Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Imaging flow cytometry for the characterization of extracellular vesicles

Joanne Lannigan [a,*], Uta Erdbruegger [b]

[a] University of Virginia, School of Medicine, Flow Cytometry Core, 1300 Jefferson Park Avenue, Charlottesville, VA 22908-0734, USA
[b] University of Virginia, Department of Medicine/Nephrology Division, 1300 Jefferson Park Avenue, Charlottesville, VA 22908-0133, USA

ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 15 September 2016
Accepted 30 September 2016
Available online xxxx

Keywords:
Extracellular vesicles
Imaging flow cytometry
Imagestream
Exosomes
Microparticles

ABSTRACT

Extracellular Vesicles (EVs) are potent bio-activators and inter-cellular communicators that play an important role in both health and disease. It is for this reason there is a strong interest in understanding their composition and origin, with the hope of using them as important biomarkers or therapeutics. Due to their very small size, heterogeneity, and large numbers there has been a need for better tools to measure them in an accurate and high throughput manner. While traditional flow cytometry has been widely used for this purpose, there are inherent problems with this approach, as these instruments have traditionally been developed to measure whole cells, which are orders of magnitude larger and express many more molecules of identifying epitopes.

Imaging flow cytometry, as performed with the ImagestreamX MKII, with its combination of increased fluorescence sensitivity, low background, image confirmation ability and powerful data analysis tools, provides a great tool to accurately evaluate EVs. We present here a comprehensive approach in applying this technology to the study of EVs.

1. Introduction

Extracellular vesicles (EVs) are small membrane-bound particles that are generated by both prokaryotic and eukaryotic cells following some sort of stimulation, stress or activation. They are potent bio-activators and inter-cellular communicators and of great interest in studying their role in health and disease. These small biological particles are generally categorized as three types: 1) exosomes (40–100 nm), which are formed within the endosomal compartment as multi-vesicular bodies, and subsequently released from the cell after fusing with the plasma membrane; 2) microvesicles or microparticles (100–1000 nm), which are the result of plasma membrane blebbing/budding and subsequent release from the cell; and 3) apoptotic bodies (1000–3000 nm), which are released by cells undergoing apoptosis [1–9]. Although these different vesicle types are often characterized based on their size range, currently there are no specific markers that can conclusively identify one type of vesicle from another. Therefore we will refer to these small biological particles as extracellular vesicles or EVs [5].

EVs are known to carry cargo important to many biological processes; i.e., miRNA, mRNA, DNA and a variety of signaling proteins believed to be contained within the cytosolic compartment carried within the vesicle or on their membrane. Many of these vesicles also carry membrane proteins derived from parent cells from which they were shed. In addition to being associated with normal homeostasis, EVs have also been shown to be involved in a large number of pathological processes, including infectious, autoimmune, cardiovascular and other inflammatory diseases, cancer, and a variety of coagulopathy disorders. In many of these diseases the type and quantity of these EVs are being indicated as important novel biomarkers as they can be diagnostic and predictive of disease, but also have a potential functional and therapeutic role [6,8,10–27]. As a result there is a strong interest in being able to phenotype and enumerate EVs from biological samples. Additionally there is a great deal of interest in understanding and controlling their biological activity with potential for use as therapeutics. Hence many researchers are exploring a variety of ways to phenotype, enumerate, size and sort EVs.

One of the most popular approaches for making these measurements is flow cytometry, as it is a high throughput way to quantify and phenotype large number of vesicles and is a readily available technology in most research institutions. However, most flow cytometers were designed to measure biological particles in the cellular range (2–30 μm) with measurement sensitivities capable of measuring much larger numbers of fluorescent molecules than would traditionally be found on the surface of very small particles such as EVs. This has been a major challenge to the field to date. There are specialized flow cytometers that have been developed to make measurements consistent with the level of sensitivity

* Corresponding author.
E-mail addresses: jl7fj@virginia.edu (J. Lannigan), ue2u@virginia.edu (U. Erdbruegger).

http://dx.doi.org/10.1016/j.ymeth.2016.09.018
1046-2023/

necessary for sub-micron particles however they are not widely available [28–30]. Traditionally, flow cytometers utilize laser scatter of particles as a way to trigger measurements, with a requirement for exceeding a threshold set to eliminate background "noise". This approach works well for cells, however, falls short of sensitivity when attempting to measure sub-micron particles [31,32]. EVs below 500 nm scatter laser light in the range of electronic noise and buffer alone, making it difficult to resolve particles in this size range using scatter thresholding [33]. It is also important to realize that due to their low refractive index, lipid based vesicles scatter much less light than particles with higher refractive indices such as polystyrene beads. Studies have shown that most commercially available cytometers cannot easily detect single particles smaller that 500 nm using a scatter trigger [4,28,31,33–35]. Due to their very small size, EVs also present a problem with coincidence detection at concentrations traditionally found in biological samples, especially from diseased subjects. It is this coincidence or "swarm" detection" which has made detection of biological particles less than 500 nm possible, however, since this form of detection is not of single vesicles it directly impacts on the accuracy of the phenotype and enumeration of these vesicles [36]. Some have proposed the use of fluorescence rather than scatter triggering to increase the separation of the signals of the EVs above the background [32,33,37–39]. While this does help resolve the populations above background, it requires the use of a pan fluorescent marker for all EVs and still does not address the problem of coincidence at higher concentrations of vesicles.

Imaging flow cytometry (IFC) as found in the ImagestreamX MKII (ISX, EMD Millipore, Seattle, WA) has all of the advantages of traditional flow cytometry; ability to measure scatter and multiple fluorescent markers in a high throughput manner, while also providing the added value of being able to see the images of the cells/particles being measured [40]. The ISX does not require triggering, has no dead time, and measures all objects in the core continuously with no coincidence issues [40,41]. The ISX uses charge coupled device (CCD) cameras for signal detection, as opposed to photomultiplier tubes (PMT). CCDs have a larger dynamic range, significantly lower "noise", and a larger quantum efficiency compared to PMTs, making them more suitable for measuring small amounts of light [40–42]. The ISX uses Time Delay Integration (TDI) as a read out of pixel intensities from the CCD. The advantage of this approach over conventional flow cytometry or strobed illumination imaging is the longer signal integration times (in the range of milliseconds vs. microseconds), with no readout noise penalty, coupled with the slower flow rates leads to increased sensitivity [41,43,44]. In contrast to conventional flow cytometry, there is 100% duty cycle with TDI data collection, resulting in every object that flows through the system being imaged, negating the need for separate data acquisition trigger [44]. This approach makes this technology inherently more sensitive for detection and eliminates much of the noise associated with traditional flow cytometry measurements.

Although coincidence detection is not an issue with the ISX MKII, even when there is a coincident event detected in a single image frame, it can easily be identified with imagery tools and eliminated from the analysis [42]. We, and others have previously reported [42,45], using IFC, the many limitations of using traditional flow cytometry for measuring EVs. Here we provide a simple approach to measuring EVs using IFC with an ISX MKII.

2. Materials and methods

2.1. Sample collection/storage

It is well known that pre-analytical factors such as procedures of collection, handling, and storage affect the quantity, type and quality of EVs in a sample. All reagents used in isolation, as well as labeling, of EVs should be free of contaminating vesicles, especially with culture media that has serum-based additives. A great deal of work has been published in this area, with a focus on efforts toward standardization, especially when working with biological samples [46–63]. Efforts should be made to handle samples consistently and in a manner which is least likely to produce artifacts in numbers and characteristics of the EVs. A comprehensive discussion of these challenges can be found in a variety of publications [9,34,54,56,58,60,64–67]. Table 1 provides considerations for sample collection for different types of body fluids.

2.2. Isolation of EVs

There are many different approaches to isolating or enriching EVs. The method of choice will vary depending on a) the origin of the sample (plasma, urine, culture media, ascites, etc.), b) the size/density of the vesicles you are interested in and c) downstream analyses to be performed on the EV preparation. The most commonly used method is differential centrifugation,

Table 1
Sample collection considerations for various body fluids.

Body fluid	To consider	Comment	References
Blood, plasma preferred	1. Choice of anticoagulant 2. Use of large bore needle 3. Gentle process, vertical position of tubes 4. Process within 2 h 5. Mostly done at room temperature 6. Fresh samples preferred, but if samples are stored at −80C by snap freezing, platelets should be removed prior 7. If a study is performed consider also same time/diet for all samples	1. Citrate preferred 2. To avoid shear stress 3. Avoid generation of EVs	[60,66,85]
Urine	1. Ideally use of fresh voided morning urine 2. Presence of proteins (Uromodulin, Immune globulin, other proteins) 3. Different concentrations of urine, correcting e.g. with creatinine level in urine 4. If a study is performed consider same time, diet, collection type of urine for all samples 5. Depending of downstream investigation protease inhibitors might need to be added 6. Consider bacterial contamination	1. But often frozen after removal of cells with low grade centrifugation 2. Use of detergents for removal of protein 3. Correction to urine creatinine level not established, e.g. level different in acute kidney failure 4. Bacteria can generate EVs by themselves	[54,60,86]
Cell Culture supernatant	1. If serum is added to medium it needs to be depleted of EVs, some add serum free medium for the conditioning time 2. Cell death needs to be accounted for, ideally < 5% 3. Volume needed for EV generation needs to be determined for each study	1. Serum free medium itself can be stressful and cause release of EVs 2. Cell death itself can release EVs	[15,19]

Please cite this article in press as: J. Lannigan, U. Erdbruegger, Methods (2016), http://dx.doi.org/10.1016/j.ymeth.2016.09.018