Title: Next Generation Extracellular Vesicles Studies for Cancer Research

Speaker: Joshua A. Welsh

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Abstract: Extracellular vesicles (EVs) promise a powerful method to enable personalized medicine to individuals across a range of pathologies. Despite an abundance of studies investigating the use of EVs as translational biomarkers, it is impossible to interpret and reproduce most, if not all, translational studies utilizing single-EV flow cytometry published to date. The greatest barrier to rigor and reproducibility in the field is differences in the limits of detection between instruments and the lack of standardization methods and tools.

Within the Translational Nanobiology Lab at the National Institutes of Health, I have established a toolbox of software and methods to improve EV flow cytometric analysis and reporting. This toolbox establishes a framework for obtaining standardized measurements with a suite of free software tools to enable EV standardization using flow cytometry in labs worldwide, and also includes software to screen hundreds of EVs phenotyping before utilizing flow cytometers capable of single-fluorescent molecule detection. This toolbox is an integral part of the high-throughput, scalable, clinical pipeline that our group is developing to enable the use of EVs as clinical biomarkers.

I will give a brief introduction to what EVs are before covering the hurdles our lab have been working to overcome in order to develop a clinical pipeline. This will include: 1) scalable EV purification, 2) flow cytometer standardization, 3) software analysis tools, 4) protein screening tools, 5) spectral scatter labels.

Title: Conventional, high-resolution and imaging flow cytometry: Potentials, pitfalls and solutions for EV characterisation

Speaker: Jaco Botha

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Abstract: Flow cytometry (FCM) has long been a preferred method for characterising EVs, however their small size has limited the applicability of conventional FCM to some extent. Thus, high-resolution and imaging FCMs have been developed but not yet systematically evaluated. The aim of this presentation is to describe the applicability of high-resolution and imaging FCM in the context of EV characterisation and the most significant pitfalls potentially influencing data interpretation.

Methods: 1) First, we present a side-by-side comparison of three different cytometry platforms on characterising EVs from blood plasma regarding sensitivity, resolution and reproducibility: a conventional FCM, a high-resolution FCM, and an imaging FCM. 2) Next, we demonstrate how different pitfalls can influence the interpretation of results on the different cytometry platforms. 3) Finally, we propose controls, solutions or workarounds for understanding and limiting the influence of each of these pitfalls.

Results: 1) High-resolution FCM and imaging FCM displayed greater sensitivity and resolution compared to conventional FCM when measuring a mixture of nanospheres. Equally, both methods could detect larger concentrations of specific EV phenotypes than conventional FCM, where imaging FCM outperformed high-resolution FCM. Within day variability (n = 20 aliquots) was similar for conventional and high-resolution FCM, while imaging FCM had a markedly larger variability. Between day variability (n = 5 x 5 aliquots) was similar for all three platforms. 2) The three most substantial pitfalls variably influencing interpretation of results on the three platforms are non-specific binding of labels, antibody aggregates, and entities in the sample (i.e. lipoproteins) binding EV-defining dyes. 3) The most important strategies for circumventing these pitfalls are stringent matching, gating and comparison of antibodies and isotype controls, high-speed centrifugation of antibodies and labels prior to staining, and the use and interpretation of stained buffer controls and detergent treated samples.

Conclusions/summary: High-resolution and imaging FCM hold great potential for EV characterisation. However, increased sensitivity also leads to new artefacts and pitfalls. The solutions proposed in this presentation provide useful strategies for circumventing these.

Title: Using a combination of bead-based flow cytometry and imaging flow cytometry to understand Extracellular Vesicle Heterogeneity

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Abstract: Extracellular vesicles (EVs) are secreted by all cell types and can be found in all body fluids. They can be roughly classified based on their size and origin as exosomes (70-150 nm) and microvesicles (100 nm to 1 µm). However, it is nowadays commonly accepted in the field that there is a much higher degree of EV heterogeneity within these two subgroups. Also, their content, protein composition and surface signature likely is dependent on multiple parameters like the cell's metabolic or immunological status. Moreover, the protein composition and surface marker signature of EVs is further dependent on the cell type releasing them. Accordingly, EVs secreted by different normal cell types or malignant cells also will display distinct surface profiles. Until today, only few EV surface markers have been related to specific cell sources. We have recently optimized two flow cytometry based methods for EV surface marker analysis, a multiplex bead-based approach which allows robust identification of co-expressed surface marker combinations (Wiklander et al, 2018) and a method using imaging flow cytometry to quantify EV subsets at the single vesicle level (Görgens et al, in revision).

Apart from further working on method optimization and standardization, we are aiming to combine both flow cytometric approaches aiming to identify EV surface marker combinations being specific for EVs from specific cell types and/or disease-related cells such as cancer cells. In an ongoing project, we first used the multiplex bead-based assay to screen EVs isolated from 40+ different immortalized human cell lines from different tissues. Next, we have applied the same screening technology to assess surface signatures of EVs derived from diverse biological fluids of human healthy donors in order to identify differential surface marker combinations between different body fluids and estimate general donor-to-donor variation within respective sample groups. Validation of identified EV surface signatures by high resolution single vesicle imaging flow cytometry and other methods is currently ongoing.

We will show preliminary data resulting from this approach and propose that the identification of specific EV surface marker combinations will be highly relevant to further understand the molecular content and related functions of subsets of EVs in health and disease.

Title: Flow-activated nanoparticle sorting as a novel tool for the characterization of nanoparticles

Speaker: Jens Bæk Simonsen

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Abstract: I present two cases where we have used flow cytometry/FACS to analyze (1) the oligomer distribution of a mixture of gold nanoparticle oligomers and (2) the size distribution of a polydisperse artificial vesicle (liposome) sample.

Case 1. Here we show that flow cytometry can be used as a high-throughput method to determine the relative distribution of oligomeric clusters (monomers, dimers and multimers) of molecularly linked gold nanoparticles in solution at the single-particle level with good statistics. This unique information would be near impossible to obtain using traditional characterization techniques.

Case 2. Here we present a novel method to estimate the size of individual fluorescently labeled liposomes by flow cytometry. In order to do that, we prepared liposomal size-calibrators by FACS-sorting a poly disperse fluorophore-labeled liposome sample with respect to fluorescence intensity. We then evaluated the size of the FACS-sorted liposomes by nanoparticle tracking analysis (NTA). Next we correlated the NTA-derived sizes of the FACS-sorted liposomes with their flow cytometric scatter and fluorescence intensities. Our study demonstrated the poor resolution of the scattering readout and significantly better resolution of the fluorescence signal when using these readouts from a FACSAria to estimate the size of nano-sized fluorophore-labeled liposomes. Finally, we validated the size-distribution of the polydisperse liposome sample derived from the calibrated flow cytometric fluorescence intensities against standard methods including cryo-transmission electron microscopy.