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NEWSLETTER

ALL ABOUT
EXOSOMES
FEATURED ARTICLE

FAQ’S
BY MILTENYI BIOTEC’S TECHNICAL APPLICATIONS TEAM

PUBLICATION HIGHLIGHTS & REVIEWS

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In this issue...

It is with great pleasure that we announce the launch of the Miltenyi Biotec Flow Cytometry eNewsletter. This quarterly publication will include educational information on a pre-selected topic as well as FAQ’s, brain teasers and tips and tricks related to flow cytometry.

By Anne Gable, PhD
Marketing Product Manager
Miltenyi Biotec

For our first edition we have chosen the topic of “flow cytometric analysis of extracellular vesicles” (EVs). There is immense therapeutic interest in these microscopic particles due to their mechanisms of action and the different disease states that they are associated with. For example, exosomes (a type of extracellular vesicle) have been shown to carry their cargo from one cell to another, providing a potential method for drug delivery. Additionally, EVs contain biological components such as mRNAs and microRNAs that can go on to be expressed once the contents have been transferred into the target cell. There is evidence that these EV-derived circulating RNAs can even have a role in promoting tumorigenesis. However, while extracellular vesicles can be scientifically fascinating, they provide a unique challenge for researchers who are interested in their identification and isolation, as well as characterization of their contents. Because they have a large size range and are often very small (< 100nM), detection on a conventional flow cytometer can be quite difficult. In this edition, to help explain some of these complexities, we are featuring an article describing the unique considerations for extracellular vesicle analysis by flow cytometry, written by Christopher Spring. (See references on pg. 4)

Featured Scientific Article

By Christopher Spring, PhD
Director of Flow Cytometry Core Facility at St. Michael’s Hospital in Ontario, Canada

Let’s Talk Exosomes!
The study of subcellular submicron particles by flow cytometry has dramatically expanded over the past decade with the realization that this family of divergent subcellular membrane-encapsulated particles play critical roles in regulating pathophysiological processes or acting as biomarkers of disease. Once thought to be cellular “dust,” the disease states in which these subcellular particles have now been implicated in include but are not limited to cancer, sepsis, immunological disease, inflammation, neurological disease, trauma and critical illness. However, the investigation of these particles is complicated by a lack of common nomenclature in the literature. Originally these small biological particles were stratified by size and composition into large DNA-containing particles (<4000 nm; apoptotic bodies), smaller particles generated as off-buds from parental cells (100-1500nm; microvesicles/microparticles) which express cell lineage markers (and occasionally annexin V) and finally the smallest particles (<100nm; exosomes) generated inside multivesicular bodies and released on fusion with the plasma membrane. More recently the umbrella catch-all term “extracellular vesicles” has come to globally represent all these vesicles. This change to a less complicated encompassing nomenclature has been driven in part due to recently increasing complexity of nomenclature and divergent mechanistic identities of vesicles under active debate with size ranges, resource materials, mechanisms of particle generation, surface marker expression and even niche-subtype identifying terminology (oncosomes, exosome-like extracellular vesicles, extracellular vesicle, etc) being far from standardized. Recognizing naming of these vesicles under active debate for our purposes we will use the term “extracellular vesicles” as it is favoured by the International Society of Extracellular Vesicles (ISEV). However we will discuss challenges, controls, tips and tricks for successfully analysing all of the above mentioned subcellular, submicron membrane enclosed biological particles, whatever terminology may be applied.

The assessment of EVs has become increasingly popular via flow cytometry given the ability to rapidly assess large numbers of particles across a range of sizes and interrogate multiple phenotypic markers simultaneously. This has placed flow cytometry as preferable over secondary, supportive analysis measures including nanoparticle tracking analysis, dynamic light scattering, electron microscopy, cryoEM and resistive pulse sensing as they are hampered by issues of cost, throughput, quantitation, etc. While flow cytometry has major advantages it is also limited by a complex EV-specific workflow that can be challenging to the uninitiated cytometrist. Below we will outline the nature of several of these challenges as well as some recommendations on how to circumvent them for meaningfully sample analysis. Unlike cellular populations, which are distinct and well resolved from...
instrument noise, EV populations are typically characterized by a heterogeneous “smear” of a continuous population distribution. Therefore the smallest particles (which are more abundant) cross over into unresolvable overlap with instrument noise. How and to what extent small EVs overlap with machine noise is related to the specific optics and cleanliness of the cytometer, electronics and the refractive index of the EVs being sampled. The challenge here in part is how to place appropriate gates to exclude larger, non-EV material on the upper end and limit the gate in terms of smaller EVs at a reproducible spot prior to running into instrument noise. The most common gating method is to use a number of various sized reference beads (spherotech, megamix, apogee mix, etc.) made of different materials and fluorescence signatures. The best reference beads contain fluorescent particles and nonfluorescent particles, ideally created from a substance closely matched to the refractive index of EVs (i.e. silica) so that the observed “size” is remotely related to your EV “size” because they scatter light similarly (Figure 1). More ideal gating references would be made of biological materials with matched refractive indices shared by EVs. Several attempts to date (i.e. LMBs, nanoE, etc.) in this regard have yet to create complete biological reference material sets.6,7

Once a gating strategy has been decided upon it is important to rule out a few key artefacts including swarm detection (or coincidence) and false positive events using a control sample in some basic pilot experiments. In EV flow it is of particular importance to rule out swarm populations of multiple EVs traversing the interrogation point at the same time. This is exaggerated in these types of experiments since EVs are so much smaller than cells and capable of dwelling in the laser spot with other particles so much more easily than with cells (Figure 2). As a result, EV samples should be analysed at serial dilutions to monitor the FSC and SSC medians within dilutions to ensure they do not decrease with dilution. If they do change significantly this may indicate swarm or “invisible swarm” detection depending on the nature of the change. It is also critical to ensure analytical plots for fluorescent or FSC/SSC parameters are displayed on signal Height (H) and not the traditional Area (A) measurement since signal pulses are not Gaussian like with slit-scanning cells but rather resemble a wide mesa with the height “saturating” throughout a portion of the EV transit time since beam heights are much “taller” than EV diameters.

With appropriate gates and validated dilution ranges established, it is crucial to discount any measurements throughout the first 5 second of sample uptake for accurate, stable measurements. It is recommended to measure samples over a minimum of 1 minute to ensure good representative sampling although excessive sampling times sometimes result in software read errors, particularly with high concentration EV samples. Samples should be collected on the slowest possible settings to maximize enumeration and characterization of these small targets.

Lastly, elimination of false positive events posing as EVs from buffers (salt crystals, protein aggregates, etc.) that would be fluorescently negative and those that would be fluorescently negative...
positive (i.e. antibody aggregates if you are staining the EVs) is important. To limit this all buffers (and potentially antibodies) should be fresh and filtered. Creating a library of “non-EV containing” buffers and reagents or antibodies can help discriminate between valid EV observations and obligatory false positive events that share the approximate forward and sideward scatter properties as EVs, and as such could be inappropriately counted as EV events instead of being “blank subtracted.” Running a reference EV sample followed by lysing EVs with the addition of 0.1% TritonX-100 or other detergents like NP-40 and re-running the sample should reveal the difference between the EV-plus-background sample and the background-alone sample, respectively.

**Conclusion**

There are several other critical steps including preanalytical sample prep, instrument maintenance and QC, methods of enumeration, fluorescence and light scatter sensitivity, laser source selection, etc which dramatically influence EV flow cytometry experiments, but the above set of controls and considerations is very achievable with a very basic EV sample in order to quickly and efficiently eliminate many key pitfalls that would otherwise derail EV experiments indefinitely.

**Featured Scientific Article References**


**References - In this issue... (from page 2)**

EXOSOMES

How do I prepare my sample for the exosome isolation?
Exosomes are small extracellular lipid-based vesicles ranging from 30 to 100 nanometers in diameter. Since they are extracellular, no cell lysis is necessary. Typical starting materials are cell culture media, ascites fluid, urine, blood plasma, and milk. The general principle is to get a cell and debris-free supernatant as starting material usually by sequential centrifugation steps.

Cell culture media, urine or ascites fluid: 1. 300xg for 10 minutes, 2. 2000xg for 30 minutes and 3. 10,000xg for 45 minutes. Blood Plasma (EDTA or citrate anti-coagulant): 1. 2000xg for 30 minutes and 2. Dilute supernatant with equal volume PBS 3. 10,000xg for 45 minutes Milk: 1. 300 × g for 30 minutes and 2. 3000 × g centrifugation 3. filtered sequentially through 1.2-, 0.8-, 0.4-, and finally 0.2-μm filters to remove cell debris 4. 10,000 × g for 30 min Always centrifuge at 4 degrees and if large particles are persistent, filter the supernatants as needed.

Post-isolation assays with the exosomes?
Due to their small size, capturing and examining exosomes has proven challenging. Besides isolation of intact exosomes that can be analyzed for protein and nucleic acid contents by standard methods, we offer solutions for analysis by flow cytometry. Detection of single exosomes is very difficult via flow cytometry, but it is possible to gain insights about a population of exosomes. Proteins on the surface of exosomes can be stained with fluorochrome-conjugated antibodies to characterize populations originating from a particular cell type. The MACSplex Exosome Kits allow qualitative and semi-quantitative detection of 37 exosomal surface epitopes plus two isotype controls. The MACSplex Exosome Kit comprises a cocktail of various fluorescently labeled bead populations, each coated with a specific antibody binding the respective surface epitope. The 39 bead populations can be distinguished by different fluorescence intensities detected by flow cytometry (PE and FITC channels). In combination with the Express Modes of the MACSQuant®, the MACSplex Exosome Kit is optimized for automated acquisition and analysis of the samples.

Can I do the data acquisition and analysis on the MACSQuant® Analyzer? Do I need a specific software version/Express Mode Version?
Yes, with the MACSQuant Analyzer Express Mode in MACSQuantify® Software version 2.8 or higher and at least Express Mode version 28.1.15443. Instrument Settings are automatically uploaded when using the MACSplex Express Modes with MACSQuantify Software version 2.8 or higher.

Analyze the file by selecting the file name in the samples tab, right click and select “View with Analysis. MACSplex_Exosome.” Please note that when you are performing the data analysis on a PC, make sure that the MACSQuantify Software is set to the correct instrument configuration, the MACSQuant instrument that was used for data acquisition. Please visit, www.goo.gl/8gOPQe for data analysis and troubleshooting.

Do I need a MACSQuant® Analyzer to do the data acquisition and analysis?
No. We have instructions on how to do it using different flow cytometers. However, it is obviously easier to do it on the MACSQuant Analyzer. Please visit, www.goo.gl/E1i83dt for the MACSplex kit general instructions.

Have more questions? Email us at, macs@miltonyibiotec.com

Plasma extracellular vesicles (pEVs) are strongly elevated in HIV infection and do not decline after anti-retroviral therapy. These pEVs contain viral accessory proteins as well as pro-inflammatory effectors, raising the hypothesis that they play a role in chronic HIV infection.


Mesenchymal stem cells (MSC) have demonstrated significant neuroprotective and axogenic effects on retinal ganglion cells in blindness and degenerative eye disease. In a rat optic nerve crush model, exosomes were isolated from bone marrow derived MSC and applied to primary retinal cultures, which resulted in significantly improved functional activity.


This review discusses recent scientific findings and technological advances for cell processing with regards to the development of cellular therapies for treatment of severe GvHD, life-threatening infections, and relapsed leukemia. It includes an overview of the therapeutic potential for MSC-derived extracellular vesicles.


Isolation and analysis of extracellular vesicles can be quite difficult. This article describes a new flow cytometric technique to investigate up to 39 different surface markers in one sample, allowing for easy screening of surface markers on populations of extracellular vesicles.


In this article, a broad protein characterization of EVs from plasma of melanoma patients and healthy donors as well as from T cells, B cells, natural killer (NK) cells, monocytes, and monocyte-derived dendritic cells (moDCs) was performed. Results indicate that plasma EVs from healthy donors and melanoma patients display differential surface markers, suggesting an effect of melanoma cells on the vesicle secretion or vesicle protein loading by blood cells.
Reader Naming Contest - Win an Apple Watch™

Help us with a new title for our Flow Cytometry Newsletter. Please submit answers by June 30, 2017

We want to draw on the creativity of all our readers and select an appropriate name for our quarterly flow cytometry eNewsletter. Submit your entry at the www.miltenyibiotec.com/flow-newsletter and the winner will WIN an Apple Watch! We are looking for a name that encompasses the diversity of research fields and applications that use flow cytometry for their experiments.

Submit your entry for a chance to win an Apple Watch* (valued at $250.00). One entry per person. Please email your submissions to macs@miltenyibiotec.com

Brain Teasers & Games - Test Your Knowledge!

Sudoku:
The object is to fill all empty squares so that the numbers 1 to 9 appear exactly once in each row, column and 3x3 box, and the sum of the numbers in each area is equal to the clue in the area's top-left corner*.

Word Search:
Print out and complete. Circle the 18 words listed below. Words appear straight across, backward, straight across, up and down, down and up, and diagonally*.

*To view answer key, please visit www.miltenyibiotec.com/flow-newsletter/