Chapter 19

Imaging of Isolated Extracellular Vesicles Using Fluorescence Microscopy

Dmitry Ter-Ovanesyan, Emma J. K. Kowal, Aviv Regev, George M. Church, and Emanuele Cocucci

Abstract

High-resolution fluorescence microscopy approaches enable the study of single objects or biological complexes. Single object studies have the general advantage of uncovering heterogeneity that may be hidden during the ensemble averaging which is common in any bulk conventional biochemical analysis. The implementation of single object analysis in the study of extracellular vesicles (EVs) may therefore be used to characterize specific properties of vesicle subsets which would be otherwise undetectable. We present a protocol for staining isolated EVs with a fluorescent lipid dye and attaching them onto a glass slide in preparation for imaging with total internal reflection fluorescence microscopy (TIRF-M) or other high-resolution microscopy techniques.

Key words Exosomes, Extracellular vesicles, EVs, Imaging, Microscopy, TIRF

1 Introduction

Over the last decade, interest in extracellular vesicles (EVs) has significantly grown due to their role in normal physiology, as well as pathological processes. EVs are thought to signal with recipient cells by interaction with their surface ligands or by transferring their contents (such as RNA or protein) into the recipient cell's cytoplasm [1]. Furthermore, as EVs are secreted into all biological fluids, they also hold great promise as novel biomarkers [2].

One of the major challenges in understanding the role of EVs in intercellular communication is characterizing the composition of individual EVs. When EVs are isolated in bulk, information regarding EV heterogeneity is lost. Existing EV isolation techni-

Dmitry Ter-Ovanesyan and Emma J.K. Kowal contributed equally to this work.

Winston Patrick Kuo and Shidong Jia (eds.), *Extracellular Vesicles: Methods and Protocols*, Methods in Molecular Biology, vol. 1660, DOI 10.1007/978-1-4939-7253-1_19,

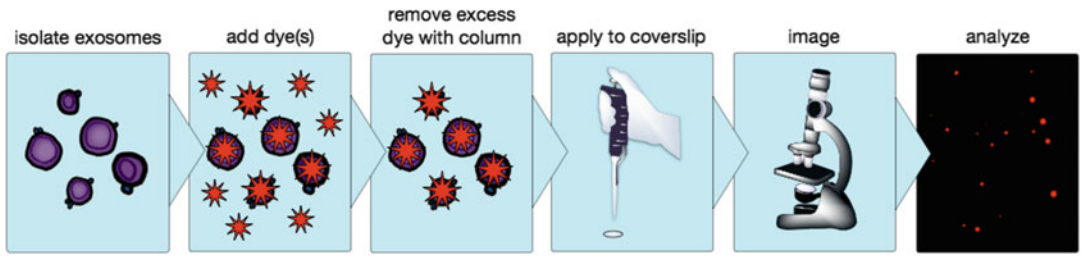

Fig. 1 Conceptual overview of the protocol. Schematic of EV staining and imaging procedure

ques, such as ultracentrifugation [3], result in a diverse mixture of vesicles from different cellular compartments [4] and are often contaminated by nonvesicular components such as extracellular protein complexes [5].

In recent years, high-resolution imaging has contributed greatly to the understanding of cell biology, permitting the detailed visualization of complex processes such as endocytosis [6], or the composition of single objects such as viruses [7]. In particular, total internal reflection fluorescence microscopy (TIRF-M) has been widely applied since it permits a high signal to noise ratio, the detection of single molecules, and a facile calibration of measurements to the single molecule level. These approaches can be efficiently applied to the study of EVs and identify different subpopulation of EVs present in a given sample. Although flow cytometry-based methods are able to characterize EV subpopulations, using flow cytometry for EV detection presents many technical challenges [8, 9] and is much less sensitive than TIRF-M.

Here, we provide a protocol for imaging isolated EVs by fluorescence microscopy. Although we have found TIRF-M to be best suited for these studies, other microscopes such as confocal [10, 11] and STED microscopes [12] can be used as well. We provide detailed instructions for staining EVs with a fluorescent membrane dye and attaching them to a glass slide (Fig. 1). This basic approach can be easily combined with other more specific staining, such as antibodies or nucleic acid dyes to study the heterogeneity of EV composition. Though colocalization does not strictly indicate that a given molecule is encapsulated within the lipid membrane, since it could also be adhered to the outside of the vesicle, use of this technique after various enzymatic treatments could provide this information. Additionally, EVs isolated from cells expressing fluorescent fusion proteins can be visualized using high-resolution microscopy [10, 11]. In conclusion, the high sensitivity of TIRF-M and its ability to make quantitative measurements provides a highly promising approach to study EV composition, as well as other diffraction-limited objects such as viruses or nanoparticles.

2 Materials

Store all materials at room temperature unless otherwise stated.

1. Glass coverslips. # 1.5 round 25 mm glass coverslips (Warner Instruments).
2. Coverslip rack. Teflon rack to vertically hold glass coverslips during cleaning procedures (C-14784, Thermo Fisher Scientific).
3. Coverslip holder for imaging. Attofluor Cell Chamber (A-7816, Thermo Fisher Scientific).
4. Total Internal Reflection Microscopy System. The imaging setup described here is composed of a Zeiss Axio Imager Z2 microscope equipped with a 63× Oil immersion objective (1.46 NA, Carl Zeiss) and a TIRF slider with manual angle and focus controls (Carl Zeiss). The illumination is supplied by solid state and argon ion lasers at 405 nm, 458/488/514 nm, 561 nm and 639 nm (power ~ 2–5 mW at the objective depending on laser). The excitation light is coupled through an acousto-optical tunable filter into a single mode optical fiber, carried to the TIRF slider and reflected into the objective using a single- or multi-band dichroic mirror (various; Semrock). The emission light is collected by the objective, passes the dichroic mirror and a single- or multi-band emission filter (various; Semrock), and is projected onto an electron multiplying charge-coupled device (EMCCD) camera (ImagEM-1K BackThinned EMCCD, Hamamatsu) for a final pixel size of 206 nm. Microscope operation and image acquisition are controlled by the Zen Blue software (Carl Zeiss). Any equivalent setup is suitable for the experiments described in this chapter.
5. EVs (*see* **Note 1**) isolated fresh, stored at 4 °C, or thawed from storage at −80 °C (*see* **Note 2**).
6. Vybrant DiD Cell-Labeling Solution (V-22887, Thermo Fisher Scientific).
7. Other fluorescent dyes (*see* **Note 3**) stored according to respective manufacturer recommendations.
8. Diluted dye stocks (*see* **Note 4**) stored according to respective manufacturer recommendations. For DiD, make a 50 nM stock in DMSO and store at room temperature in the dark.
9. EV Spin Columns, MW 3000 (4484449, Thermo Fisher Scientific) (*see* **Note 5**).
10. Fluorescent beads (*see* **Note 6**). 0.1 μm carboxylated beads (Ex/Em: 505/515 nm; F-8803, Thermo Fisher Scientific) stored at 4 °C.

11. Fluorescent bead solution. Dilute the fluorescent beads (2.10) to obtain ~4x10^7 beads in 100 μL of PBS, which according to the manufacturer (*see* 2.10) corresponds to a 10^5-fold dilution. To do this, prepare two consecutive dilutions, first 1:1000 to obtain a 1 mL stock solution, then 1:100 to obtain the working solution. Store all bead solutions at 4 °C or on ice during the experiment.
12. Bottle-top filter unit. Reusable bottle top filter unit (DS0320–5033, Nalgene) with removable membrane of 0.2 μm pore size for vacuum filtration.
13. 100% Ethanol. Filter 1 L of denatured ethanol (9401–03, JT Baker) into a bottle using a vacuum-based bottle-top filter unit (2.10). Fill a glass jar with the filtered ethanol such that it will entirely cover a rack loaded with glass coverslips (2.1 and 2.2).
14. Dulbecco's Phosphate Buffer Saline, no calcium, no magnesium (PBS, Thermo Fisher Scientific).

3 Methods

3.1 Clean Glass Coverslip Preparation

1. Place coverslips in rack (one per sample to be imaged, plus controls; *see* **Note 7**) and submerge rack in filtered 100% ethanol.
2. Sonicate for 20–30 min on low power.
3. Remove rack from ethanol and, without touching coverslips, adsorb excess ethanol by placing rack onto a Kimwipes or other tissue.
4. Place the rack with coverslips in 120 °C oven until dry; a lower temperature is acceptable but will take longer.
5. Immediately before applying sample to be imaged, expose the rack of coverslips to air plasma inside a glow discharge unit operated at 50 mA for 2 min.

3.2 Sample Preparation

1. Prepare one clean 1.5 mL tube of 50 μL PBS (*see* **Note 8**).
2. Prepare one tube of 50 μL EVs. If you are testing other dyes in addition to DiD, prepare one tube of EVs per dye and dye combination to be tested (*see* **Note 7** on experimental design).
3. Add 1 μL of 50 nM DiD stock to PBS and to EVs. If using other dyes, add to appropriate tubes, making sure to add all dyes in use to control PBS tube.
4. Tap or gently vortex tubes to mix.
5. Incubate all samples for 20 min at 37 °C in the dark.
6. While incubating, prepare spin columns (*see* **Note 5**) according to manufacturer instructions [13], reproduced here:

7. Tap the column to settle the dry gel into the bottom of the spin column.
8. Hydrate the column with 650 μL PBS.
9. Cap, vortex, tap out air bubbles, and leave at RT 5–15 min.
10. Place columns in 2 mL collection tubes and spin at 750 RCF for 2 min at RT. Keep track of the orientation of the column in the rotor.
11. Discard collection tube and immediately apply the EV sample directly to the center of the gel bed at the top of the column IMPORTANT! Do not disturb the gel surface or contact the sides of the column with the pipette tip or reaction mixture.
12. Place column in 1.5 mL elution tube and place in rotor, maintaining same orientation as previous spin.
13. Spin at 750 RCF for 2 min at RT.
14. Discard column, retrieve EV sample from tube.
15. Samples can be imaged immediately or stored at 4 °C in the dark for up to 24 h.

3.3 Sample Addition to Coverslips and Imaging

1. Tape a clean strip of Parafilm onto bench and carefully lay down coverslip(s).
2. If using beads to visualize the focal plane (*see* **Note 6**), add 1 μL working fluorescent bead solution (*see* 2.1.11) to sample and invert several times to mix.
3. Add 50–100 μL sample to each coverslip and wait for 5 min for EVs to adsorb. Monitor the coverslips to ensure that they do not dry out completely, as this will create salt crystals which will interfere with imaging. If necessary, add several drops of PBS. (*see also* **Note 8**).
4. Using tweezers, turn the coverslip on its side and dab against Kimwipes or other tissue to wick off excess liquid, again without drying completely.
5. Place the coverslip carefully into bottom of holder with sample oriented up and make sure it is centered (tap around edges with tweezers, it should not lift on opposite side; *see* **Note 9**) before screwing holder together.
6. Gently add 1 mL PBS directly to coverslip.

3.4 Imaging

1. Place the holder with coverslip and PBS on the microscope stage.
2. Find the focal plane (*see* **Note 6**). In the DiD-stained EV sample, you should see a distribution of diffraction-limited punctae across the field of view (as in Fig. 2, right panel).

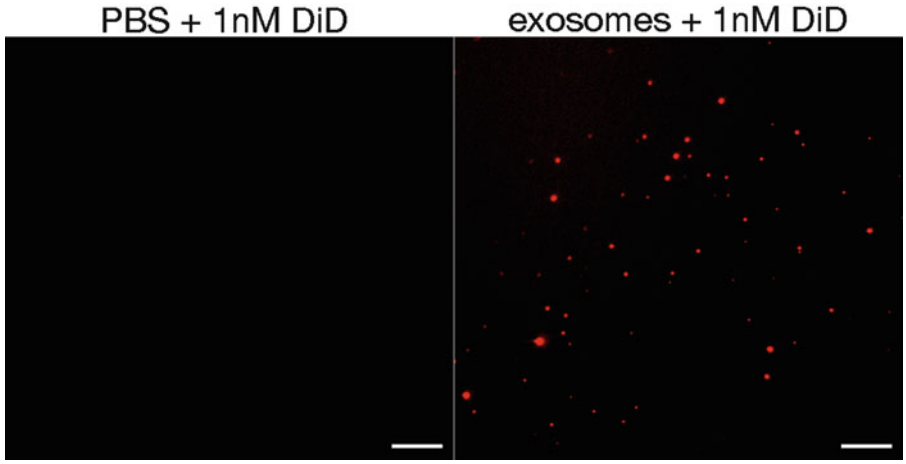

Fig. 2 Imaging of DiD-labeled extracellular vesicles. K562 EVs and PBS control incubated with 1 nM DiD, then imaged in far-red (Em filter 688/48). No signal is visible in the PBS slide while the EVs are clearly visible above background. Images shown are representative of three coverslips, twenty fields of view, in each case. (*Scale bars* = 10 μm)

3. Acquire several images in each channel of interest. Ensure that the PBS + dyes control sample is devoid of signal in each channel (as in Fig. 2, left panel).
4. For colocalization studies, ensure that vesicles labeled with one dye alone do not give signal in other channels (as in Fig. 3, bottom panels) and use caution when interpreting results (*see* **Note 10**).

4 Notes

1. EVs can be derived from any source of interest, i.e., cell culture or biological fluids. We find that EVs collected by standard ultracentrifugation protocols [3] from 250 mL of 50% confluent K562 cells over 24 h are sufficient to cover 50 coverslips with a reasonable concentration of particles/field.
2. EVs stored at 4 °C for under a week look similar to freshly isolated vesicles. For storage at −80 °C we recommend the addition of 20 mM HEPES buffer to stabilize the pH at ~7.4 over freeze-thaw cycles.
3. This protocol specifically outlines use of a lipid dye (DiD) to label EV membranes, but it can be used in combination with other dyes to label EV protein and RNA, or with transgenic cell lines to label specific molecules of interest [10, 11]. DiD in particular is conveniently combined with other probes as it emits in far-red, minimizing chance of bleed-through into other commonly used channels. Based on our tests, the spin

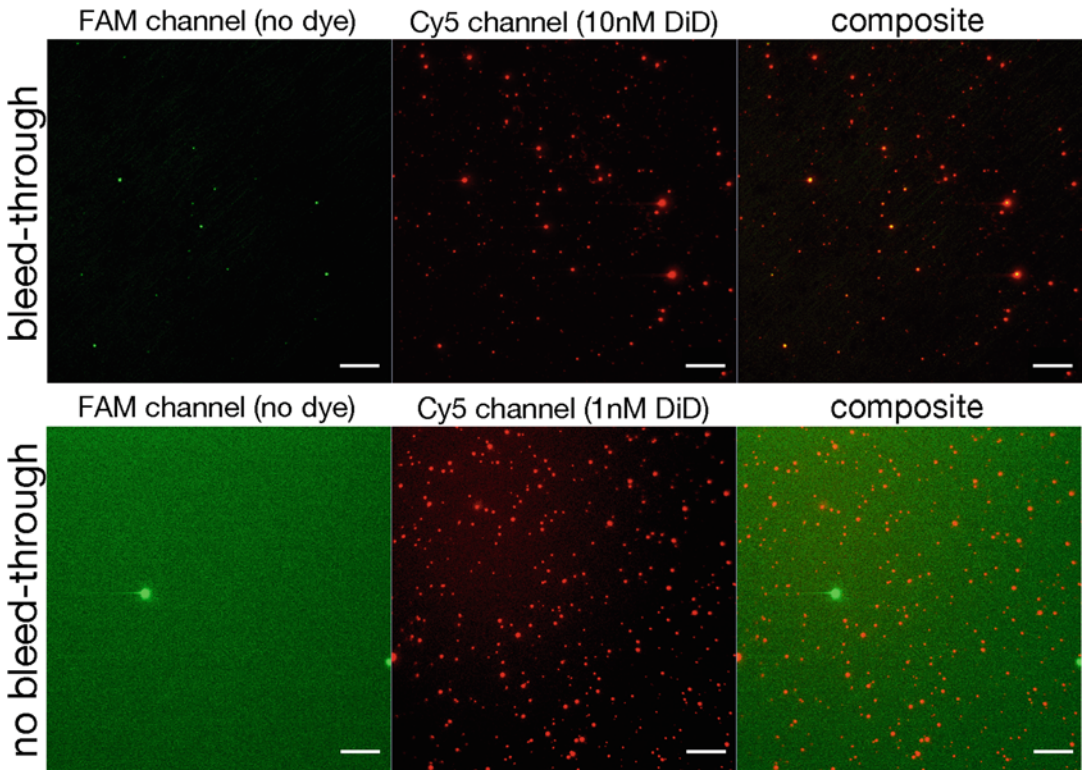

Fig. 3 Imaging of DiD-labeled EVs together with fluorescent beads. Distinguishing EV signal from bleed-through. *Top*: K562 EV sample demonstrating bleed-through between channels. *Left*: green (Em filter 525/50), *Center*: far-red (Em filter 688/48), *Right*: merge. EVs stained with 10 nM DiD appear to colocalize with objects in the green channel (emission filter) despite absence of green fluorophores in the sample. *Bottom*: K562 EV sample demonstrating no bleed-through between channels. *Left*: green (Em filter 525/50), *Center*: far-red (Em filter 688/48), *Right*: merge. Decreasing the concentration of DiD to 1 nM in the staining relieves bleed-through, while GFP-coated beads are still visible. Green channel is deliberately overexposed to demonstrate absence of any signal from DiD-stained objects. Images shown are representative of three coverslips, twenty fields of view, in each case. (*Scale bars* = 10 μm)

columns (2.1.9) did not effectively clear unbound fluorescent antibodies. When using multiple dyes or labeling strategies it is important to perform controls outlined in **Note 7**.

4. We recommend making dye stocks at a concentration equal to the desired final concentration times the volume of the sample, such that each dye can be provided to each sample in 1 μL of stock solution—for example, use a 150 nM stock of DiD with 30 μL EV samples for a final concentration of 5 nM. This is both for convenience and to avoid disrupting vesicle membranes with high concentrations of stock solvent.
5. The purpose of these spin columns is to remove contaminating fluorescent components from the dyed sample (i.e., free or aggregated dye, which should be in complexes smaller than

3 kDa). We found that the spin columns were more effective than dialysis and more convenient than ultracentrifugation.

6. It can be difficult to find the correct focal plane while imaging using signal from the vesicles alone, especially for initial experiments, and so we recommend the use of beads (with Ex/Em spectra distinct from any dyes in use) to aid in finding the correct plane. This is particularly crucial for control experiments with no labeled vesicles (*see* **Note 8**).
7. As EVs are diffraction-limited objects and this technique is very sensitive, there are many potential sources of false positive signal. It is important that for a given set of parameters (EV and dye concentration, laser power, TIRF angle, gain and exposure time) a number of controls is performed, and several images are taken across the coverslip in each channel of interest and at the correct focal plane (*see* **Note 9**). A sample of PBS and dyes only (no EVs) should be prepared and imaged to ensure that the dye itself is not forming micelles or aggregates which escape the spin column, as these would appear as fluorescent punctae indistinguishable from EVs (*see* Fig. 2, left vs. right). If objects are seen in this control, potential fixes are: decreasing the concentration of dye, cleaning and handling coverslips meticulously, filtering PBS to ensure it is free of contaminants, loading the spin column without disturbing the gel bed, or centrifuging dye stocks at high speed to remove precipitates. For colocalization experiments, a separate aliquot of EVs should be labeled with each dye alone and imaged to ensure that there is no bleed-through of fluorescence from the expected channel into another (*see* Fig. 3, top vs. bottom). If bleed-through is seen, exposure time may be lowered in the channel where bleed-through is observed or the concentration of the dye is decreased. Another potential solution is finding a narrower emission band filter for that channel.
8. The manufacturer's protocol [13] for the EV Spin Columns suggests a sample volume of 20–100 μL, but in our hands, samples lower than 40 μL applied to the column do not consistently elute at input volume and often come out 50% more dilute (i.e., 20 μL input yields 30 μL or more stained sample). This should be taken into account when considering desired final sample concentration for imaging.
9. If the coverslip is not flat it will be difficult to keep the focal plane while panning across the sample. If repeated adjustments of the coverslip in the holder do not fix a tilted sample, check that the microscope stage is flat.
10. The diffraction of light limits the resolution of imaging instruments. Therefore objects smaller than the diffraction limit, which approximately corresponds to half of the emitted

wavelength in a fluorescent sample, corresponds to the point spread function of the imaging system. Since a large fraction of EVs is smaller than the diffraction limit (~250 nm) more than one vesicle might be present in a single detected point spread function. Thus, this technique cannot strictly distinguish between one vesicle and two vesicles stuck together. Furthermore, as small EVs are diffraction-limited objects, colocalization does not strictly indicate that a given molecule is encapsulated within the lipid membrane, since it could also be adhered to the outside of the vesicle. Use of this technique in concert with enzymatic protection assays would be necessary to elucidate the contents of EVs.

Acknowledgment

This work was supported by US National Institutes of Health National Human Genome Research Institute Grant P50 HG005550, The Ohio State Cancer Center (P30-CA016058), and an intramural fund (IRP46050-502339) granted to EC.

References

1. Tkach M, Thery C (2016) Communication by extracellular vesicles: where we are and where we need to go. Cell 164(6):1226–1232
2. Revenfeld AL, Baek R, Nielsen MH, Stensballe A, Varming K, Jorgensen M (2014) Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. Clin Ther 36 (6):830–846
3. Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol Chapter 3:Unit 3.22
4. Cocucci E, Meldolesi J (2015) Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends Cell Biol 25 (6):364–372
5. Cvjetkovic A, Lotvall J, Lasser C (2014) The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. J Extracell Vesicles 3
6. Cocucci E, Aguet F, Boulant S, Kirchhausen T (2012) The first five seconds in the life of a clathrin-coated pit. Cell 150(3):495–507
7. Chou YY, Vafabakhsh R, Doganay S, Gao Q, Ha T, Palese P (2012) One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. Proc Natl Acad Sci U S A 109(23):9101–9106
8. van der Pol E, Coumans F, Varga Z, Krumrey M, Nieuwland R (2013) Innovation in detection of microparticles and exosomes. J Thromb Haemost 11(Suppl 1):36–45
9. Nolan JP, Moore J (2016) Extracellular vesicles: great potential, many challenges. Cytometry B Clin Cytom 90:324–325
10. Fang Y, Wu N, Gan X, Yan W, Morrell JC, Gould SJ (2007) Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biol 5(6):e158
11. Lai CP, Kim EY, Badr CE, Weissleder R, Mempel TR, Tannous BA, Breakefield XO (2015) Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. Nat Commun 6:7029
12. Koliha N, Wiencek Y, Heider U, Jungst C, Kladt N, Krauthauser S, Johnston IC, Bosio A, Schauss A, Wild S (2016) A novel multiplex bead-based platform highlights the diversity of extracellular vesicles. J Extracell Vesicles 5:29975
13. Exosome Spin Columns. https://tools.thermofisher.com/content/sfs/manuals/MAN0008464_Rev01_PI_08Aug2013.pdf. Accessed 4 Apr 2016