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Flow cytometry analysis of fluorescently-labelled extracellular vesicles.

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Flow cytometry analysis of fluorescently-labelled extracellular vesicles.

Abstract:

In the recent decade, there was a rise of interest in extracellular vesicles (EVs) due to their potential as biomarkers and drug delivery. However, for better understanding of extracellular vesicles standardized isolation and detection protocols that would take into account their heterogeneity and small size are needed. The present thesis studies the efficiency of studying fluorescently tagged extracellular vesicles by flow cytometry and the challenges of this method. Taken together, the findings in this thesis suggest that flow cytometry has the potential to be utilized as an EVs analysis method but after all, further studies are needed for enhancing small particles detection and reading noise decreasing.

Keywords: Extracellular vesicles, Cop5Ebna, flow cytometry, fluorescent labelling, MAGE-A, MAGE-A4, MAGE-A10, GFP, mCherry.

CERCS: T490 Biotechnology, P310 Proteins, enzymes

Fluorestsentselt märgistatud ekstratsellulaarsete vesiikulite voolutsütomeetriline analüüsimine

Viimase kümnendi jooksul on ekstratsellulaarsete vesiikulite (EV), kui potentsiaalselt heade biomarkerite ja ravimikandjate, vastu tõusnud suur teaduslik huvi. Kuigi standartsed EV-de puhastamise ning tuvastamise meetodid, mis võimaldaksid EV-sid paremini tundmaõppida, vajavad veel väljatöötamist. Käesolevas uurimustöös hinnatakse voolutsütomeetria sobivust fluorestsentselt märgistatud EV-de analüüsimiseks ning tuuakse välja selle puudused. Kokkuvõtvalt, uurimustöö tulemuste põhjal võib väita, et voolutsütomeetria on potentsiaali olla sobiv meetod EV-de uurmiseks, kuid on vaja teostada edasisi uuringuid väikseste partiklite tuvastamise parandamiseks ning taustamüra vähendmisek.

Võtmesõnad: Ekstratsellulaarsed vesiikulid, COP5EBNA, voolutsütomeetria, fluorestsents märgistamine, MAGE-A, MAGE-A4, MAGE-A10, GFP, mCherry.

CERCS: T490 Biotehnoloogia, P310 Proteiinid, ensüümid

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TERMS, ABBREVIATIONS AND NOTATIONS

EDTA - Ethylenediaminetetraacetic acid

ESCRTs - endosomal sorting complexes required for transport

EV – extracellular vesicles

FACS - Fluorescence-Activated Cell Sorting

FBS - Fetal bovine serum

FSC - forward scatter

FSC-A - FSC-Area

FSC-H - FSC-Height

GFP- green fluorescent protein

GFP-H - GFP-Height

ILV - intraluminal vesicle

IMDM - scove's Modified Dulbecco's Medium

MAGE-A10 - melanoma-associated antigen A10

MAGE-A4 - melanoma-associated antigen A4

MVB - multivesicular body

NTA - nanoparticle tracking analysis

NTA - Nanoparticle tracking analysis

PBS - Phosphate buffered saline

SSC - side scatter

UC - ultracentrifugationn

INTRODUCTION

Extracellular vesicles (EVs) are derived from cell vesicles of typically 30–100 nm in diameter size that are present in various biological substances such as blood, urine, amniotic and other biological fluids, sparking the interest of their potential usage as biomarkers. Thus, the ability to identify EVs within body fluids would benefit cancer, cardiovascular disease, neurodegenerative conditions and other disease syndromes.

Extracellular vesicles are classified into three following groups: exosomes, microvesicles and apoptotic bodies. Although exosomes and microvesicles have a distinct from each other biogenesis pathways, in reality, characterization and analysis of particles are confusing. As extracellular vesicles field is still in its youth, no standard isolation or detection protocols exist, which complicates vesicles research. Thus, a current challenge is to adopt defined methods for analyses of EVs as they are often decelerated with the size of particles and difficulty of heterogeneity among extracellular vesicles classes.

Flow cytometry is a technology design of identifying a significant number of cells ' light absorption and fluorescence characteristics. In order to perceive if flow cytometry method is sufficient for extracellular vesicles' analysis, we developed a study in which extracellular vesicles from eukaryotic COP5EBNA cell line are first fluorescently labelled with GFP, GFP MAGEA4, GFP MAGEA10, mCherry, mCherry MAGEA4 and mCherry MAGEA4. Then vesicles are isolated from the medium by ultracentrifugation protocol and analysed by flow cytometer on amount of fluorescently tagged EVs of 100-400 nm size and larger than 400 nm vesicles. Another objective is to observe the interactions between MAGE-A proteins and EVs.

The work has been carried out at the University of Tartu Institute of Technology.

1 LITERATURE REVIEW

1.1 Extracellular Vesicles

The term extracellular vesicles are used broadly as lipid- delimited particles that are derived from the cell membrane and used for cell-to-cell communication, coagulation and other pathophysiological processes. Although previously apoptosis was known to be a mean of cell-to-cell communication, ever since it was discovered that almost all cell types (Simons, Raposo, 2009) can extract EVs in a healthy environment.

Extracellular vesicles are classified into three following groups: exosomes, microvesicles and apoptotic bodies. They take part in the different biological process such as coagulation (Mackman, 2007) and cell-to-cell communication. (Raposo, 2013)

Historically, the first observation of EVs in a form of procoagulant platelet-derived particles took place in 1946 by Chargaff and West (Chargaff, West, 1946). Albeit being considered just a waste product at first and being referred as ‘platelet dust’ the scientific interest in these particles increased during the next years as it was discovered that they could transport nucleic acids of interest(Valadi et al, 2007), thus insinuating a future method of therapeutics as well as biomarker perspective. Following the discovery of RNA transfer by EVs, these vesicles have been further analysed in various biological fluids. (Ronquist and Brody. 1985), (Andre, et al. 2002; Pisitkun, et al. 2004; Caby, et al. 2005)

1.1.2. Classification of extracellular vesicles

Typical size of extracellular vesicles varies from 30 to 2,000 nm in diameter. As it is still an emerging field, a certain irregularity in the nomenclature of EVs among scientific papers could be witnessed.

Largely accepted today nomenclature is based on biogenesis origin, size and density of extracellular vesicles. Another nomenclature consists of naming vesicles on the basis of their origin and function, such as octosomes that originate from cancer cells or proctasome from prostate. It has been suggested to use term EVs broadly to all three groups of secreted vesicles: exosomes (40-120 nm), microvesicles (50 – 1000 nm) and apoptotic bodies (50-5000 nm). Microvesicles are considered to have the highest heterogeneity, in some

classifications viewed to contain apoptotic bodies as both are formed by budding of the cell membrane. In contrast, exosomes are produced via endosomal compartments through the formation of multivesicular bodies (MVBs).

1.2 Biogenesis of Extracellular Vesicles

1.2.1 Biogenesis of exosomes

Exosomes are formed as an end product of the endocytic pathway that performs either as recycling of membrane molecules by mean of early endosomes or as degradation machinery by lysosomes and late endosomes.

During the maturation of early endosome into late one, an assemblage of intraluminal vesicles (ILVs) into the lumen of endosome takes place. Subsequently, various proteins, lipids and nucleotides are packed into it. Due to the origin of exosomes, membrane-associated proteins as Rab GTPases, as well as Alix and TSG101 usually are implemented as markers (Pereira-Leal, Seabra, 2001). During the formation of ILVs, membrane proteins (CD9, CD63 and CD81 for example) reestablish into tetraspanin-enriched microdomains.

The late endosome is termed a multivesicular body (MVB) after budding of intraluminal vesicles (Johnstone, et al. 1987). Later on, MVB can follow different routes, one of which is degrading by means of fusion with lysosomes. Another is merging with plasma membrane while releasing ILVs out into extracellular space, being now essentially called exosomes (Trams, et al. 1981, Harding, et al. 1983).

Protein delivery to differentially fated ILVs in either degradation or secretion, occur by means of various sorting pathways, consequently suggesting the presence of several varieties of MVEs. Post-translational modification pathway is regulated by ESCRT machinery (endosomal sorting complexes required for transport) (Hierro A, 2004). This results in current consideration of MVB-formation routes to be ESCRT-dependent and ESCRT-independent.

There is another and much more imminent exosome production route next to the "classic pathway" of exosome origin. Exosomes are released from the plasma membrane of SupT1, Jurkat and other T cell lines consequent to HIV infection or presence of Nef protein, along with spontaneous delivery Fang et al., 2007; Lenassi et al., 2010). As alternative endosomal pathway vesicles contain the same membrane proteins and other

classic endosomal pathways labels, they are considered interchangeable to previously described exosome formation route. It is currently not discovered if these kind of exosomes are present in the other than T cell lines exosomes or in vivo.

1.2.2 Biogenesis of microvesicles

Microvesicles also called as ectosomes (Stein and Luzio, 1991) in literature, is a term used for vesicles that are released from the plasma membrane during cell stress. Formation of microvesicles - currently less understood than exosome biogenesis - arises by budding and fission of the plasma membrane that is archived by enzymatic activity and alterations in the cytoskeleton.

Biogenesis of microvesicles is believed to be dependent on lipid distribution (Hankins et al., 2015) through the plasma membrane, specifically relocation of phosphatidylserine to the outer leaflet (Akers et al., 2013). It was also noted that high concentrations of calcium trigger a phospholipid reposition that leads to the MV formation (Pasquet et al., 1996).

Although the formation of microvesicles and exosomes is well distinguished (Figure 1), common intracellular mechanisms and sorting machinery hamper the distinguishing between classes. In this work, the focus is on exosomes and microvesicles which come from live cells.

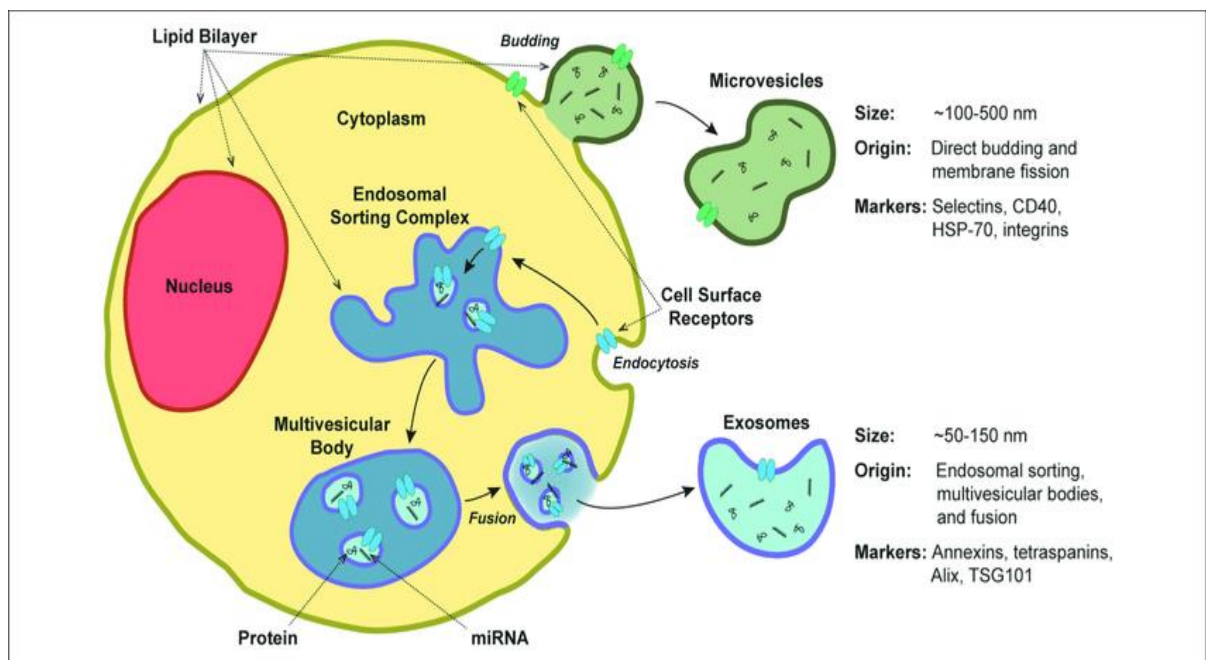


Figure1. Microvesicles are formed by direct budding and fission from the plasma membrane (100–500 nm in diameter). In contrast, exosomes are released via the endosomal-sorting complex, where intraluminal vesicles formed by the inward budding of the endosomal membrane are packaged within multivesicular bodies. These bodies then fuse with the plasma membrane to release their enclosed exosomes (~50–150 nm). Both exosomes and microvesicles contain miRNA and protein cargoes (Blaser, Aikawa, 2018,)

1.3 MAGE family

MAGE (melanoma-associated antigens) gene family was first described in 1991 when three samples of the same genus were found from samples taken from melanoma patients. The gene family was discovered because they encoded cancer antigens that spontaneously induced cytotoxic (living cells) T-lymphocyte response in the tissues of melanoma patients. (Van der Bruggen et al., 1991)

The first detected tumour antigen was named melanoma antigen 1 or MAGE-1 (later the corresponding antigen was named MAGE-A1 (De Plaen et al., 1994). Now, MAGE antigens are divided into two large groups, MAGE-I (cancer antigens) and MAGE-II (ubiquitous antigens), depending on their tissue-specific expression, structural differences and location on the chromosome. (Sang et al., 2011) The MAGE-I group includes the genera MAGE-A, MAGE-B and MAGE-C, the antigens of which are all located in the human X chromosome (De Plaen et al., 1994).

It was suggested that MAGE-A proteins (preferably A1, A4 and A10) could be the main targets of immunotherapy as they are likely to affect tumour development the most. (Daudi et al., 2014) The MAGE-A4 protein is localized in the cytoplasm and nucleus of the cell (CTdatabase).

1.4 Isolation of Extracellular Vesicles

The main isolation challenge is the small size of extracellular vesicles below the detection range of many established techniques of detection. Also, separation process recovery and contamination cannot be accurately measured and there is yet to be formed standardized isolation protocol.

Additionally, isolation of EVs is conditional on vesicle class. Regarding the sample type, two primary classes can be identified: extracellular vesicles isolated from cell culture media and extracellular vesicles isolated from body fluids. Naturally, specific requirements of these types must be taken into account when conducting research. One of these provisions to consider when isolating EVs from media is the presence of additional EV source, such as fetal bovine serum (FBS) or other supplements that are added to media (Théry et al, 2006). Generally used isolation methods are ultracentrifugation with/without a density gradient, ultrafiltration, size exclusion chromatography, affinity capture of magnetic/non-magnetic beads and polymer-based precipitation.

1.4.1 Differential centrifugation with ultracentrifugation (UC)

Extracellular vesicles are differentiated from cells and isolated through differential centrifugation in many EVs research. For sedimentation of particulate matter, such as EVs in solution, high acceleration of centrifugal force is applied. The separation of the different kinds of EVs in a study is based on size and density, with bigger and denser components relocating aside from the centrifuge axis.

The traditional differential centrifugation process includes a progression of expanding centrifugation steps starting with a 300-500 g spin pursued by a turn of 2,000 g to get rid of dead cells and cell debris. (Théry and others, 2006). The supernatant is then spun at 10,000×g to pellet larger EVs. This fraction is sometimes referred to as the MV pellet or simply the 10,000×g EV pellet (Théry et al, 2006). Numerous different protocols exist and implemented by researches, though for the most part, the protocols follow the scheme by Raposo et al, who purified exosomes from the conditioned culture media of transformed human B cell lines. (Raposo et al, 1996) Quite often, a density (20–60%)/cushion (30% sucrose) gradient step is added to UC protocols. It is required for detachment of proteins with non-EV density from the rest of the sample. (Webber, Clayton, 2013)

Likewise, there are various issues that come with centrifugation performance. It can be challenging to remove all cells from biological fluids, for example in the situation with blood centrifugation as platelets and apoptotic bodies size can interfere with bigger particles. When the centrifugal force applied to remove cells is too high, cells may fragment or become activated. Washing of vesicle pellets will often result in the loss of vesicles, resulting in variable yields. For instance, approximately 40 to 60% of platelet-derived vesicles are lost at every washing step, whereas vesicles from erythrocytes are

unaffected (M. C. L. Schaap and R. J. Berckmans). In addition, high UC acceleration of 100,000 to 200,000×g may result in vesicle fusion and contamination of the pellets with proteins, thus hampering TEM and proteomic studies (Bard et al., 2004; Rood et al., 2010; Gyorgy et al., 2011b).

1.4.2 Filtration and/or size exclusion based techniques

Based on their size, chromatography particles are differentiated in size exclusion, moving at different rates through the filtration column. Larger particles are going to go quicker through the size exclusion column stationary phase (gel) as the elution velocity is unique. Commonly size exclusion chromatography is coupled with low-speed centrifugation to purify EVs from cell debris and filtration of samples to pre-concentrate the EVs. As with other methods, chromatography has its own limitations. The high force driving EVs through filtration column may alter the structure of vesicles and eventually cause rapture into smaller particles. Albeit it is also considered to yield near complete removal of soluble proteins and most kinds of lipoprotein from plasma while being significantly faster and easier (Boing AN et al, 2014).

The sample is processed through a semipermeable membrane in ultrafiltration. Filter-based methods will not enrich EV populations unless low-molecular-weight filters (e.g. Centricon units) are used for concentration. As with other size based techniques, the main issues are swamping and shear pressure, that can cause undesirable modifications in characteristics such as extracellular vesicle morphology.

1.4.3 Affinity binding techniques

The presence of characteristic surface proteins, lipids and polysaccharides on certain EV classes is the basis for immunoaffinity isolation. Due to EVs similarities with their parent cells, the correct selection of antibodies can lead to either enrichment of preferred vesicles (positive selection) or negative selection against undesired vesicles. Magnetic beads, highly porous monolithic silica microtips and other materials are employed for this purpose (D. W. Greening, 2015). This approach evaluates particular subpopulations of EVs without taking their size or density into consideration and has the potential to increase specificity

(Tauro BJ et al., 2012). Typically made of polystyrene, for example, surfactant-free latex beads are a good alternative as their covalent linkage preserves them from aggregation while binding to Evs regardless of morphology of exterior marker or vesicle size.

1.4.4 Microfluidics

Microfluidic techniques are another rapidly gaining consideration group of EV isolation practices that has certain benefits as they provide a considerable decrease in the amount of sample and time, making them a potential tool for high-performance clinical diagnosis. Exosomal purification based on microfluidics can be done by means of immunoaffinity, molecular sieving or capture on nanoporous structures (S. S. Kanwar et al., 2014). Nevertheless, in comparison with conventional isolation's high sample amount absorption and rate of captured EVs, microfluidics methods still require future optimizations. (D. Ingato et al., 2016)

1.5 Detection methods of microparticles

1.5.1 Flow cytometry

Flow cytometry is a technology that can detect light absorption and fluorescence properties of a large number of cells and is considered one of the most typically used techniques for analyzing nanoparticle-sized particles. Such a technique is commonly applicable in research groups and enables multitudes of vesicles to be analyzed quickly in a sample and a large range of samples. In addition, flow cytometry is optimal for evaluating the expression of particular EV indicators by using particular fluorochrome-conjugated antibodies for classifying as well as quantifying vesicle populations correspondingly.

Conventional methods in flow cytometry are polystyrene and silica beads of defined size. Although convenient for establishing detection thresholds and as a set standard for flow cytometry this is still an impediment as certain properties (eg. refractive index) of beads and EVs contrasts.

The main challenge of isolation of the small size of extracellular vesicles is them being under the detection range of many established detection methods. Consequently, determining amount of recovery and contamination during isolation is laborious, with having no standardized isolation protocol. The interrelated difficulties of the detection and isolation of vesicles partly explain the differences in classification criteria and clearly exposes one of the main issues to be solved by the research field.

1.5.1.1 Signal detection and analysis

Light signals are generated as particles pass through the laser beam in a fluid stream. These light signals are converted to electronic signals (voltages) by photodetectors and then assigned a channel number on a data plot. First is forward scatter (FSC) parameter that measures scatter onward the path of the laser. Due to light diffraction around the cell, forward scatter can detect the diameter of cells, thus being used in selection cells based on size. Although particles of nano-size generate signals that depend on the shape of the cell as well as the refractive index (Lacroix, 2010). Meanwhile, side scatter (SSC) detects light at a ninety-degree angle relative to the laser. It detects the inner complexity of cells attributable to the reflection of the laser from intracellular compositions. Light scatter intensity is a complex function of wavelength, particle size and shape, angle of collection, and refractive index. In a flow cytometer, FSC and SSC are measured simultaneously and the combination of two parameters delivers a foundation for cell analysis. (Shapiro, 1985) As in recording multiple detectors are measuring particle emission, gating method is used to separate populations of interest. A gate is a fixed data boundary on cells that help to further investigate particular cytometric events.

The pulse is formed by three components—a pulse height (-H), width (-W) and the pulse area (-A) that is aggregated from the height and the width. Commonly, it is the pulse area that is reported, although each component has pros and cons. By combining these elements, cells can be gated for different purposes. For instance, FSC-Height (FSC-H) by FSC-Area (FSC-A) is a commonly used gate for removing coincident events (failure to achieve single cell signal, the pulse is generated by two cells instead).

1.5.2 Nanoparticle tracking analysis (NTA)

Tracking analysis of nanoparticles makes it possible to detect fluorescence or dispersion from a single extracellular vesicle. However, label-stained extracellular vesicles such as green fluorescent protein (GFP) or fluorescein isothiocyanate-conjugated antibodies, phycoerythrin, and so on have their dye bleached either before the EVs reach the microscope's field of view or before the EVs can be tracked for an adequate length of time to accurately estimate their size. On the other side, the information acquired from flow cytometry is within ~1 μs, which is shorter than the time needed to bleach the dye. (Tatischeff I et al., 2012) NTA limits are speed and efforts involved with regard to flow cytometry in diagnostic usage.

1.5.3 Tunable Resistive Pulse

This technique is centred on several autonomous forces, being electrokinetic and nanopore-driven fluidic forces (Kozak D et al., 2012).

EV characterization challenges with tunable resistive pulse sensing include cluttering nanopore with large vesicles and limiting the use of the same buffer elements with calibration beads as the EV sample buffer. A "spiking" approach can be perceived to resolve this by adding the calibration beads to the measurement sample. (From Vrij J and others, 2013)

2 THE AIMS OF THE THESIS

- Aim nr. 1: Evaluate the suitability of flow cytometry for analysis of fluorescently la-belled extracellular vesicles.
- Aim nr. 2: Production of extracellular vesicles carrying GFP and mCherry fused MAGE-A4 and MAGE-A10 proteins through eukaryotic cell culture and their flow cytometric analysis.
- Etc.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Cell culture and medium

Cell line COP5EBNA (University of Tartu, Estonia), a mouse fibroblast cell expressing an Epstein-Barr virus nuclear antigen, was used in these experiments.

COP5EBNA cells were cultured in Iscove modified Dulbecco medium (IMDM) with 4.5 g/L glucose & L-glutamine without sodium pyruvate, 1% penicillin (100 U/ml) and streptomycin (100 ng/ml). All cells were cultured at 36o C in a 5% CO2 atmosphere (Panasonic MCO-19AIC (UV)-PE incubator). For extraction of cells, washing with PBS and collecting with trypsin/EDTA were implemented. Medium, used after transfection of plasmids contains a pre-prepared bovine serum that was centrifuged overnight in an SW28 rotor at 100 000g to release exosomes, and then filtered and added to the medium. This medium is referred as exo-free later in text.

3.1.2 Plasmids

Plasmids pQM-MAGE-A4, pQM-MAGE-A10, pQM mCherry, pQM-MAGE-A4-Cherry, pQM-MAGE-A10-Cherry, pEFGP-C1 are from the University of Tartu Technology Institute.

3.1.3 Transfection of eukaryotic cells

An electroporation method was used to introduce the plasmids into the cells. Cells were harvested from tissue culture, the medium was aspirated and the cells were washed with 2-3 ml of PBS. Subsequently, the cells were removed from the dish with 1 to 2 ml of trypsin solution (0.05% trypsin, 0.02% EDTA in PBS) and loaded into a centrifuge tube with the addition of trypsin equivalent IMDM medium. Cells were centrifuged for 5 min at 1000 rpm at 20° C (Eppendorf centrifuge 5810R). The supernatant was aspirated and 250 µl of medium per poration was added to the cells. An electroporation cuvette was loaded with 5 µl of carrier DNA (salmon sperm carrier DNA) and the required amount of DNA to be

examined. They were mixed with 250 μl of cell suspension. Electroporation was performed on a BioRad GenePulser Xcell apparatus at a capacity of 975 μF at 230 V, 4 mm cuvette parameters. Electroporated cells were transferred to 15 ml tissue culture tubes, pre-plated with 4 mL of medium with a pre-prepared bovine serum that was centrifuged overnight in an SW28 rotor at 100 000g to release exosomes, the serum was filtered and added to the medium. Tubes were centrifuged for 5 min at 1000 rpm at 20° C (Eppendorf centrifuge 5810R). The supernatant was aspirated; the cells resuspended in 1ml of exo-free IMDM medium and transferred to 100 mm diameter tissue culture plates containing 8 ml of the medium. The electroporated cells were grown for 72 hours at 37° C in a 5% CO2 atmosphere (Panasonic MCO-19AIC (UV)-PE incubator).

3.1.4 Isolation of extracellular vesicles

For isolation of EVs ultracentrifugation was implemented. After cells were grown for 72 h, media containing extracellular vesicles was collected for further centrifugation and one plate containing each transfection was analyzed with FACs.

First, to separate media from the dead cell and cell debris, it was centrifuged at 300×g for 5 minutes at 4° C (Eppendorf centrifuge 5810R) and poured into a new 50 ml tube. In the second step, the vesicles were centrifuged at 2000×g for 20 min at 4° C (Eppendorf centrifuge 5810R). At this stage, apoptotic bodies in the medium and larger membrane complexes and extracellular vesicles were precipitated. The precipitate (2K pellet) was suspended in 200 μl PBS. The vesicles were then centrifuged at 17 000×g for 15 min at 4° C in Optima L-90K Ultracentrifuge (Beckman Coulter) to separate medium sized extracellular vesicles or microvesicles. The supernatant is further purified in the ultracentrifugation tube for 20 min at 16 500 g 4° C (SW28 rotor). Pellet is dissolved in 200 μl of PBS. 2K and 16K pellets were centrifuged at 17 000×g for 15 min. The precipitate is suspended in 100 μl PBS.

The precipitate (120K pellet) separated in the fourth centrifugation step was suspended in 3 ml PBS and centrifuged at 120 000×g for 1.5 hours with a Beckman Coulter ultracentrifuge (SW55 rotor). The precipitate was suspended in 100 μl PBS.

For cell flow cytometric analysis, the cells were washed on a plate with PBS and removed from the plate with 1 ml PBS / EDTA. Subsequently, the cells were suspended, transferred to microcentrifugation tubes and centrifuged for 5 min at 4000 rpm. The separated precipitate was suspended in 1 ml of PBS.

3.1.5 Fluorescence-Activated Cell Sorting (FACS)

EGFP expression was analyzed by flow cytometry using an Attune NxT Acoustic Focusing Cytometer (Invitrogen Thermo Fisher Science) with associated software.

For EVs analyses of each fluorescently labelled vesicle, 5 µl of EV samples were mixed with 500 µl of filtered PBS in a new tube. Before recording, flow cytometer was washed with 1 ml of filtered PBS at 1000 µl/min. Flow cytometry parameters used in all experiments were designed by Dr Kurg's laboratory in the University of Tartu Technology Institute: FSC 100 Log, SSC 460 Log, BL1(GFP) 500, Threshold SSC 0.2, ASF 1.27. The result plots were applied with gates of different particle sizes (0.1-1 µm) by the use of FACS calibration beads. The gating was previously performed by Eve Toomsoo.

3.2 RESULTS

3.2.1 Flow cytometric analysis of cells

Mouse fibroblast COP5EBNA cells were transfected with plasmids pQM-MAGE-A4, pQM-MAGE-A10, pQM mCherry, pQM-MAGE-A4-Cherry, pQM-MAGE-A10-Cherry, pQM-EFGP-C1 to generate extracellular vesicles. Transfected cells were allowed to grow and to express fluorescent proteins GFP and mCherry and MAGE-A antigens fused with the named fluorescent proteins for 72 hours. The medium was then collected from the cells and extracellular vesicles were separated by centrifugation.

In order to verify successful transfection and expression of fluorescent proteins, cells were taken to Attune NxT Acoustic Focusing Cytometer for cell analysis with following settings: FSC 50V, SSC 320V, BL1 (EGFP) 260V and YL2 (mCherry) 400V.

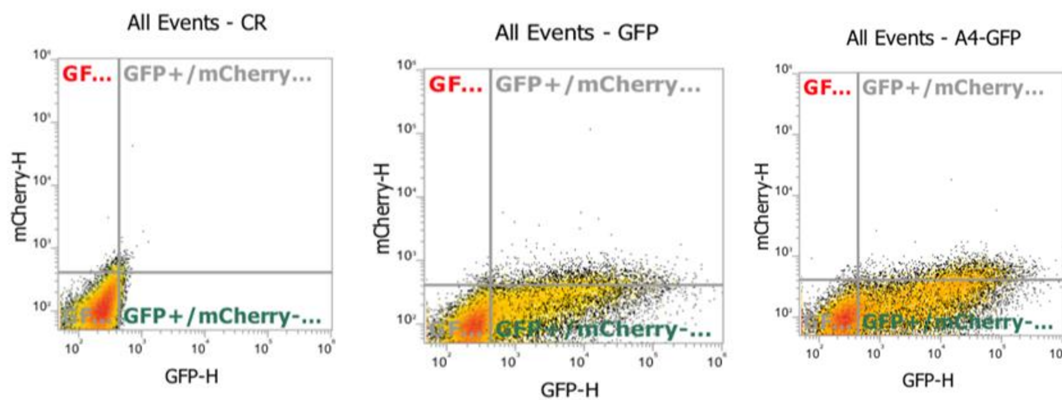


Fig 1. Density plots of COP5EBNA cells after 72 hours with GFP, GFP-MAGEA4 and a negative control (CR in plot headline). mCherry and its fusion proteins are not included.

Table 1. GFP, GFP-MAGEA4 and MAGEA10 fusion proteins fluorescence percentage in gated events.

Name	GFP+/mCherry- (%)
Carrier	1.080
GFP	39.020
GFP MAGE-A4	49.987
GFP MAGE-A10	42.827

Table 2. mCherry, mCherry-MAGEA4 and MAGEA10 fusion proteins fluorescence percentage in gated events.

Name	GFP-/mCherry+ (%Gated)	GFP+/mCherry- (%Gated)
mCherry	22.310	0.186
MAGE-A4-Cherry	23.282	0.153
MAGE-A10-Cherry	17.189	0.160

As it can be seen from Table 1 and 2, MAGEA4 melanoma-specific antigens had highest amount of labelled vesicles among gated events with GFP-MAGEA4 showing best expression.

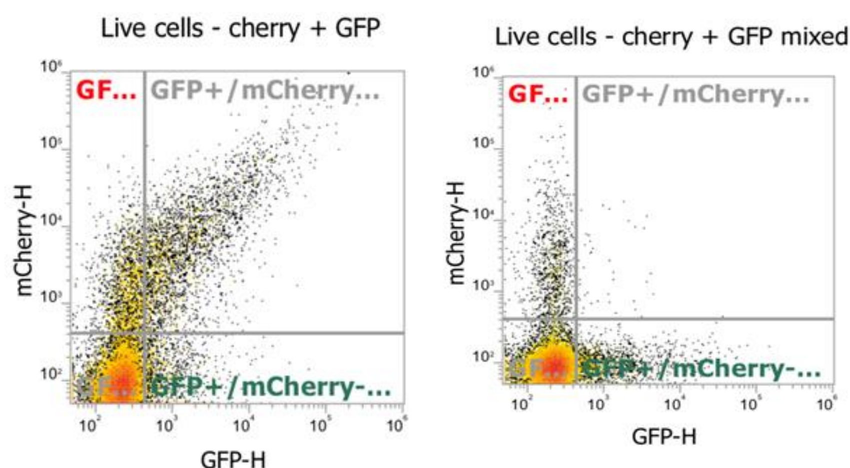


Fig 2. COP5EBNA cells with mCherry and GFP signals from cells transfected with mCherry and GFP plasmids (on the left) and a mixture of cells transfected with only one plasmid.

In order to test expression of two fluorescence proteins transfected together and compare levels of co-expression in single plasmid transfection, only GFP transfected cells were mixed in ratio 1:1 with single mCherry ones (Fig2).

Table 3. Comparison of the amount of mCherry and GFP fluorescent proteins in two plasmid transfected cells and a mixture of cells transfected with only one plasmid.

Name	GFP-/mCherry+ (%Gated)	GFP+/mCherry- (%Gated)	GFP+/mCherry+ (%Gated)
GFP, mCherry transfection	10.055	4.978	13.214
GFP, mCherry mixture	5.036	7.304	0.177

As can be seen from both table3 and fig2, transfection of two different fluorescent proteins leads to high co-expression levels which can further contribute to noise levels can misinterpret data. Similar experiments with mixing mCherry MAGEA4 and GFP MAGEA10, as well as mCherry MAGEA410 and GFP MAGEA4, showed similar results (not presented).

3.2.2 Analysis of EVs

To study the expression of the fluorescent proteins in Cop5 cells and their secreted EVs, an experiment was performed in which plasmids containing the DNA sequences of the GFP, Cherry and their respective fusions of MAGE-A4 or MAGE-A10 antigens were introduced into the cells. After DNA electroporation, the cells were grown in a 37° C incubator at 5% CO2 for 72 hours. The medium was then harvested from the cells and extracellular vesicles of different sizes were separated from it by centrifuging the medium at four different speeds. In addition, cells were harvested from the plate with 1 ml PBS-EDTA solution. Of the resulting vesicles 100 µl were analyzed by flow cytometric analysis at 25 µl/min with settings FSC 100V, SSC 460V, BL1 (EGFP) 500V and YL2 (mCherry) 560V. For all experiments data analysis comprised of log plots SSC-H versus FSC-H and SSC-H versus fluorescence (GFP-H or mCherry-H).

In order to identify presence of vesicles of 100-400 nm and more than 400 nm sizes, all samples were gated based on data from measurement of calibration beads. Fig3 is the recording of filtered PBS, serving as an illustration of the percentage of background noise from the solution. Fig4 is a Carrier (CR) as a negative control of fluorescence.

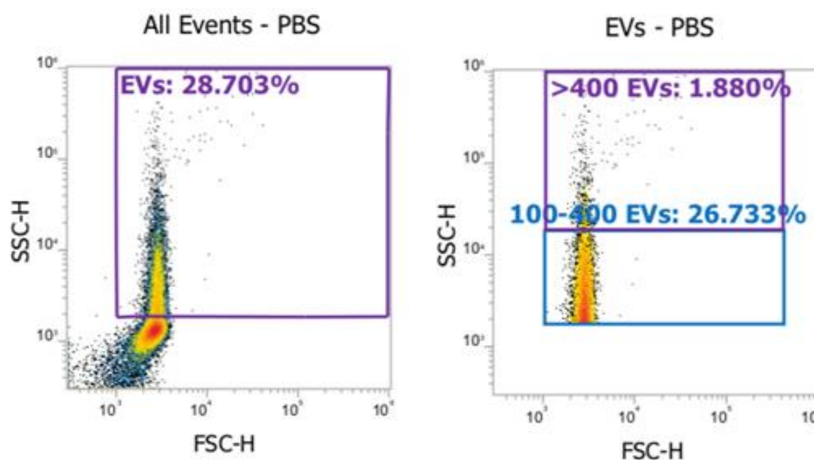


Fig 3. Density plot of filtered PBS solution in FACS.

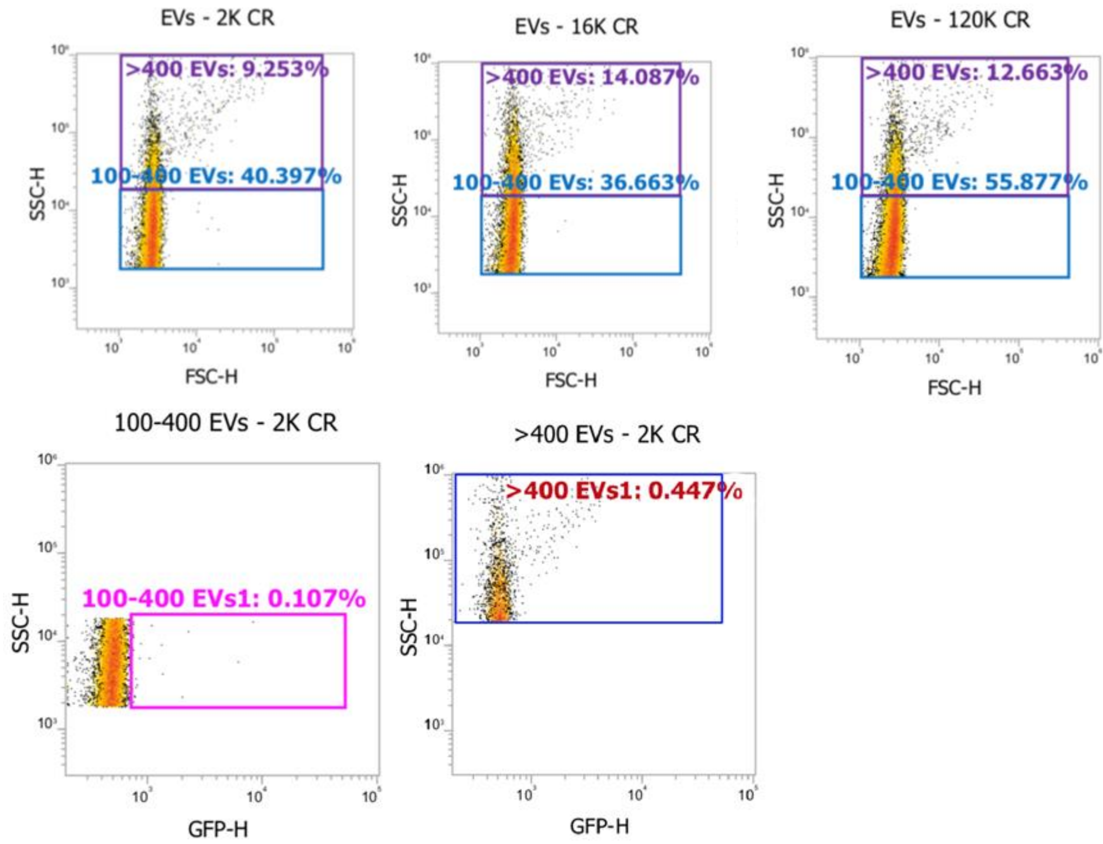
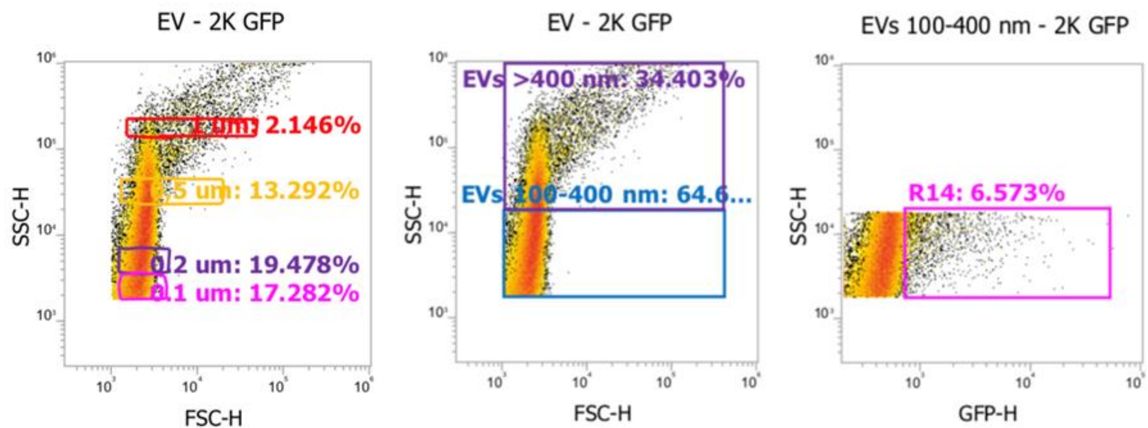


Fig 4. Carrier (CR on plot) EVs samples that was gated according to their size into EVs in range 100-400 nm and larger than 400 nm. Lower figure is an example of negative control for auto-fluorescence.



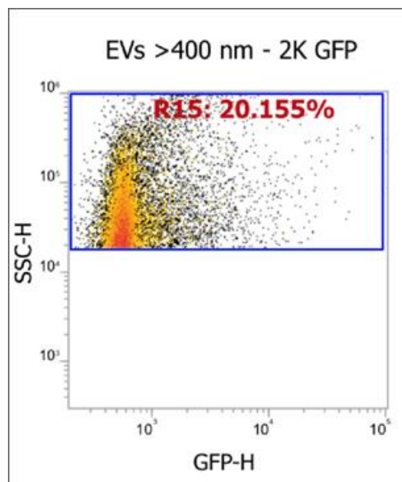


Fig 5. 2K of GFP-labelled extracellular vesicles gated according to their size with bead reference and then gated to identify fluorescence presence. R14 is a gate designed to include only fluorescence in vesicles of size in the range of 100-400 nm, whereas R15 illustrates fluorescence in vesicles bigger than 400 nm.

All labelled vesicles have been depicted in density plots with gates based on size: 100-400 nm and bigger than 400 nm, each plot was further gated by fluorescence presence by placing negative controls in order to exclude particles that do not contain GFP or mCherry proteins as illustrated with pure GFP-labelled vesicles on Fig 5.

In total, two experiments were conducted. Data A from first experiment contains 2K, 16K and 120K of GFP, GFP-MAGEA4 and MAGEA10 fusion proteins. Data B from second experiment is comprised of 2K and 16K sizes of above mentioned proteins together with mCherry, mCherry-MAGEA4 and MAGEA10 fusion.

Table 4. Percentages of total EVs in samples, 100-400 nm, bigger than 400nm and their respective GFP fluorescence expression for 2K, 16K, 120K of Carrier (CR) and GFP-labelled vesicles.

	2K Carrier (%)	16K Carrier (%)	120K Carrier (%)	2K GFP (%)	16K GFP (%)	120K GFP (%)
EV presence	49.880	51.117	68.833	46.607	48.303	73.723
100-400 nm EVs	80.988	71.725	81.177	66.092	85.47	76.6
100-40 nmEVs GFP	0.264	0.127	0.167	4.8	2.842	20.421
>400nm EVs	18.551	27.558	18.397	33.186	14.098	22.6
>400nm EVs GFP	4.827	2.130	1.974	14.93	7.244	40.774

Table 5. Percentages of total EVs in samples, 100-400 nm, bigger than 400nm and their respective GFP fluorescence expression for 2K, 16K, 120K of GFP-MAGEA4 and GFP MAGEA10 labelled vesicles.

	2K GFP-MAGE-A4 (%)	16K GFP-MAGE-A4 (%)	120K GFP-MAGE-A4 (%)	2K GFP-MAGE-A10 (%)	16K GFP-MAGE-A10 (%)	120K GFP-MAGE-A10 (%)
EV presence	50.743	57.630	77.237	51.640	60.370	68.963
100-400nm EVs	64.078	70.195	81.943	73.878	75.243	76.118
100-400nm EVs GFP	19.047	32.960	62.922	25.4	22.7	10.655
>400nm EVs	35.230	29.190	17.587	25.607	24.250	23.201
>400nm EVs GFP	37.125	59.738	85.325	62.1	32.21	28.6

The amounts of fluorescently labelled vesicles can be compared between tables 4 and 5. Presence of MAGEA4 and MAGEA10 in EVs were coordinated with being the highest as in COP5EBNA cells. With the decrease in size, fluorescence increased in GFP expression, notably 120K of the highest fluorescence. In particular, MAGEA family proteins have a better expression than pure GFP or mCherry, with MAGEA4 showing the largest percentage of 85 in 120K extracellular vesicles.

Table 6. Percentages of total EVs in samples, 100-400 nm, bigger than 400 nm and their respective GFP and mCherry for 2K, 16K, 120K of mCherry-labelled vesicles.

	2K mCherry (%)	16K mCherry (%)	2K mCherry-MAGE-A4 (%)	16K mCherry-MAGE-A4 (%)	2K mCherry-MAGE-A10 (%)	16K mCherry-MAGE-A10 (%)
EV presence	67.677	80.278	52.855	60.897	37.054	52.436
100-400 EVs	59.030	88.670	69.281	79.034	56.960	78.466
100-400 GFP	0.439	1.519	1.867	1.313	1.006	1.811
100-400 mCherry	0.245	0.385	0.206	0.228	0.113	0.216
>400 EVs	39.852	10.814	29.789	20.335	42.201	20.818
>400 EVs GFP	3.705	0.805	5.355	1.240	8.963	1.964
>400 EV mCherry	1.695	0.342	1.090	0.307	1.054	0.393

However, in the case of mCherry, the fluorescence expression rate was disproportionally

low as it can be seen from Table 6, despite the percentage of EVs in samples was quite distinguishable. Additionally, GFP expression was shown in many mCherry samples, providing fluorescent expression even higher than mCherry.

3.3 DISCUSSION

This Bachelors thesis was focused on the suitability to analyse extracellular vesicles by flow cytometric methods and challenges for obtaining credible results. Flow cytometry is a powerful and promising tool for analysis of extracellular vesicles in clinical research. However, as currently, with most EVs analysis methods, certain drawbacks exist and can be broadly divided into two groups. One obstacle lies in EVs size range being 30-2,000 nm in diameter, while most of the conventional flow cytometers can't detect particles smaller than 300 nm, thus resulting in low detection efficiency of < 2%, failing to capture most of the extracellular vesicles. Additionally, according to Mie theory intensity of light scatter signals depends on the relationship between the size of the particle to the wavelength of the laser. More specifically, particles with diameters that are larger than the wavelength of the laser will scatter light with a different pattern than particles that are smaller than the wavelength of the laser. (van der Pol E et al., 2010). To address this, individual researchers have developed optimized flow cytometry strategies. For instance, advancement in detection of small particles called "dedicated flow cytometry" is described by Vendula Pospichalova et al. and utilizes larger spectrum of light angles such as SALS (small angle light scatter), MALS (middle angle light scatter), and LALS (large angle light scatter) for relative particle sizing. Another strategy is called nanoscale flow cytometry (Aizea Morales-Kastresana et al, 2017) that implements certain software and hardware modifications designed to enhance EVs detection and low-end resolution. They stress the importance of having better control systems to be able to discriminate extracellular vesicles from noise while not disregarding them as cell debris. Moreover, these controls should be checked daily to examine how the instrument is performing for stable sample measurements. The coincidence of events is a well-known problem in flow cytometry in general, but it tends to show itself even more with small vesicles as it increases the tendency that many of them can get through simultaneously. This can lead to samples to show a bigger amount of larger particles as small ones aggregate together. Despite inconsistency with our results, a certain differentiation between vesicles of varying size can be seen. This suggests that the isolation protocol by ultracentrifugation can be further considered for research purposes. In all samples analysed the presence of particles in 100 - 400 nm size range was higher than in case of larger vesicles. Despite inconsistency with our results, a certain differentiation between vesicles of varying size can be seen. This suggests that the isolation protocol by ultracentrifugation can be further considered for

research purposes. In all samples analysed the presence of particles in 100 - 400 nm size range was higher than in case of larger vesicles.

The second problem is the presence of background noise from buffers, optics and etc. As can be seen from results, even filtered PBS solution still gives quite a large reading of noise for almost 30 per cent. In order to solve the problem of aggregating EVs, several other solutions have been used in research groups. It is also critical to perform data collection at a low flow rate for downgrading fluorescent noise, which could be result of particles moving out of laser spot`s range thus resulting in lower light scattering signal when recording at higher flow rates.

Another factor that needs to be considered in flow cytometry of vesicles is the choice of relevant markers (tetraspins, lipophilic dyes, etc.). In our experiments, COP5EBNA cells were shown to be successfully transfected with plasmids GFP, mCherry, their fusion proteins MAGEA4 and MAGEA10, and that the protein expressed from the plasmid switches to the naturally occurring EVs. In both GFP and mCherry fusions, MAGE-A4 acts more efficiently than MAGE-A10. The reason may be that MAGEA4 is a cytoplasmic protein that binds to the extracellular vesicles re-leased from the cell but MAGE-A10 is a nuclear protein that does not come into contact with extra-cellular vesicles in the cell enough to be present in their composition. Core localization may result in the protein not being released in large quantities from the cellular outlet because it is not located at the site where the cellular material is incorporated into the vesicles. In addition, while GFP and GFP-MAGEA4 were more efficiently expressed on smallest particles, GFP-MAGEA10 shows more expression in larger particles than in 120K.This behaviour can also be seen in a recent paper (Kurg, in press), although at this point, the current results do not give a definite answer to the question why this may be the case.

CONCLUSION

Notwithstanding the challenges that the small size of EVs and the background noise in readings present, the principles of flow cytometry are generally applicable to EV analysis and it is expected that as commercial instruments designed to measure small, dim particles become available, flow cytometry-based approaches will play an important role in understanding the origins, functions, and diagnostic and therapeutic significance of EVs in health and disease.

In addition, accurate, unbiased comparison of instrument detection capabilities would require concentration standards, and configuration and acquisition settings for all instruments probably can be further optimised for reducing noise. Thus, the data presented herein cannot provide final conclusions about the performance or limitations of an instrument model. However, the data presented in this thesis demonstrate that fluorescently-labelled EVs can be used for future studies aiming to further analyse different sized EVs. Here, our results indicate that the Attune NxT has a sufficient sensitivity to detect fluorescently labelled EVs at the differential sizes.

SUMMARY

Extracellular vesicles (EVs) are a family of small membrane particles that carry cell information that produces them. There is considerable investment in studying the role of extracellular vesicles in intercellular communication and implementing them for diagnostics in cancer, cardiovascular, and other biomedical areas. However, with an increase in the involvement of these particles in intercellular communication, and in utilizing their diagnostic and therapeutic possibilities in medical applications, comes the need for the accurate evaluation of their biochemical and physical properties. More precise high-throughput techniques are highly desired to investigate vesicles in order to further characterize and discriminate extracellular vesicles.

In this thesis, we have been able to demonstrate that fluorescent labelling of extracellular vesicles has been more successful with GFP and its MAGEA fusions than with mCherry proteins and highlight the presence of background noise from PBS solutions in cytometric flow data. We also showed that the protocol for ultracentrifugation displays adequate size separation of extracellular vesicles, which was detected in a flow cytometer by bead-based gating.

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