Oct 23, 2020

Counting particles and EVs on Astrios EQ with spike in reference beads

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1 Works for me dx.doi.org/10.17504/protocols.io.bj6rkrd6

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ABSTRACT

There are different methods to quantify the particle count in a solution. Common counting methods are resistive pulse sensing (RPS) and nanoparticle tracking analysis (NTA). RPS uses the Coulter principle to derive size from the measurable changes in electrical impedance produced by nonconductive particles suspended in an electrolyte. NTA uses tracks the movement of particles by Brownian motion allowing the calculation of their diameter using the Stokes-Einstein equation. Concentration can also be calculated by using the number of particles tracked per detection window volume. This technique requires the use of a specific instrument that may not be available to the broad scientific community on regular basis. Here, we describe an alternative method for counting particles in a solution, where spherical polystyrene fluorescent beads of known concentration are used as spike in reference materials. This method however depends upon the accuracy of the bead stock concentration.

DOI

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PROTOCOL CITATION

Aizea Morales-Kastresana, Joshua A Welsh, Jennifer Jones 2020. Counting particles and EVs on Astrios EQ with spike in reference beads. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bj6rkrd6

KEYWORDS

Astrios EQ, jet-in-air, small particle, flow cytometry, extracellular vesicles, flow virometry, nanoFACS

CREATED

Aug 22, 2020

LAST MODIFIED

Oct 23, 2020

PROTOCOL INTEGER ID

40881

PARENT PROTOCOLS

In steps of

Detection and Sorting of Extracellular Vesicles and Viruses using nanoFACS

MATERIALS TEXT

Reagents:

- 200 nm fluorescent beads: e.g. red (Thermo Fisher Scientific, Cata. F8794) or yellow-green (Thermo Fisher Scientific Cat. F8811)
- EVs in DPBS
- $Ca^{2+}Mg^{2+}$-free Dulbecco's Phosphate Buffer Saline (DPBS, Gibco)
- FACS Clean
- FACS Rinse

Hardware:

- FACS polystyrene tubes
- Orthogonal EV particle counter e.g. resistive pulse sensing device or nanoparticle tracking analysis device.

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

There are different methods to quantify the particle count in a solution. Common counting methods are resistive pulse sensing (RPS) and nanoparticle tracking analysis (NTA). RPS uses the Coulter principle to derive size from the measurable changes in electrical impedance produced by nonconductive particles suspended in an electrolyte. NTA uses tracks the movement of particles by Brownian motion allowing the calculation of their diameter using the Stokes-Einstein equation. Concentration can also be calculated by using the number of particles tracked per detection window volume. This technique requires the use of a specific instrument that may not be available to the broad scientific community on regular basis. Here, we describe an alternative method for counting particles in a solution, where spherical polystyrene fluorescent beads of known concentration are used as spike in reference materials. This method however depends upon the accuracy of the bead stock concentration.

1 Prepare a stock of 200 nm polystyrene reference beads by diluting 50 µL of Fluosphere beads in 50 ml of DPBS (1000-fold dilution) and keep this solution as a '*Big Stock'*. Determine the particle concentration of the beads in the '*Big Stock'*using NTA or any other method.

Companies provide an estimated concentration of beads in solution, based on the size and weight. This tends to be a rough estimation, we therefore recommend preparing a 10,000x dilution of 'Big Stock' beads in DPBS to perform quantification of particles per ml. Polystyrene beads are stable in DPBS, with no significant loss of particles and fluorescence over time. Polystyrene particles can therefore be quantified once and used in the next couple of years. As a reference, the authors' 'Big Stock' of 200 nm yellow-green beads listed in the Material section is at ~1.8 x 10^{10} particles/ml, measured by NTA. It is worth noting that every time an aliquot is taken from the 'Big Stock' its concentration will likely decrease. It is therefore recommended that several smaller 'Big Stock' aliquots to ensure better consistency over long periods of time.

2 Vortex the bead stock solution for 5 seconds.

3 Prepare a working stock of beads by diluting them 100-fold in DPBS (~1.8 x 108 particles/ml). Keep this working stock for the whole experiment.

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4 Prepare EVs in a known volume of DPBS and annotate the dilution, if any. Note that if coming from Basic Protocols 1 and 2, expect some EV loss during the protocol and dilute accordingly.

5 Vortex the working stock of beads for 5 seconds.

6 Spike the beads by diluting them 100-fold in the EV prep. The final concentration of reference beads, if following previous example, will be 1.8 x 106 particles/ml. Mix the sample.

7 Run the EV sample containing the spiked reference beads as described in Basic Protocol 4.

200 nm polystyrene beads may overlap in scatter with some EVs. The authors therefore use 200 nm yellow-green beads if EVs are stained PE or APC, or 200 nm red beads if EVs are stained with CFSE or FITC. This allows differentiation of the EV and bead populations more easily and will provide a more reliable count.

8 Record the EV and bead event count as shown.

The detectable concentration is dependent upon the limit of detection of the instrument and how the gating strategy was defined. In accordance with the MIFlowCyt-EV framework, parameters being used to gate EVs and the threshold of the instrument should be calibrated into standard units to allow the detectable concentration to be defined in standard units that can be validated(Welsh et al., 2020). See dx.doi.org/10.17504/protocols.io.bjcqkivw *for information on how to calibrate fluorescence and light scatter parameters into standard units.*

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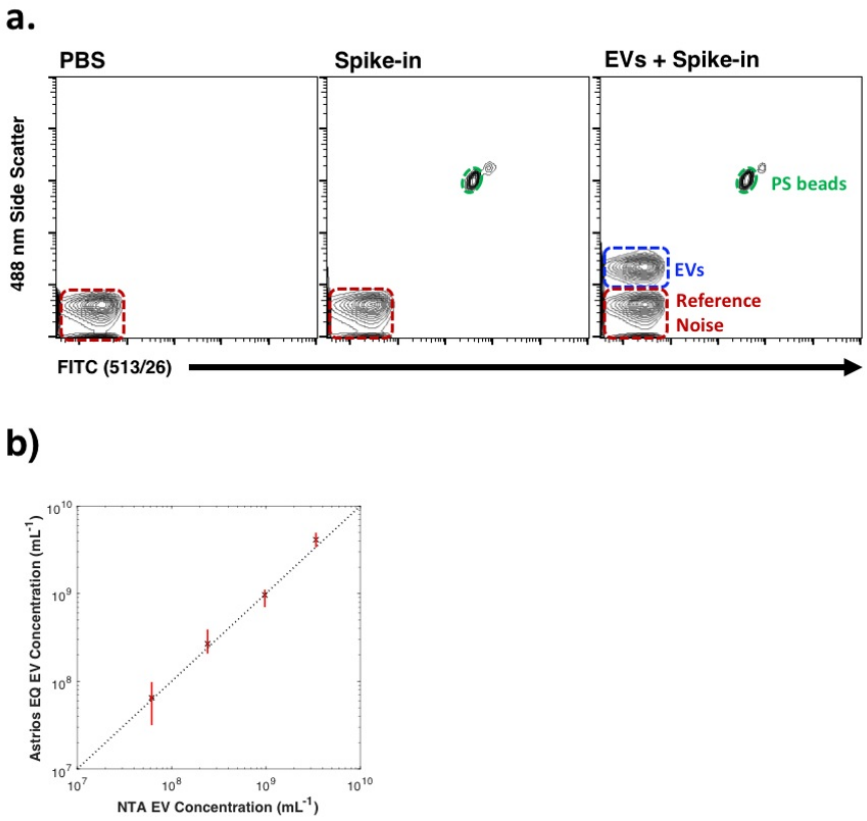

EV concentration of a solution can be calculated using beads as spike in references. a) nanoFACS plots depicting FSC and SSC signals in the 488 nm laser channel for control PBS, 200 nm polystyrene spike in beads and DC2.4 EVs + spike in beads. b) Determined concentration of DC2.4 EVs from nanoparticle tracking analysis and Astrios EV using calculated based on reference bead counts. Experiments were performed in triplicate (median ± 25th, 75thpercentile plotted for NTA and Astrios EQ data).

9 Multiply the EV count by the bead concentration before dividing by the bead count, as shown in the following **equation:**

$$Detectable\ EV\ Concentration = \frac{(EV\ Gate\ Count \times Bead\ concentration)}{(Bead\ Gate\ Count)}$$

Note: If there is any noise or background source in the EV gate, these background counts must the subtracted to approximate EV counts. A clear example of this background would be any events that we get when running clean PBS. In that case use ***equation****:*

$$Detectable\ EV\ Concentration = \frac{(\ (EV\ Gate\ Count - Background\ Count}{(Bead\ Gate\ Cou}$$

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