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Antigen-specific staining of EV markers with fluorochrome-conjugated antibodies

Aizea Morales-Kastresana[1], Joshua A Welsh[1], Jennifer Jones[1]

[1]Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

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Translational Nanobiology Section

Joshua Welsh

ABSTRACT

The use of antibodies for EV staining and analysis using flow cytometry poses challenges due to the relatively large size of antibodies and fluorochrome-conjugates in comparison to the majority of EVs diameter. A conventional IgG measures ~3 x 11 nm, not including the conjugated fluorochrome(s) (Reth, 2013). Antibodies conjugated to fluorescent proteins such as PE and APC (250 and 105 kDa, respectively) are considerably bigger. Furthermore, it is known that commercial antibody preparations contain some aggregates that can be misidentified as positive events if not removed (Gagnon & Beam, 2009). Therefore, choosing a method that efficiently eliminates antibody aggregates and discriminates labeled EVs from unbound antibody conjugates is crucial. Some groups have reported successful unbound antibody removal after gradient ultracentrifugation in sucrose or iodixanol or repetitive ultracentrifugation (Groot Kormelink et al., 2016; Higginbotham et al., 2016; van der Vlist et al., 2012). In this protocol, we use high-speed centrifugation to remove antibody aggregates and size exclusion chromatography (SEC) to wash the unbound antibody after staining.

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Detection and Sorting of Extracellular Vesicles and Viruses using nanoFACS

MATERIALS TEXT

Reagents

•Ca2+ Mg2+-free Dulbecco's Phosphate Buffer Saline (DPBS, Thermo Fisher Scientific)
•Anti-MHCII-PE antibody (Biolegend, Cat. 107608)
•Fc Block (anti-CD32) (BD Pharmingen, Cat. 553142)
•10 mL qEV-Original (or Sepharose-2B) size exclusion chromatography columns (Izon Bioscience)
•EV sample in DPBS with a measured concentration (particles/ml)

Hardware:

•Round-bottom 2 ml microfuge tubes (Eppendorf)
•Rack or equivalent to hold the chromatography columns
•Micropipettor and tips
•Rack or similar to hold the chromatography columns
•Micropipettor and clean tips
•Airfuge (Beckman Coulter, Cat. 347854)
•A100/18 rotor (Beckman Coulter, Cat. 347593)
•Plastic rotor lids for A100/18 rotor (Beckman Coulter, Cat. 339643)
•Thin wall polypropylene tubes for A100/18 rotor (Beckman Coulter, Cat. 342630)
•Gentle mixer
•Sharp tweezers

DISCLAIMER:

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

ABSTRACT

The use of antibodies for EV staining and analysis using flow cytometry poses challenges due to the relatively large size of antibodies and fluorochrome-conjugates in comparison to the majority of EVs diameter. A conventional IgG measures ~3 x 11 nm, not including the conjugated fluorochrome(s) (Reth, 2013). Antibodies conjugated to fluorescent proteins such as PE and APC (250 and 105 kDa, respectively) are considerably bigger. Furthermore, it is known that commercial antibody preparations contain some aggregates that can be misidentified as positive events if not removed (Gagnon & Beam, 2009). Therefore, choosing a method that efficiently eliminates antibody aggregates and discriminates labeled EVs from unbound antibody conjugates is crucial. Some groups have reported successful unbound antibody removal after gradient ultracentrifugation in sucrose or iodixanol or repetitive ultracentrifugation (Groot Kormelink et al., 2016; Higginbotham et al., 2016; van der Vlist et al., 2012). In this protocol, we use high-speed centrifugation to remove antibody aggregates and size exclusion chromatography (SEC) to wash the unbound antibody after staining.

1 Wash one qEV column per EV preparation with 20 mL of DPBS. Never allow the columns to become dry.

2 Pipette 1x10^9EVs in a 10 µL volume of DPBS and add 2 µg of Fc Block reagent to block Fc receptors. Incubate with no agitation for 10 minutes at room temperature.

Note: *The presence of Fc receptors on EVs is not well documented. However, adding Fc Block will not only block putative Fc receptors, but also serves as a source of protein to block other non-specific binding sites of fluorescent antibodies.*

3 In a 1.7 mL microfuge tube, pipet 1.5 µg of fluorochrome-conjugated antibody and add DPBS to a finale volume of 120

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µL per sample. Mix by pipetting up and down. Prepare a master mix if multiple samples are to be stained with the same antibody.

Note: This antibody quantity is a reference starting point when testing a new antibody. Antibody titration is recommended to achieve optimal staining and avoid the use of unnecessary material. Many anti-human antibodies are provided in a test volume format (µL per test) rather than in concentration (µg mL^{-1}).

4 Transfer the 120 µL of the antibody solution to an airfuge tube and mark one side of the tube with a waterproof marker. Place the tube with a corresponding balance into an A100/18 rotor, with the mark facing up. Place a lid on the rotor.

Note: The mark is a reference for the location of the antibody aggregates after airfuging. Using the rotor cover can reduce sample evaporation during centrifugation.

5 Place the rotor into the airfuge and close the airfuge lid tightly. Open the air source to centrifuge until the gauge reads 22 psi (~130,000 RCF) and leave for 5 minutes.

Note: In order to avoid extreme heat during centrifugation, airfuge step can be performed in a cold room. Alternatively, the authors cool down the rotor before using it.

6 When the airfuge step is complete, use sharp tweezers to remove the tubes from the rotor and place them on the corresponding rack.

7 Gently pipet off the top 70 µL of solution and add it on top of the EV solution.

Note: Antibody aggregate pellets cannot always be observed. For that reason, leaving a reasonable volume in the bottom of the tube and using the top part of the solution is recommended.

8 Incubate the EVs with antibody 15-30 minutes in the dark at room temperature whilst being gently agitated.

Note: As with CFSE, time is a parameter that can be modulated to increase the labeling with antibodies. The authors have observed slight improvements of staining with certain epitopes when increasing the staining period up to 1 hour.

9 Prepare collection tubes for 12 fractions. To facilitate the visualization of eluted sample, use a marker to draw a line indicating the volume of each fraction (500 µL) on the side of the collection tubes.

10 Add DPBS to the EV prep to a final volume of 500 µL and proceed to remove unbound antibody using SEC with qEV columns. Samples that are not going to be immediately loaded on the columns can be stored at in the dark at 4ºC.

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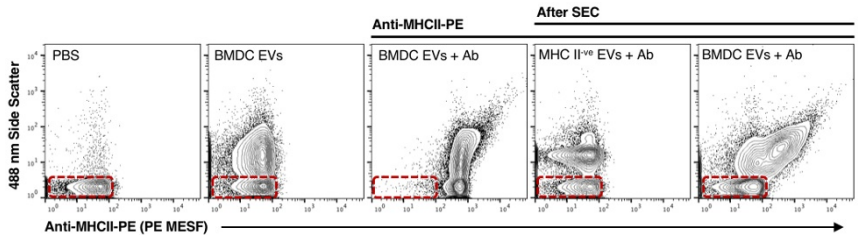
11 Wait until all of the DPBS used for pre-washing the column has entered the column bed. Immediately load 500 µL of the sample and simultaneously start collecting 500 µL fractions.

12 Keep adding DPBS (500 µL each time) and whilst collecting fractions. Stained EVs will start eluting in fraction 7, with the majority in fractions 8-9. For maximum recovery, harvest fraction 10 too.

13 Store EVs at 4ºC and in the dark until performing flow cytometric analysis. Alternatively, some antibody-fluorochrome conjugates can resist one freeze/thaw cycle and therefore, labeled EVs can be stored at -80ºC if being analyzed at a later date.

14 qEV columns can be stored at 4ºC and reused with extensive washing. Authors recommend washing them with a minimum 50 ml of DPBS, to elute as much remaining antibody as possible, followed by 10 ml of 20% ethanol diluted in DPBS to keep the columns aseptic during storage. When reusing a column, wash 40 ml of DPBS to make sure that any traces of ethanol are removed.

15 Proceed to analysis.



Representative contour plots show PBS, unstained, and MHCII-stained BMDC EV (before and after SEC) and control DC2.4 EVs that lack of MHCII on their surface. Red box indicates system reference noise.

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