Ultrastructural analysis of exosomes by immunolabeling and negative staining

Introduction

Exosomes are 30–150 nm nanovesicles with sophisticated RNA and protein cargo, constantly secreted by all cells *in vitro* and *in vivo*. Exosomes are formed within multivesicular bodies, and after fusion of the multivesicular bodies with the plasma membrane the vesicles are released into the surrounding environment.

Currently, there is no gold standard for identification and characterization of exosomes. Rather than relying on one method for verification, a set of different methods is recommended, including electron microscopy (EM), various instruments for analysis of nanosized objects (e.g., ZetaView™, qNano™, and NanoSight™ systems), and confirmation of protein markers like CD63 by western blot. EM, in combination with immunolabeling and negative staining, can be efficiently used for advanced and extensive analysis of extracellular vesicles, as it allows very accurate determination of the size and shape of the nano-objects and confirms the presence of certain markers. For exosome verification or in-depth analysis, care must be taken when preparing the vesicles for ultrastructural analysis. Here we present a protocol for immunolabeling and negative staining of purified exosomes for downstream EM analysis.

Exosomes, like most biological materials, show little contrast, which is a challenge in terms of visualization and evaluation. In the case of light microscopy, contrast can be enhanced by adding stains that absorb certain wavelengths and enable a colored image. Since resolution is limited by the wavelength of the visible light, EM is required for observation of exosomes—the energy source, in this case electrons, has very short wavelengths, providing the resolution needed for ultrastructural analysis. For more in-depth characterization of the exosomes, immunolabeling can be added to the protocol.

Materials and methods

SW480 cells (American Type Culture Collection) were cultured to confluence in RPMI 1640 medium (10% fetal calf serum, 1 mM sodium pyruvate) in bottles at 37ºC, 5% CO_2. The medium was replaced with 50 mL of fresh medium when cells reached confluency, and after 7 days the conditioned medium. Conditioned medium was harvested and centrifuged twice (300 x *g*, 10 min, 2–8ºC; 2,000 x *g*, 30 min, 2–8ºC) to remove cells and debris. Invitrogen™ Total Exosome Isolation Reagent was added to the medium, which was then incubated at 4ºC overnight and centrifuged (10,000 x *g*, 1 hr, 4ºC) to precipitate exosomes. Purified exosomes were resuspended in PBS. Immunolabeling, negative staining, and EM were performed using glow-discharged carbon-coated copper grids, uranyl acetate, rabbit anti-mouse antibody, and protein A gold (CMC Utrecht, Netherlands). The stained and labeled exosomes were analyzed on a Philips™ CM100 100 kV acceleration voltage electron microscope.

Results

Contrast

The contrast in EM is mainly obtained by electron scattering, as electrons are not absorbed by biological material. Adding an electron-dense stain, however, will increase the contrast. Such staining solutions are usually compounds of heavy metals of high atomic numbers, used in a method termed negative-staining EM. In EM, heavy metal precipitation provides the contrast. If the particle of interest itself is coated with stain (positive staining), fine details may be obscured; therefore, negative staining is often preferred. Negative staining leaves the particle untouched, with the stain molded around the particle and outlining its structures. Only electrons passing through the specimen are involved in the formation of the final image. In positive staining with a solution of a heavy metal salt, the structures will appear dark on a bright background. In negative staining, the object is unstained but is embedded in a dried film of the heavy metal salt. The specimen appears light on a dark background.

Negative staining solutions

Negative staining solutions typically penetrate hydrophilic regions and substitute water. These solutions dry faster than the specimen itself, creating a viscous cap protecting the specimen. The rate of penetration is determined by the size and charge of the staining ion. There are several advantages of using such a protocol: it does not require any protein–stain interactions, it is a simple procedure, the relationship between electron scattering and image intensity is favorable, the negative-staining solutions have high melting points and are stable in the electron beam, and it is frequently used in high-resolution studies of viruses and exosomes. There are several commonly used negative-staining solutions, such as aqueous uranyl acetate (can be used in combination with methyl cellulose), neutral phosphotungstic acid, and ammonium molybdate.

Optimization of negative staining

- Glow discharging of carbon-coated copper grids improves adsorption by making the surface hydrophilic. The drop of preenriched exosomes used can be as small as 5–10 µL. Cover the grids during incubation with a lid to avoid any disturbance of the grid.
- The concentration of exosomes on a grid can be increased by incubating the grid on a drop of exosomes for 10 minutes, removing the grid for a few minutes, and then putting the grid back on the same drop of exosomes. The exosomes will accumulate at the water–air interface and can be picked up by the grid in several steps.
- For double labeling, different sizes of protein A gold are used, such as 5 nm, 10 nm, 15 nm, and 20 nm.
- To obtain the best possible contrast and resolution, several different solutions should be tested.
  - Aqueous uranyl acetate: A 1–3% solution of uranyl acetate dissolved in water can be used to negatively stain many samples. The stain has a low pH, so this solution is not recommended for particles that are unstable in acidic conditions.
  - Neutral phosphotungstic acid: A 1–3% solution of phosphotungstic acid is made in water, and the pH is adjusted to 7 using sodium hydroxide. This is a useful stain for many samples but is especially good for viruses that dissociate at low pH. The stain produces less contrast than uranyl acetate.
  - Ammonium molybdate: A 1% solution of ammonium molybdate is made in water. This solution has also been used to negatively stain thawed thin cryosections of fixed cells.
- Remove excess methyl cellulose and uranyl acetate using filter paper and placing the grid perpendicular to the filter paper. Barely touch the grid to the filter paper, and move the grid until no more methyl cellulose is removed. There should only be a thin layer of methyl cellulose covering the exosomes on the grid. Alternatively, use only uranyl acetate.

Resolution

For microscopes using visible light, the wavelength of the incident light is fixed. It is the light-collecting properties of the lens that determines the resolution of the instrument (Figure 1). However, resolution is limited by the opening angle (half) of the objective (sin 90° = 1) and the refractive index of the immersion medium (n = 1 for air, n = 1.5 for oil). For detailed analysis of exosomes, higher resolution is needed. One way of increasing the resolution is to reduce the wavelength of the incident "light" by using electrons (Figure 1). A shorter wavelength will increase the resolution significantly, enabling ultrastructural analysis of the exosomes. By increasing the voltage, the electrons can be further accelerated and the wavelength reduced, yielding increased resolution (high-resolution EM with 200 kV instruments).

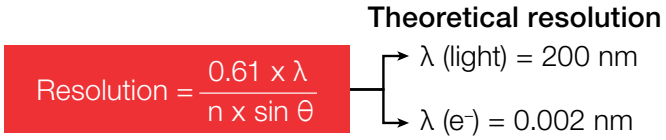

Figure 1. Resolution is determined by the wavelength of light and the numerical aperture (NA) of the lens. NA = n x sin θ, where n is the refractive index of the immersion medium and θ is half the opening angle of the objective.

The workflow

Exosomes can be characterized in detail by performing immunolabeling of the surface markers, followed by EM (Figure 2). The workflow is fast and can be performed at room temperature (RT) on a clean surface (e.g., Parafilm™ wrap). The starting material can be exosomes preenriched by precipitation using the Total Exosome Isolation Reagent, or isolated using an ultracentrifugation protocol. A drop of exosomes is placed on a clean surface. Typically, 5–10 µL of exosome suspension is sufficient. The exosomes will accumulate at the water–air interface and attach to the surface of the copper grid when the grid is applied to the drop. The grid is incubated for 15 min and then blocked with PBS containing 0.5% BSA for 10 min. The grid is then transferred to a drop of primary antibody solution (e.g., CD9 or CD81 antibody) for 30 min. It is extremely important to wash the grid carefully to remove the unbound primary antibody. This is done using 3–4 drops of PBS, one drop at a time, over a total incubation time of 10 min. If the primary antibody is of mouse origin, a bridging antibody (rabbit anti-mouse) is recommended. The grid is incubated on a drop of rabbit anti-mouse antibody solution for 30 min. This step is again followed by extensive washing in PBS (3–4 drops, one drop at a time, over a total of 10 min). Finally, the grid is incubated with protein A gold for 30 min followed by extensive washing in PBS (4 drops for a total of 10 min) and ultrapure water (4 drops for a total of 10 min). The labeled exosomes are now ready for negative staining using 0.3% uranyl acetate and a 15 min incubation.

Figure 2. Workflow for immunolabeling and electron microscopy of exosomes.

Ultrastructural analysis of exosomes

Negative staining of exosomes provides several advantages. It is a very simple procedure that does not require any protein–stain interactions. The relationship between electron-scattering properties and image intensity is very favorable. The melting point of the heavy metal salt is high, and thus the salt is stable in the electron beam. These properties make negative staining suitable for analysis of small nanosized objects such as exosomes. By introducing immunolabeling into this workflow, ultrastructural analysis can be combined with detection of exosomal surface markers such as CD81 (Figure 3). Prior to in-depth analysis, it is critical to optimize the antibody concentration used for detecting exosomal markers. With the correct dilution of antibody, the labeling (gold particles) will appear only on the vesicular structures and will be absent on the rest of the grid surface. Since the structures themselves are not stained, the exosomes will appear light on a dark background, providing more accurate structural information. The exosomes may sometimes appear collapsed, resembling erythrocytes. These exosomes are less well supported by the surrounding fluid, resulting in folding and an apparent increase in size. In addition to structural analysis and identification of exosomal surface proteins (10 nm gold particles, Figure 3), it is also possible to perform stereological analysis, which can provide quantitative information on the number of exosomal proteins on each structure (e.g., number of gold particles per diameter or per area).

Conclusions

The workflow presented here provides a fast and convenient method for detailed examination of exosomes or other extracellular vesicles by EM. By combining EM with simple exosome purification and immunolabeling strategies, detailed analysis of exosome structure and composition can be obtained.

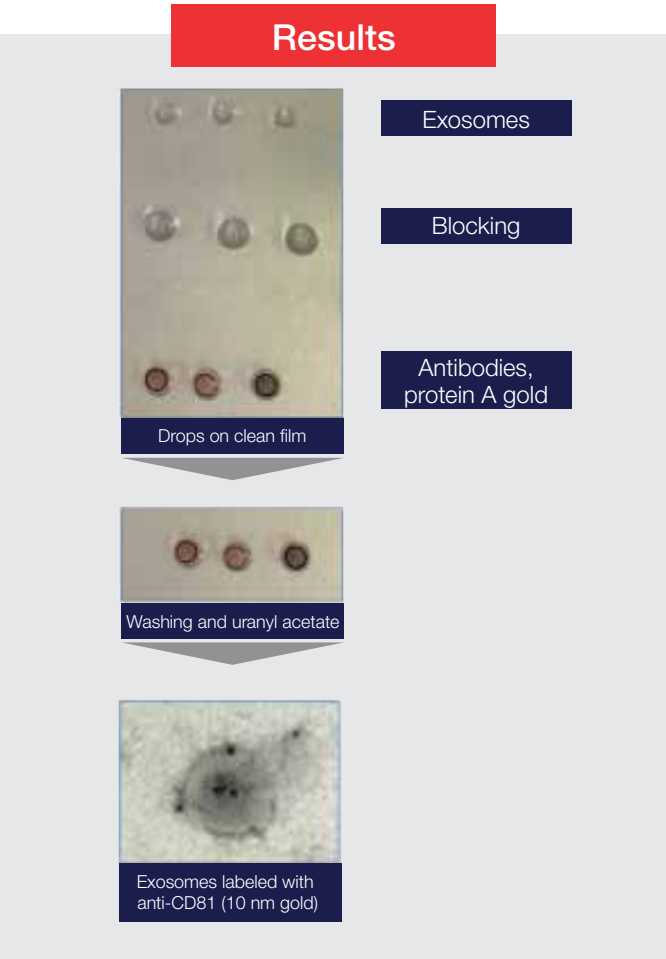

Figure 3. Exosomes derived from SW480 cells and labeled for CD81.

Ordering information

Product	Cat. No.
Total Exosome Isolation Reagent (from cell culture media)	4478359
CD81 Monoclonal Antibody (M38)	10630D
CD63 Monoclonal Antibody (Ts63)	10628D
CD9 Monoclonal Antibody (Ts9)	10626D

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