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Enumeration of extracellular vesicles by a new improved flow cytometric method is comparable to fluorescence mode nanoparticle tracking analysis

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Abstract

Extracellular vesicles (EVs) play a role in a variety of physiological and pathological processes. However, use of EVs as biomarkers has been hampered by limitations of current detection and enumeration methods. We compared fluorescence-threshold flow cytometry (FT-FC) to nanoparticle tracking analysis (NTA) for enumeration of cell culture-derived EVs. FT-FC and NTA utilising fluorescence mode (F-NTA) enumerated similar numbers of EVs stained with a membrane dye PKH67. Both methods were sufficiently sensitive to detect cell-derived EVs above the background of culture medium. NTA in light scatter mode (LS-NTA) detected 10-100× more particles than either fluorescence-based method but demonstrated poor specificity. F-NTA appeared to have better sensitivity for <100 nm vesicles, however, the FT-FC method combined direct enumeration of EVs with high sensitivity and specificity in the >100 nm range. Due to wider availability and higher degree of automation and standardisation, FT-FC is a reasonable surrogate to emerging F-NTA for quantification of EVs.

Key words: Extracellular vesicles; Flow cytometry; Nanoparticle tracking analysis; PKH67; Enumeration

Introduction

The study of extracellular vesicles (EVs) is a rapidly growing field in biology and medicine. EVs are small (<1 μm in diameter) bilipid membrane bound vesicles including exosomes, ectosomes, microvesicles, microparticles and apoptotic bodies among other vesicle fractions, which can be released into blood and other body fluids potentially by all cell types during cell activation, intercellular interaction and cell death.[1] Attempts to classify these heterogeneous vesicles by size or biogenesis have not been universally accepted and many questions concerning biogenesis, transport and other processes remain unanswered. Similarly, there is no consensus on markers that identify the origin of vesicles once outside their cell of origin. For example, exosomes have traditionally been defined as originating from multivesicular bodies and being smaller than 100-nm in size, whilst microvesicles are considered to be larger than 100-nm and formed by direct budding from plasma membranes. However, accumulating

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experimental evidence suggests that particles less than 100-nm in diameter can bud from plasma membranes, and that EVs with exosome markers can be larger than 100-nm.[2] Despite these uncertainties, scientific interest in EVs has greatly expanded recently as EVs are recognised as central players in intercellular communication. Furthermore, EVs are increasingly reported to have roles in many physiological and pathological processes including intercellular communication, coagulation, angiogenesis, cell survival, inflammation and immune response modulation.[1,3-5] Thus, EVs have significant potential as biomarkers for disease diagnosis, prognosis and monitoring. One particular area of interest is the potential use of EVs as diagnostic and prognostic biomarkers in cancer and cancer-associated thrombosis.[6-11] As of February 2015, there were 53 clinical trials registered with ClinicalTrials.gov (search terms: biomarker AND (microparticles OR exosomes OR microvesicles)) exploring use of EVs as biomarkers or therapeutic agents.[12]

A major obstacle to translation of EVs as biomarkers for clinical use has been difficulty establishing standardised methods to measure EVs, primarily due to their small size, most being smaller than 300-nm in diameter.[13-15] This is well documented[15] and includes problems with sensitivity in measuring sub-micron particles as well as specificity when size is used as main identification criteria. Several optical and non-optical techniques have been modified or developed for quantitative and qualitative assessment of EVs including flow cytometry, dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), electron microscopy (EM) and atomic force microscopy (AFM).[16]

Flow cytometry remains the most commonly available and widely used approach for enumeration and qualitative analysis of single vesicles. Benefits include availability, high throughput and opportunities to explore multiple parameters using combinations of dyes and fluorophores. Traditionally, forward scatter signal in response to a 488 nm laser is used to define EVs by size, with direct limitations including inability to resolve structures below 200 nm in diameter,[17] false positive signal from detection of protein aggregates,[18] and "swarm" detection, referring to registration of multiple vesicles simultaneously identified as single event signals.[19,20]

Recently, a protocol,[21] based on original work from Nolte-'t Hoen and colleagues,[22] was published for fluorescence-triggered flow cytometry characterisation of fluorescent EVs using membrane dye PKH67, sucrose density gradient fractionation, and a customised high-end cytometer (BD-Influx; BD Biosciences). This method overcame several problems associated with conventional light scatter-based flow cytometry. Using membrane dye coupled with sucrose gradient fractionation, interference by protein aggregates or calcium-monophosphate precipitates was eliminated and specificity was significantly enhanced. Increased sensitivity for detection of small particles was achieved by triggering event detection on fluorescence signal rather than light-scatter, enabling easy detection of fluorescent beads down to 100-nm in diameter.

However, many of these gains were dependent on substantial customisation and around 3 h of flow cytometer preparation time. Customisation steps included configuration of pulse-processing channels, changes to the FSC obscuration bar, replacement of standard tubing lines, removal of the polarisation unit and neutral density filter in front of the FSC-PMT and installation of 488/10 nm band-pass filter in front of the FSC-PMT. Preparation time is not insignificant and involves laser calibration, with regular monitoring of sheath and sample pressures using an external pressure meter to ensure stable flow rate and reproducible enumeration.

These modifications made this approach impractical in our busy multi-user facility. Therefore, we sought to adapt this method to a commercially available flow cytometer, without need for end-user modifications to create a flow cytometry method for a multi-user environment whilst retaining sensitivity and specificity of the original method. Importantly, we also aimed to develop a simple automated and reproducible protocol for enumeration of fluorescently labelled EVs isolated from cell-conditioned supernatants. In this study, we chose a system with low level EV production to ensure that the method was robust at lower limits of detection. We compared results with those obtained using NTA in light scatter and fluorescent modes.

Methods

Materials

Extracellular vesicle isolation and labelling

For EV production, HEY human ovarian cancer cells were plated in RPMI medium containing 5% of vesicle depleted FBS. Culture medium not previously exposed to cells, but otherwise treated identically to cell-conditioned medium, was used as negative control. Cell-conditioned and control media (22 mL) were collected after 72 h at ~80-90% confluency.

EVs were isolated from cell culture supernatant and control medium by differential centrifugation at 4 °C, (300 g for 5 min, 3000 g for 10 min and 10,000 g for 20 min). EVs were pelleted from the 10,000 g supernatant by ultracentrifugation at 100,000 g for 120 min in a 45Ti rotor (Beckman Coulter Inc, Fullerton, USA). Pellets were washed in 1 mL of 0.1 μm filtered PBS and supernatant removed by centrifugation (TLA-110 rotor; Beckman; 110,000 g, 60 min). Finally, washed pellets were resuspended in 20 μL of filtered PBS supplemented with 0.2% vesicle depleted bovine serum albumin (BSA).

Freshly isolated EVs were fluorescently labelled and fractionated as described by Nolte-'t Hoen and colleagues.[21,22] Briefly, EVs stained with 7.5 μM PKH67 (Sigma Aldrich) membrane dye were mixed with 2.5 M sucrose and overlaid with linear sucrose gradient (2.0-0.5 M sucrose in PBS) in a SW40 tube (Beckman). Following isopycnic sucrose density gradient centrifugation (192,000 g, 16 h) twelve 1-mL fractions were collected starting from the bottom of each gradient. Fraction density was measured using a digital refractometer (HI96801, Hanna Instruments, Australia). Multiple aliquots of each gradient fraction were stored at −80 °C before analysis.

Fluorescence-triggered flow cytometry (FT-FC)

The BD LSRFortessa flow cytometer (BD Biosciences, Australia), equipped with a 100 mW blue 488 nm laser and a photo multiplier tube (PMT) for detection of forward scatter (FSC) (FSC-PMT), was used to detect fluorescently labelled EVs. A high throughput 96-well plate sampler with volumetric