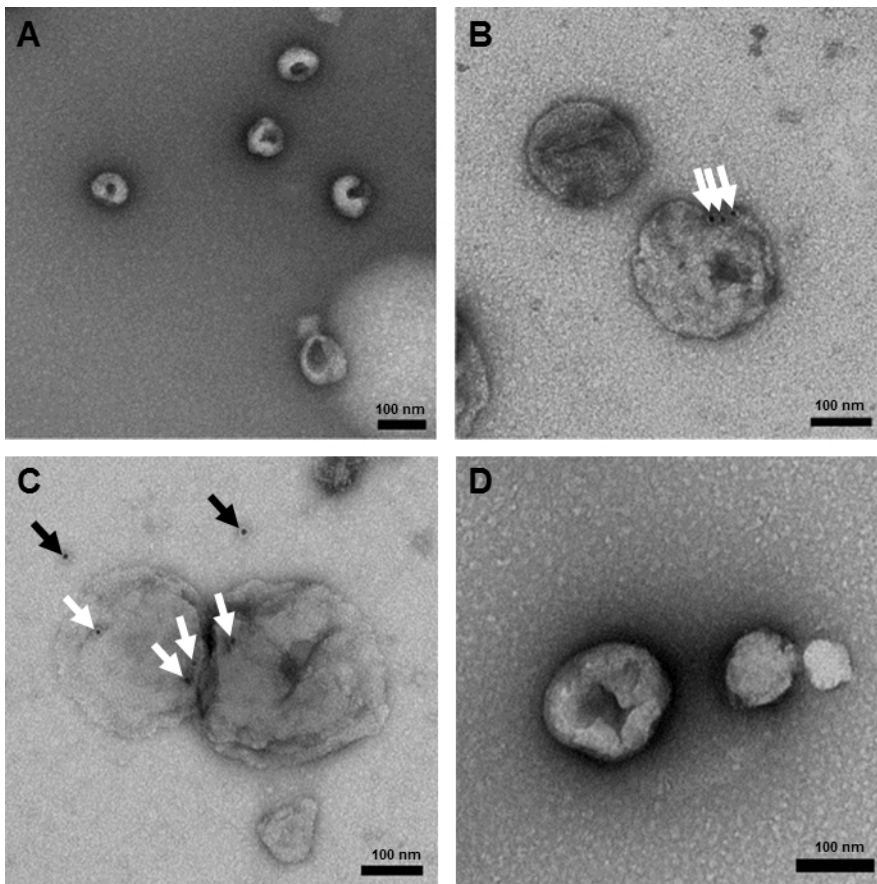


Figure 2: Negative staining and whole mount immuno-staining. (A) Exosome morphology is observed by negative staining. Exosomes shows their cup-shaped morphology. (B, C) Whole mount immuno-gold staining shows the location of specific protein (anti human CD274; PD-L1) in exosome. White arrows indicate the location of the gold. Black arrows indicate the location of the background signal. (D) Negative control of immunostaining result. Isotype control was used by primary antibody in this immunostaining process. Scale bar = 100 nm.

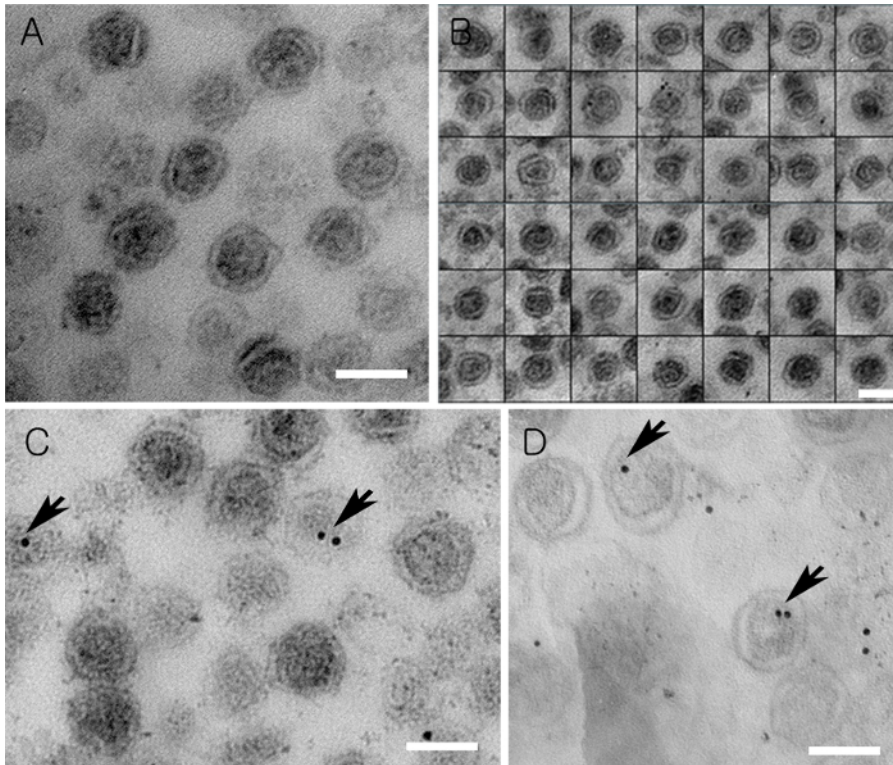


Figure 3: Electron micrograph of sectioned exosomes. (A) Exosomes' round shape morphology. **(B)** Boxed exosome using the "boxer" tool in EMAN program. **(C, D)** Immuno-gold labelled exosome with heavy metal staining. Black arrows indicate gold particles **(A-D)** Scale bar = 100 nm. [Please click here to view a larger version of this figure.](#)

Discussion

This article presents a protocol for observing the detailed exosome's structure and labeling of its specific proteins. Negative staining has been considered as the best method for exosome imaging¹⁷. This conventional technique has shown exosomes' cup-shaped structure. However, this cup shape is a form of artefact that can occur due to the drying process. Cryo-TEM results have shown that exosomes have a perfectly spherical structure in aqueous solution^{25,26}. The cryo-TEM technique is a very powerful method for examining the natural structure, but it is difficult to apply the immuno-gold method. Raso and co-workers pointed out drying in the conventional method (whole mount-negative staining method) resulted in a cup-shaped morphology. In this study, the standard method for cell preparation (routine EM; fixation, dehydration, embedding, and sectioning) was applied to reduce the exosome drying effect. The routine EM using plastic embedding can avoid or reduce artefacts (changes in volume and shape) caused by denaturation. For chemical fixation, glutaraldehyde (GA) was used for the cross-linking (covalent interactions between amino groups). Usually, osmium tetroxide (OsO₄) is used for fixation of lipids as well as improved contrast.

In this study, negative staining helped to confirm the typical morphology of exosomes that has been reported in previous papers^{21,27,28,29}. In addition to negative staining, block sectioning is also a good method for observation of exosomes (**Figure 2**). While sectioned images showed the inner structure of exosome, in contrast negative stained TEM showed mainly the surface of the exosome. In sectioned images (**Figure 3**), the vesicles showed the lumen structure that is known as a structural feature of exosomes³⁰. In this protocol, we used block sectioning, immuno-staining, and TEM imaging. Specially, the block sectioning is useful for analysis at a later time when images may be required at different magnifications, for different microscopy technique, and for other analyses such as immuno-gold labelling. After sectioning, sections on the grid can be stored in the grid box, which can be used for immuno-staining for classification of exosomes. For example, we detected the known exosome markers¹⁴ in the isolated exosomes to study the functions of exosomes.

Immunogold labeling methods have some technical difficulties, including no labeling or high background labeling. When experiencing no labeling, we recommend checking the primary antibody concentration and primary incubation times. In the case of low concentration of primary antibodies, antibody titration may be helpful to find the optimized immunoreactivity. It may also be helpful to increase the incubation times to 4 °C overnight. In contrast, if the concentration of the primary antibody is too high or the incubation time is too long at the high temperature, the background signal will be increased.

Disclosures

The authors have nothing to disclose.

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