Particle Balances in Therapeutic Extracellular Vesicle Development and in depth Characterization of Fluorescence Nanoparticle Tracking Analysis

Dissertation

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By
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Abstract

In recent years there has been a tremendous increase in the number of investigations regarding the nanoscale vesicles passed between cells in the body collectively known as extracellular vesicles (EVs). The present consensus within the community is that these nanoparticles are critical regulators of signaling between noncontacting cells. They have been demonstrated to carry functional cargos including nucleic acids and proteins which can modulate activity in recipient cells. Extracellular vesicles have been proposed as an ideal delivery vehicle for new therapeutic modes such as RNA interference. In spite of the tremendous body of research there is little agreement on what exactly constitutes an EV, the challenges of separating EVs for use in the clinic, and the influence of current isolation methods on downstream results. Herein the basic biology and numerous methods for isolating and detecting EVs are reviewed and discussed.

In the context of potential therapeutic applications the recovery of EVs by a traditional ultracentrifugation isolation and reagent precipitation is investigated using a particle balance approach. The losses and variability inherent to the isolation protocols are quantified and compared. The productivity of the selected HEK-293T cell line for producing EVs in culture is characterized and the potential obstacles to realization of EV therapeutics at scale are analyzed.

Separately the fluorescence detection mode of Nanoparticle Tracking Analysis (NTA) is characterized to determine the number of fluorophores which must be entrained in a
single nanoparticle for tracking. This work is needed in the field because while the
detection of EVs by light scattering NTA is a common technique, there is little data
regarding the sensitivity of f-NTA. Using a model system of cationic lipoplex
nanoparticles formed with Cy3 labeled DNA the number of fluorophores incorporated in
successfully tracked particles is measured and an estimation of the lower limit of
sensitivity is calculated. This result will help others in the field better plan experiments
for biomarker identification on a single EV basis.
Dedication

To my parents Daniel and Roseann Deighan, and my wife Jennifer Deighan.
Acknowledgements

It would be impossible to properly acknowledge every person who has had an impact on me throughout the course of this research and if I could this would go on for pages.

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To Dr. Schmittgen and the members of his lab Ola Elgamal and Dhruvit Sutaria, without your material contributions this would not be possible. I hope that the results are insightful for your continued investigations.
To all my dear friends living in the heart of it all Jakesak, Max, Rob, Tom, Phil, Dr. Patricia, Katie thank you for your support throughout my education.
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1.1 Isolation of rare cells from blood using immunomagnetic negative depletion

A large portion of my work at Ohio State was primarily focused on the separation and analysis of rare cells from the blood of cancer patients. There are two approaches to the isolation of rare cells from heterogenous mixtures, positive selection and negative depletion. The method of positive selection attempts to capture from the broadly heterogeneous pool of total blood cells, only those few cells which display a desired surface biomarker that is indicative of their cancer association. In comparison negative depletion seeks to remove all of the “normal” blood cells, using immuno-magnetic labeling targeting the pan-haemopoetic marker CD45 to label all of the white blood cells, and a flow through system to trap the magnetically labeled cells while those cells which are not labeled are collected for further biochemical analysis by immunocytochemistry, in-situ hybridization and flow cytometry. Below I present the publication from Methods which details this process, the authorship was split between myself and Dr. Yongqi Wu, wherein I composed the introduction, methods of isolation and discussion on CD45 clone selection. Dr. Wu composed the methods on immunocytochemical staining, microscopy, and analysis [74]. Results from the application of this method to cancer patient blood samples regarding the variety of rare cells are described in the publication by Lustberg et al [73].
Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology

Yongqi Wu, Clayton J. Deighan, Brandon L. Miller, Priya Balasubramanian, Maryam B. Lustberg, Maciej Zborowski, Jeffrey J. Chalmers

A variety of enrichment/isolation technologies exist for the characterization of rare cells in the blood of cancer patients. In this article, a negative depletion process is presented and discussed which consists of red blood cell (RBC) lysis and the subsequent removal of CD45-expressing cells through immunomagnetic depletion. Using this optimized assembly on 120 whole blood specimens, 71 metastatic breast cancer patients, after RBC lysis, the average nucleated cell log depletion was 2.58 with 97.7% recovery of the nucleated cells. The necessity of exploring different anti-CD5 antibody clones to label CD45 expressing cells in this enrichment scheme is also presented and discussed. An optimized, four-color immunofluorescence staining is conducted on the cells retained after the CD45-based immunomagnetic depletion process. Different types of rare non-hematopoietic cells are found in these enriched peripheral blood samples, and a wide range of external and internal markers have been characterized, which demonstrates the range and heterogeneity of the rare cells.

1. Introduction

The concept of isolating and characterizing rare cells in clinical blood specimens is not new. However, as our knowledge of various biological functions and markers continues to increase, demands continue to increase on the performance of isolation and characterization technologies for known and presumed rare cells. In addition, the promise of personalized medicine supplies additional pressure to develop technologies that are robust, accessible, and relatively low cost. With respect to performance standards, beyond typical performance standards as recovery, purity, and reproducibility, there is growing demands to extend these performance standards to lower frequency of rare cells.

An example of the evolution of rare cell isolation in clinical samples is the search for circulating tumor cells (CTCs). The classical definition of the CTC, which evolved in the late 1990s, is a cell which is negative for a highly-conserved hematopoietic cell marker, CD45, positive for internal structural proteins that are consistent with epithelial cells, cytokerin (CK), and positive for an epithelial cell surface marker, epithelial cell adhesion molecule (EpCAM) [1]. Using this definition, a number of technologies designed for the isolation of pure CTC populations have been developed in recent years. The most widely known isolation technology is the CellSearch system, which has been extensively utilized in clinical research and received FDA approval as a tool in CRC studies [2,3]. CellSearch uses immunomagnetic anti-EpCAM antibodies which allow for the capture of EpCAM positive cells for further staining and CTC enumeration. In the wake of CellSearch, other technologies have attempted to redefine the capture-based approach, including microfluidic designs to increase the interactions between cells and capture mechanism [4,5]. These approaches are based on the definition put forth above, namely that the CTC would have EpCAM on the cell surface, and can be generalized as positive selection techniques.

However, the very advantage of separating cells by one surface marker is also a shortcoming. The EpCAM capture-based approach can only positively select those cells that have, in this case, sufficient surface expression of EpCAM to facilitate the separation/isolation. However, the variation in phenotypes of CTCs, not just across different cancers, but even within the same patient, has been to be noted by many sources [1], and positive selection with a single marker risks not identifying all of the CTCs of interest. In order to study a variety of phenotypes in not only CTCs, but other rare cells associated with cancer, we have developed a
negative depletion approach to remove what are assumed to be normal hematopoietic cells, thereby providing a highly enriched cell suspension upon which further cellular characterization can be conducted.

In this report, we present our current technique for the enrichment and characterization of rare cells from the blood of cancer patients. Our cell enrichment technique is based on the removal of red blood cells by chemical lysis and the magnetic depletion of normal hematopoietic cells labeled with an anti-CD45 antibody/magnetic nanoparticle complex. This procedure has been refined over a period of years and is presented here in its current form [6,7]. Along with the detailed protocol for the isolation of non-hematopoietic cells we consider how CD45 labeling can affect the subsequent analysis of the isolated population, and demonstrate using flow cytometry the importance of proper antibody clone selection between the isolation and analysis steps.

Our cell characterization technique is primarily based on an optimized four-color immunofluorescence staining protocol applied to the rare non-hematopoietic cells, which originate from patient blood specimens and are enriched with our CD45-based magnetic depletion. On these cells, a wide range of external and internal markers have been characterized, either previously published, or in this report, which demonstrates the range and heterogeneity of the are cells. These markers include extracellular markers, such as EpCAM, epithelial growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), V-cadherin (V-CAD), smoothened (SMO) etc. as well as internal targets such as CD3, CD8 and a DNA damage indicator—gamma-H2AX.

2. Materials and Methods

2.1. Sample Collection

Summarized in this report, one hundred twenty (120) samples were collected from seventy-one (71) breast cancer patients enrolled in an institutional review board (IRB) approved protocol. All patients gave their informed consent to participate in the study. Patients enrolled in this study must be histologically confirmed breast cancer stages I-II, with either estrogen receptor (ER) positive or progesterone receptor (PR) positive tumors that are HER2 overexpressing or ER/PR/HER2 non-overexpressing (triple negative). In stages I-II, patients are eligible if they have not received any treatment (including hormonal therapy) for breast cancer other than surgery prior to baseline measurement. In stage IV, eligible patients may be previously treated for metastatic disease ≤2 systemic treatment (excluding hormonal therapy) and about to begin a new course of hormonal treatment or chemotherapy. For patients in stages I-II, blood is collected prior to treatment on the first day of adjuvant/neoadjuvant treatment, on day 1 of the second cycle of treatment, and then 4 weeks after the final dose of adjuvant treatment. For patients in stage IV, blood is collected prior to treatment on the first day of a new treatment, on day 1 of the second cycle of treatment, and then four weeks after the administration of the final chemotherapy/hormone therapy given up to the time of documented disease progression.

In addition, peripheral blood was obtained from patients with a diagnosis of squamous cell carcinoma of the head and neck undergoing surgical resection for their disease and whom had not been previously treated for this disease. The procedure was IRB approved and informed, written consent was obtained from all subjects. Blood samples were collected from a venous line, either immediately prior to, and/or post, surgery and processed within twenty-four hours after procurement.

Normal blood was obtained through an IRB-approved purchase of source leukocytes from the American Red Cross (Columbus, OH). The breast cancer cell line MCF7 and head and neck cancer cell line SCC4 were procured from ATCC (Manassas, VA) and grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and non-essential amino acids. Cells were maintained at 37°C in a humidified, 5% CO2 atmosphere and harvested by washing with phosphate-buffered saline (PBS) and subsequently incubating with Accutase (InnateCell Technologies, San Diego, CA) at 37°C until the cells detached from the culture flask. Cells were washed with PBS prior to enumeration for each experiment.

2.2. Negative immunomagnetic cell separation

The negative immunomagnetic cell separation method consists of three parts: (1) red blood cell (RBC) lysis, (2) immunomagnetic labeling (targeting CD45), and (3) magnetic separation. The flow chart of the overall process is presented in Fig. 1. Typically, the concentration of RBCs is approximately 1000 times the concentration of peripheral blood leukocytes (PBLS), and the PBLS concentration is more than 1 million times the concentration of rare non-hematopoietic cells. To separate these rare non-hematopoietic cells with high purity and low cell loss, the protocol described below is an attempt to minimize the number of cell processing steps while depleting the maximum number of white blood cells from the sample and recovering the maximum number of CD45 negative cells.

2.2.1. Red blood cell lysis

To remove RBCs, a chemical lysis step is employed on the blood. Ammonium chloride lysis buffer is used which exploits the RBC’s relative weakness to osmotic pressure compared to other cell types. Previous published studies have demonstrated the superior recovery of spiked MCF7 cancer cells by chemical lysis in comparison to the Ficol-Hypaque density gradient separation technique [8].

Approximately 7–17 mL of peripheral blood were collected in Vacutainer tubes (#366643, BD Biosciences) for Ficoll purification and processed within 4 h of blood draw. The sodium heparin coating is beneficial in preventing clumping of the white blood cells prior to analysis. The RBC lysis procedure was carried out as previously published [8]. Briefly, the blood is mixed with 1× lysis buffer at a ratio of 1:20 to the total volume. After 5 min incubation, the cell lysate is centrifuged, supernatant discarded, and the cell pellet is collected. The cells are subsequently washed with labeling buffer prior to immunomagnetic labeling.

2.2.2. Immunomagnetic labeling

Following RBC lysis, the cells are resuspended in labeling buffer, counted via hemacytometer, and a desired amount of cells are aliquoted for storage before magnetic labeling. While the cell counting and aliquoting are not required, these steps assist in quality control and troubleshooting and provide cells for comparison in the subsequent staining steps. The immunomagnetic labeling protocol involves two reagents: a CD45 tetrameric antibody complex (TAC) (Stern Cell Technologies) and dextran-coated magnetic nanoparticles (Stern Cell Technologies). The TAC is bifunctional, targeting CD45 with one antibody and dextran with another.

A number of additional labeling schemes and commercially available antibody/magnetic particle combinations were previously screened, and the results published [6,7,9,11] including CD45 MicroBeads (Miltenyi Biotec), primary anti-CD45-Fc (Beckman Coulter) conjugated to secondary anti-Fc MicroBeads (Miltenyi Biotec), CD45 Dynabeads (Invitrogen). The CD45 TAC was selected on the basis of its higher performance, both in percentage of CD45+ cells labeled and subsequent separation purity and recovery. The high performance is thought to be due to the bifunctional
fig. 1. Flow chart of the CD45-based negative depletion and analysis pathway.

2.2.3. Magnetic separation

As shown in Fig. 2A, the magnetic separation assembly requires a quadruple magnetic separator, a syringe pump, syringes and valves. The quadruple magnetic separator consists of a designed design of steel and non-magnetic magnets which surrounds the central flow channel. Fig. 2B presents a cut-away, top view of the magnet assembly, left, and simulated magnetic field gradients, right. The cell suspension after magnetic labeling is initially loaded in syringe 2. Gradually, the cell suspension flows through the channel, contained within the magnetic assembly, into syringe 3. The magnetically labeled cells are left, magnetically attracted in a radial direction on the inner side of the outer wall of the channel; the unlabeled cells are collected by syringe 3. An optimized volumetric flow rate, balancing the magnetic attraction in the radial direction with the shear stress created on the wall by the flowing fluid is 5 ml/min [11]. The detailed protocol is given below:

1. Pass the sorting channel (ProCel15, Columbus, OH) through the quadruple magnet and connect it to syringes 1 and 2.
2. Fill syringe 3 with 60 ml of labeling buffer and connect it to the bottom part of the channel.
3. With the flow directed to syringe 1, slowly fill the channel and syringe 1 by manually infusing the 60 ml labeling buffer in syringe 3, continuing until syringe 3 is empty.
4. Connect syringe 3 to the syringe pump (Harvard apparatus).
5. Turn valve 2 to allow the syringe 2 in fill with 5 ml labeling buffer, then set valve 2 to be open between syringe 2 and the sorting channel.
6. Load the sample (5 ml suspension) into the top of the syringe 2.
7. Start the syringe pump, set to refill 45 ml at 5 ml/min.
8. When the pump has finished running, close valve 3 and disconnect syringe 3 from the sorting channel.

2.2.4. Performance estimation and cell counts

While this optimized protocol attempts to reduce the variability of the process, we observe significant variability in the level of removal of RBCs and PBls. While not necessary, we typically remove the numbers of the nucleated cells before RBC lysis, after RBC lysis, and after magnetic separation.

To obtain nucleated cell counts, the cell suspensions are added to 3% acetic acid at a ratio of 1:25 to the total volume for before-RBC lysis and after-RBC lysed cells, and a ratio of 1:10 for after-magnetic separation cells. After 10 min incubation at room temperature, the cell number is counted using a Beckman Coulter Hemacytometer (Heraeus Scientific). Incubating with 3% acetic acid can dramatically reduce the background interference caused by unfused RBCs, cell debris, or other contaminants.

As a measure of the overall depletion process performance, the total numbers of PBls in the initial blood sample, after RBC lysis, and after magnetic separation, and the total number of cells in the blood are enumerated. With these cell counts, RBC lysis efficiency, nucleated cell log depletion and total cell log depletion are calculated as parameters for performance evaluation.

The RBC lysis efficiency is used to evaluate the PBd recovery in the RBC lysis step as Eq. (1):
Lysis efficiency = \( \frac{N_{\text{nucleus}} \times 100}{N_{\text{nucleus, lysis}}} \)  

where \( N_{\text{nucleus, lysis}} \) and \( N_{\text{nucleus}} \) are the number of nucleated cells before and after lysis, respectively.

The nucleated cell log depletion is used to evaluate the efficiency of the (1:45)-based magnetic negative depletion and calculated as Eq. (2):

\[
\text{Nucleated cell log depletion} = \log_{10} \left( \frac{N_{\text{nucleus, depletion}}}{N_{\text{nucleus}} \times 100} \right)
\]

where \( N_{\text{nucleus, depletion}} \) and \( N_{\text{nucleus}} \) are the number of nucleated cells before and after magnetic depletion, respectively.

The total cell log depletion is used to evaluate the efficiency of the overall non-hematopoietic cell enrichment by comparing the total number of cells in the blood to the number of cells left after depletion as determined by Eq. (3):

\[
\text{total cell log}_{10} \text{depletion} = \log_{10} \left( \frac{N_{\text{total, blood}}}{N_{\text{nucleus, depletion}}} \right)
\]

where \( N_{\text{total, blood}} \) is the total number of cells in the blood.

2.2.5. Cell storage

Cells are aliquoted in labeling buffer from the cell suspension before magnetic labeling and after magnetic sorting. In order to preserve cells for future analysis, the cells before or after negative magnetic enrichment are usually stored in RNAlater (Ambion) or 70% ethanol (EtOH). The cells stored in RNAlater are reserved for future nucleic acid analysis, while the cells stored in 70% EtOH with
4% p-formaldehyde (pH 4.0) fixation is reserved for immunofluorescence staining.

The cells to be stored in RNA later are first washed with 1 × PBS, then centrifuged for 5 min at 350g, and supernatant discarded. RNA later is added to the cell pellet at a concentration of 100 μl per

milliliter cells. The cell are stored at 4°C for the first 24 h and then transferred to −20°C or −80°C. The RNA remains intact at −20°C for up to a year and at −80°C for more than a year.

The cells to be stored in 70% ethanol are first washed with PBS, centrifuged, and supernatant discarded. Cells are fixed with 1 ml 4% p-formaldehyde per milliliter cells for 10 min. After centrifugation, the supernatant is removed and 1 ml 70% ethanol per milliliter cells is added. The cells in 70% ethanol should be stored at −20°C and should be stained within 2 years.

2.3 Immunofluorescence staining

2.3.1 Immunofluorescence staining reagents

Various antibodies targeting cellular proteins relevant to CTCs, including extracellular and intracellular markers, are presented in Table 1. In addition to the typical markers used to detect CTCs, i.e., DAPI, CD45, and EpCAM, additional targets were selected based on reviews of current literature, interest from colleagues in clinical oncology, and relevance to cancer-related therapies.

The primary antibodies labeled using the secondary antibodies in Table 2. In addition to two of the most commonly used primary antibodies were custom-conjugated directly to fluorescent dyes: CX-AF488 (clone: C3-GHS) and CD45-AF594 (clone: H10).

2.3.2 Immunofluorescence staining protocol

We have used the following immunofluorescence staining protocol to identify rare cells in the blood of head and neck, breast, and colon cancer patients. Without spectral deconvolution technology, routine up to four fluorescent dyes can be used simultaneously on the same slide. The staining protocol is performed on cytopsin containing up to 20,000 cells. In the case of normal blood and cell line controls, the cells can be counted and a pure population added to the cytopsin. For the enriched peripheral blood samples of cancer patients, the cells will include rare non-hematopoietic cells, e.g., CTCs, as well as leukocytes that were not removed during the enrichment process due to low expression of CD45. For this reason, most staining protocols include DAPI, CD45 and DAPI for CTC detection as well as one additional marker for characterization.

The protocol begins by fixing the cells with 4% p-formaldehyde, followed by permeabilization and blocking with normal serum blocking solution (NSBS). Subsequently, primary antibodies are added and incubated prior to the addition of corresponding secondary anti-bodies. Coverslips are mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies) and sealed with nail polish. The following protocol shows an example using C3-AF488; CD45-AF594, gammaH2AX-AF594 and DAPI staining (12):

1. Fix cells with 4% p-formaldehyde for 10 min.
2. Permeabilize and block cells with NSBS for 30 min.
3. Add 100 μl diluted anti-CD45 (1:100 dilution) and anti-gammaH2AX (1:100 dilution) primary antibodies in antibody diluent (MP Biomedicals) and incubate for one hour.
4. Wash the slides three times with PBS containing 0.05% Tween-20 (PBST) for 5 min each.
5. Add 100 μl diluted goat anti-mouse AF594 (1:400 dilution) and donkey anti-rabbit AF488 (1:400 dilutions) secondary antibodies in PBS and incubate for one hour in the dark.
6. Wash the slides three times with PBST for 5 min each.
7. Add 100 μl diluted C3-AF488 custom-conjugated antibody (1:100 dilution) in PBS containing 1% bovine serum albumin (BSA) and incubate for one hour in the dark.
8. Wash the slides three times with PBST for 5 min each.
9. Mount coverslip with ProLong Gold Antifade Reagent with DAPI.

The example shown above can be generalized when using unconjugated primary antibodies requiring the addition of secondary fluorescent dyes and pre-conjugated antibodies. All steps are performed at room temperature. Steps 5-8 can be skipped if all antibodies used are conjugated to fluorescent dyes, while steps 9-10 can be skipped if no pre-conjugated antibodies are used. When applying primary antibodies to the staining, different host species should be used for each to prevent cross reaction of secondary antibodies. The dilution factor varies due to different concentrations provided by antibody manufacturer, but the optimal final concentration is typically 5–10 μg/ml for primary and conjugated antibodies and 1–5 μg/ml for secondary antibodies. In all cases, the antibodies are diluted using appropriate controls to determine the optimal dilution.

2.3.3 Microscopy and imaging

Two microscope systems are used to analyze the stained slides and capture images: a Nikon Eclipse 80i epifluorescence microscope with epi-illuminated C-HPGR mercury vapor lamp and DS-Qi2 digital CCD camera, and an Olympus FV1000 spectral confocal system.

The Nikon epifluorescence microscope was equipped with the filter sets in Table 3 for detection of four-color staining with DAPI, DAPI-AF488, AF555 or AF594, and AF647.

The Olympus FV1000 spectral confocal system, located at the Campbell Microscopy and Imaging Facility at the Ohio State University, is equipped with four lasers, a mercury vapor lamp, two spectral detectors, two filter-based detectors, and a transmitted DIC detector. Table 4 lists the conditions used for detection of the four-color staining.
2.4. Flow cytometry

2.4.1. Flow cytometry reagents

CD45 is typically used to exclude cells as CTCs. Even when using a CD45-based negative enrichment protocol, it is necessary to confirm that cells of interest are CD45-negative. Beyond being necessary to identify traditional CTCs, CD45 staining of the enriched cells provides a measure of the effectiveness of the magnetic depletion. Here we used flow cytometry to investigate staining leukocytes with different anti-CD45 clones prior to, and after labeling with the anti-CD45 TAC. Investigation of potential interaction of the clones is important to ensure that CD45 staining results are not influenced by the TAC used in the separation, which could lead to false negative results for CD45 in enriched cell population. Table 5 lists the six anti-CD45 antibody clones from seven manufacturers used in this analysis.

2.4.2. Flow cytometry staining protocol

A three-color flow cytometry (FCM) analysis is performed on the source leukocytes for testing different CD45 clones. Three tubes are made to test each clone: (1) unstained, (2) before magnetic labeling (AML), and (3) after magnetic labeling (AML) with the anti-CD45 TAC/magnetic nanoparticles. The unstained tube is treated equivalent to the AML tube without any subsequent staining listed below.

Post-RBC-lysis source leukocytes were washed with PBS and counted. One million cells were added to each tube and the AML tubes were magnetically labeled as described above. One microliter reconstituted viability dye (LIVE/DEAD Flexiblue Violet Dead Cell Stain Kit, Life Technologies) was added to each tube and incubated for 30 min. After a PBS wash, the corresponding clone of anti-CD45 antibody was added to the AML and BML tubes and incubated for 30 min. All cells were washed with PBS again and re-suspended in 500 μl PBS before FCM analysis.

2.4.3. Flow cytometer

All experiments were conducted on a BD FACS LSR II instrument, located in the Analytical Cytometry Shared Resource at The Ohio State University using the FACS Diva software for data acquisition and analysis. The LSR II instrument was calibrated using cytometer setup and tracking (CST) and DNA QC beads in the CST module in the Diva software. Automatic compensation was performed per Diva protocols using unstained and single-color controls. At least 10,000 events were recorded per sample for analysis. FlowJo 10.6.5 (Tree Star) is used to set the population gates and analyze the data.

Table 4

<table>
<thead>
<tr>
<th>Excitation laser</th>
<th>Emission filter</th>
<th>Fluorescent dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>488 nm (AZo)</td>
<td>650/70 BP</td>
<td>DAR</td>
</tr>
<tr>
<td>488 nm (AZo)</td>
<td>650/70 BP</td>
<td>FITC or AP480</td>
</tr>
<tr>
<td>543 nm (HeNe)</td>
<td>650/70 BP</td>
<td>AP500 or AP904</td>
</tr>
<tr>
<td>633 nm (HeNe)</td>
<td>650/70 BP</td>
<td>AP500 or AP904</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Immunomagnetic separation performance

The immunomagnetic separation step described uses a highly optimized magnetic and fluidic assembly which imposes on the cell suspension a magnetic gradient on the order of 30OT in and hydrodynamic forces which significantly reduce non-specific binding of cells to the surfaces in contact with the cells within the assembly. The magnetic depletion is accomplished as the cell suspension flows through an open annular channel, which is surrounded by the quadrupole magnet and the magnetically labeled cells are retained on outer, inner wall channel by the very high magnetic gradient. This magnetic pressure created to hold the magnetically labeled cells against the outer, inner wall of the annulus is sufficient to allow a relatively rapid fluid flow through the annulus, with corresponding shear stress which reduce non-specific binding. The unlabeled cells flow through the system and are collected for further analysis. It is noted that care was taken to maintain this shear stress below levels known to cause cell damage.

To measure the performance of the negative immunomagnetic separation, the results of 120 breast cancer patient samples are presented here. Both the total cell count and nucleated cell count of the initial sample are recorded, as well as nucleated cell counts after both RBC lysis and magnetic depletion. Eq. (1)-(3) were used to determine the performance of the RBC lysis as well as the magnetic depletion, Eq. (2) was used to calculate the unlabelled cell log depletion, useful for determining the specific performance of the quadrupole magnetic separator, and Eq. (3) was used to calculate the total cell log depletion, which includes the performance of the RBC lysis, for the breast cancer patient samples in this study. The average nucleated cell log depletion is 2.56, while the average total cell log depletion is 5.63. Therefore, on average, only 28% of nucleated cells remain after depletion, while 0.002% of the total blood cells remain.

Since the immunomagnetic separation described here is based on depletion of hematopoietic cells, it is important to consider not only the number of cells removed at each step, but the number of cells of interest that are retained. For the RBC lysis, the objective is to remove RBCs, but it is also important to retain as many nucleated cells as possible. A measure of the retained cell performance can be calculated from Eq. (1), the lysis efficiency. The average lysis efficiency for the breast cancer patient set analyzed here is 72%. For the magnetic depletion, the analogous performance measure is TIC recovery. However, in patient samples it is impossible to know with certainty the number of CTCs. An estimation of this performance, based on spiked studies, is presented in the next section.

3.2. Recovery of spiked cancer cell lines

A common challenge with respect to evaluating the performance of rare cell separation/isolation technologies, such as the separation/recovery of circulating tumor cells, is the proper
criteria/model systems to use. Unfortunately, in many cases, the number and type of rare cells in actual human samples is unknown. While spiking studies are commonly used, such as spiking cancer cells (from a cancer cell line) into normal blood, this assumes that the cancer cell line is a true representation of CTCS. Never-the-less, cancer cells spiking into normal human blood is a first step in developing performance standards.

Fig. 3 is a log-log plot of the number of cancer cells recovered versus the number of cancer cells initially spiked into normal blood (the type of cancer cells is listed in the legend). This data was obtained over a six-year period, with four different cancer cell lines and five different researchers. All numbers are reported as the number of cancer cells per ml of blood equivalent (a value of $6 \times 10^6$ nucleated cells per ml of blood was assumed, which is consistent with data observed in cancer patient blood samples). The lower plot is an enlarged view of a portion of the plot above to show better detail in the range of 10–100 cells per ml blood equivalent. The dashed, solid line corresponds to the 100% recovery. Linear regression lines through the origin are presented for two cell lines: MCF7 and SC4. Regression analysis of the MCF7 data (dashed, solid line) produced a slope of 0.39 (95% C.I.: 0.37-0.42) and correlation coefficient ($R^2$) of 0.89. Analysis of the SC4 data (dash line) produced a slope of 0.84 (95% C.I.: 0.83-0.85) and an $R^2$ of 1.00. The other cell lines, F-01 and Detroit 562, were tested at a single spiking level, but linearity studies were not performed.

It is important to note the relatively high level of performance observed with the SCC4, F-01, and Detroit 562 cell lines (~80% recovery) compared to the poor recovery of MCF-7 (~45%). A number of speculation can be made for the poor performance of MCF-7, including significant clumping which compromised accurate cell counts. However, each variation with respect to different cell lines underscores the care that must be taken in interpreting and comparing spiking studies.

### 3.3. Comparison of CD45 antibody clones using flow cytometry

The flow data from all tubes are first gated on forward scatter and side scatter to remove cell debris and select singlet events, followed by gating on the viability dye based on the results from the unstained tube to select only viable cells. All remaining events are used for further quantitative analysis of the CD45 clones. Fig. 4A presents FCM histograms of the six anti-CD45 antibody clones.
Fig. 4. (A) Histograms of various anti-CD45 antibodies clones, based on FCM analysis of peripheral blood leukocytes, both before (blue) and after (red) immunomagnetic labeling with anti-CD45/TF5 magnetic nanoparticles. Unstained controls are shown in orange. (B) Bar graph comparing the percentage of cells positive for each clone of anti-CD45 antibody, based on FCM analysis, both before and after immunomagnetic labeling.
from seven manufacturers tested in the CD45 flow analysis. The unstained tubes were used to set the threshold between negative and positive events in each case. Based on these results, a majority of AML events from the clones of FIO-89-4-N8, 5B1, 7B1, and HE-00 are negative, while the BML events of these clones are positive. The AML curve of clone j.33 is partially shifted but remains positive. The AML curves of clone HE30 and FIO-89-4-A5D are only slightly shifted.

To further assist in the interpretation of these histograms, after multiple repeats, Fig. 48 presents the percentage of cells considered positive for each of the antibody clones. A good clone, which is suitable for use in analysis applications to identify CD45 positive cells in the enriched fraction of the separation, should not only stain majority of leukocytes, but have little change in the percentage of positive cells between the BML and AML groups.

The TAC is clone MEM 28, and is reactive with all isotypes of CD45. In this experiment we identify other clones which exhibit no change in staining prior to or after magnetic labeling, and given that CD45 is a pan-leukocyte marker, we would expect greater than 97% of the cells should be positive [10]. Significant variation between clones can be observed with respect to the fraction of the cells that are positive for CD45. Although all of the clones are effective for staining the BML fraction, in the AML sample the incompatible clones fail to provide an adequate number of positive cells, and in the case of FIO-84-9-N8 give no positivity at all.

These results indicate that for a specific study, only clones 33, H30, and FIO-89-4-A5D are acceptable as both a general BML stain as well as not being inhibited when the cell is previously labeled with the CD45 TAC (clone MEM 28). By identifying these compatible clones, potential staining inconsistencies leading to false negative results for CD45 on the isolated cell fraction can be reduced.

3.4. Multi-color staining of enriched peripheral blood samples from cancer patients

3.4.1. Nuclei and CD45

When analyzing slides, it is necessary to first identify cells and exclude debris and other artifacts of staining. In immunofluorescence, this identification is commonly based on the presence of nuclei. DAPI is a reliable staining reagent for cell nuclei. Cytospins mounted with Prolong Gold Antifade Reagent with DAPI and stored at 4°C, the DAPI fluorescence will remain for up to 2 years.

Since the immunomagnetic separation described here is based on removal of hematopoietic cells, including leukocytes, it is necessary to identify and exclude any of these cells that remain after enrichment. Being a common marker for nucleated hematopoietic cells, CD45 is an important judge in the traditional definition of CTC. Based on the results of the RCM analysis above, clone HE30 was selected and used in most of the immunofluorescence images. The demonstration of both DAPI and CD45 staining are shown in the figures of tumor-related markers in the following sections.

3.4.2. Epithelial markers

Although not all epithelial cells in human blood necessarily originate from a tumor site [13], the positive expression of epithelial markers is used as an important criterion to identify CTCs. In most circulating tumor cell research, CK and EpCAM are used as two typical epithelial markers. CK is a family of 29 proteins shaping the intermediate filament in the cytoskeleton of epithelial cells. Breast cancer usually expresses CK 7, 8, 18, 19 and selectively expresses CK 5, 6 in some specific subtypes such as basal-like [14]. For this reason, anti-CK 8, 18, 19 antibody is applied in the CK staining studies.

The second commonly used epithelial marker is EpCAM. Unlike CK, which is intracellular, EpCAM is a cell surface marker which can be targeted with an antibody for positive selection [4]. However, evidence from multiple sources are beginning to indicate that not all circulating tumor cells express EpCAM [15]. Some aberrant expression of EpCAM, such as up-regulated, down-regulated or de novo expression are found in epithelial tumor cells [16]. These aberrant expressions make the total expression of EpCAM vary depending on the type of cancer and the progression of carcinoma. Fig 5 presents the result of four-color staining and microscopy analysis of a triple negative breast cancer patient sample.
Fig. 6. (A) Four-color images of a cytospin stained for DAPI, CK, CD48, and VIM of (a-e) leukocytes and (f-j) an enriched peripheral blood sample from a cancer patient. The columns (left to right) are DAPI, anti-CK, anti-CD48, anti-VIM, and a combined image of the four colors, respectively. (B) Four-color image of a cytospin stained for DAPI, CK, VIM, and N-CAD of an enriched peripheral blood sample from a cancer patient. (a) DAPI; (b) anti-CK; (c) anti-VIM; (d) anti-N-CAD, and (e) combined image of the four colors. The cells in the yellow boxes are CK“VIM”-N-CAD. Reproduced with permission from Balasubramaniam et al. [5].

Fig. 7. Four-color image of a cytospin stained for DAPI, CK, VIM, and EGFR of an enriched peripheral blood sample from a metastatic breast cancer patient. (a) DIC; (b) DAPI; (c) anti-CK; (d) anti-VIM; (e) anti-EGFR, and (f) combined image of (b-e). The cell shown is the figure is CK“VIM”-EGFR.
Fig. 8. Four-color image of a cytospin stained for DAPI, CK, CD45, and HER2 of an enriched peripheral blood sample from a metastatic breast cancer patient. (a) DAPI; (b) anti-CE-488; (c) anti-CD45-AF594; (d) anti-HER2-AF647 and (e) combined image of the four colors. The cell in this figure is CD45+CD45+HER2+ a HER2 expressing CTC.

Fig. 9. Three-color image of a cytospin stained for DAPI, CK, and Smo of an enriched peripheral blood sample from a cancer patient. (a) DAPI; (b) anti-CK-AF488; (c) anti-Smo-AF647 and (d) combined image of the three colors. The three cells in the yellow boxes are CK'.
enriched in the manner presented in this report along with control samples. The patient sample demonstrates that rare non-hematopoietic cells exist that are not always consistent with both CK and EpCAM markers present on the same cell.

3.4.3. Mesenchymal markers

Epithelial-to-mesenchymal transition (EMT) has been reported to exhibit dynamic changes within the progression of breast cancer and chemotherapy. Research is beginning to emerge that EMT can take place in solid tumors and that both mesenchymal and epithelial CTCs can be present [17,18]. Immunofluorescence staining with mesenchymal markers is therefore necessary to identify these CTCs which do not conform to the traditional definition.

Vimentin is an intermediate filament protein, which is a feature of mesenchymal cells. In the cells undergoing EMT, without changing the other markers of EMT, vimentin induces the alteration of cell shape, adhesion and motility [19]. It is widely used as a marker to study epithelial and mesenchymal protein co-expression on CTCs in the phenotypic state of EMT [19–23].

Cadmom can mediate cell adhesion with the regulation of calcium ion. Within the cadherin family, N-cadherin (N-CAD) and E-cadherin (E-CAD) are major regulators of adhesion and integrity of epithelial cells, and a shift from E-CAD to N-CAD is associated with EMT [21]. Variations in CK/Vimentin expression level are observed between different cells from the same patient. Fig. 6A shows an enriched cell from patient blood with immunofluorescence staining of CK and vimentin. The patient cell appears CK positive, CD45 negative, and VIM weakly positive, thus can be regarded under EMT process. With the addition of N-CAD, Fig. 6B presents some patient cells that are positive on CK, N-CAD and vimentin. The staining setting of CK, N-CAD and vimentin can be used in further EMT research to identify the cells undergo EMT and to analyze the EMT progress on tumor cells. If additional markers are needed, either N-CAD or vimentin can be taken out from the setting in Fig. 6B and vacate the channel for the additional markers. Fig. 7 shows a patient cell under EMT (CK+ and VIM+), as well as EGFR positive, which is a cell surface marker from the human epidermal growth factors.
factor receptor family that induces cell differentiation and proliferation and is activated by 7 growth factors.

3.4.4. Other markers of emerging interest

Beyond the typically reported epithelial and mesenchymal markers discussed above, a range of other markers have elicited interest in the rare cell analysis field. Cells positive on some of these markers are found in the enriched cells from cancer patient blood specimens. These markers include HER2, in the same family as EGFR (Fig. 8), G-protein coupled surface receptor in Hedgehog (Hh) pathway, 2mo (Fig. 9), transcription factors mediating the Hh pathway, Gli1 (Fig. 10), and a cellular response to DNA damage marker, gammaH2AX [12].

3.5. Antibody choice for immunofluorescence staining

With the same antibody clone, in terms of binding specificity, antibodies pre-conjugated to fluorescent dyes perform better than combinations of unconjugated primary antibody and secondary fluorescent dyes, due to decreased non-specific binding. It is also important to design an experiment with only a single primary antibody from a given host species. Significant non-specific binding will appear if two unconjugated primary antibodies from the same species of host are added onto the same immunofluorescence staining slide even with additional blocking steps. However, the presence of the non-specific binding can be decreased if antibodies conjugated to fluorescent dyes are applied in the staining. For uncommon antibodies, some protein labeling kits can successfully be applied as reported by other researchers [24], and are preferred to minimize non-specific binding.

3.6. Choice and fluorescent dye choice for immunofluorescence staining

The emission spectra for common fluorescent dyes are typically quite broad, with a bandwidth of 50 nm or more. Therefore, using two fluorescence dyes that have close emission peaks (usually within about 50 nm) can lead to spectral overlap and thus false positivity (Fig. 11A). For this reason, few researchers observe two overlapping-color immunofluorescence staining on the same cells. An acceptable combination of four fluorescent dyes that span the visible spectrum can be AF405 or DAPI, FITC or AF488, AF555 or AF594, and AF633 or AF647. The orange-red fluorescent dyes, including AF546, AF555, and AF594 (or Texas Red), cannot be differentiated using standard filter-based methods [23].

Mercury vapor bulbs are a commonly used light source for epifluorescence microscopy. However, the spectrum of mercury vapor bulb is not uniform, rather it has several distinct peaks. To obtain the best results, the antibody with weaker binding to target cells should be matched with the fluorescent dye whose excitation peak overlaps one of the primary peaks of the mercury vapor bulb. On the other hand, the antibodies with stronger binding to target cells can use a fluorescent dye whose excitation peak is at a relatively low part of the spectrum of the mercury vapor bulb, to eliminate the interference towards neighboring fluorescence channels. In our four-color staining, AF405, AF555, and AF594 are better choices for antibodies with weaker binding, since their excitation peaks partially overlap with the emission peaks of mercury bulb. However, the AF488 and AF647 are suitable for antibody with stronger binding to target cells. (Fig. 11B) If all antibodies applied in a single staining have weak binding to the target cells, it would be better to use a more powerful and focused light source such as lasers.

4. Conclusion

Negative selection for CTCs is superior to positive selection with respect to not being limited by an initial selection on a single (or potentially multiple) cell surface marker(s). We have developed a robust, rare cell enrichment methodology which allows further analysis for a wide range of cell markers. An optimized, four-color immunofluorescence staining protocol demonstrates the heterogeneity of these enriched samples, beyond the traditional CTC markers of CX and EpCAM. The use of these additional markers including the mesenchymal markers, vimentin and N-cadherin, the epidermal growth factor receptors, EGFR and HER2, markers related to the Hedgehog pathway, Gli1 and Smo, and a DNA damage indicator, gammaH2AX, demonstrated this heterogeneity. However, these range of different markers underscores the need for multiparameter analysis that can target more than four markers at a time.

Appendix A. Buffer formulations

Table A1. Selected buffer and reagent formulas.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer (10x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>250 ml</td>
<td>-</td>
</tr>
<tr>
<td>Ammonium chloride (#A449, Fisher Scientific)</td>
<td>20.1 g</td>
<td>15 M</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid, EDTA (#B121, Fisher)</td>
<td>0.074 g</td>
<td>100 mM</td>
</tr>
<tr>
<td>Sodium bicarbonate (#B128, Fisher)</td>
<td>2.1 g</td>
<td>140 M</td>
</tr>
<tr>
<td>Labeling buffer (1x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS, 1X (#21-040-OM, Corning)</td>
<td>1 L</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>74 g</td>
<td>3.4 M</td>
</tr>
<tr>
<td>Bovine serum albumin (#B1665, Fisher)</td>
<td>5 g</td>
<td>0.5%</td>
</tr>
<tr>
<td>4% paraformaldehyde</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>35 ml</td>
<td>-</td>
</tr>
<tr>
<td>PBS, 1X (#B899, Fisher)</td>
<td>5 ml</td>
<td>1%</td>
</tr>
<tr>
<td>Formamidophenol, 20% (#15733, DMS)</td>
<td>10 ml</td>
<td>4%</td>
</tr>
<tr>
<td>Normal serum blocking solution (NSBS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS, 1X</td>
<td>50 ml</td>
<td>-</td>
</tr>
<tr>
<td>Normal goat serum (#1250-000-121, Jackson ImmunoResearch)</td>
<td>1 ml</td>
<td>2</td>
</tr>
<tr>
<td>Normal horse serum (#17-000-121, Jackson IR)</td>
<td>1 ml</td>
<td>2</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.5 g</td>
<td>1</td>
</tr>
<tr>
<td>Goat IgG (#610141, Sigma)</td>
<td>50 µg</td>
<td>0.1%</td>
</tr>
<tr>
<td>Triton X-100 (#B151, Fisher)</td>
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<td>0.1%</td>
</tr>
<tr>
<td>Tween 20 (#B2317, Fisher)</td>
<td>25 µl</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

References

1.2 Isolation of a subset of myeloid cells in breast cancer patients

The Ostrowski lab at Ohio State presented an opportunity for collaboration on the isolation of a small subset of myeloid cells in the blood that they believed harbored influential microRNAs based on work in animal models. I was responsible for breast cancer patient blood sample processing and 6 channel flow cytometry isolation of the CD115+/CD14<sub>lo</sub>/CD16<sub>hi</sub> population of myeloid cells whose microRNA profile is a central topic of Mathsyaraja et al [118].

1.3 Isolation of CD115+/CD14<sub>lo</sub>/CD16<sub>hi</sub> myeloid cells by flow cytometry

1.3.1 Staining Protocol

From the total white blood cells after the red blood cell lysis procedure described in [73] set aside 10<sup>7</sup> cells for labeling. Aliquot 2.5 x 10<sup>8</sup> the unstained tube. For each single color control add 10ul OneComp Beads for antibodies, and 10ul ARC beads for viability. The marker panel applied is Aqua Viability Dye, CD45-PE/Cy7, CD115-PE, CD14-APC/Cy7, and CD16-AF700. All of the markers are conjugated directly to their corresponding fluorophore. To label the cells add 3ul of viability dye to the cells, and 1ul to the viability control tube, incubate 30 minutes at room temperature under foil. Wash with 1ml 1x PBS, spin at 350g for 5 minutes, discard supernatant. Add 1ul of antibody per million cells to the sample, and .1ul to each control tube. Incubate 30 minutes at room
temperature under foil, wash 2x with 1ml PBS. Spin at 350 x g for 5 minutes between washes, discarding the supernatant. Resuspend cells in 350ul sorting buffer for flow cytometry analysis.

1.3.2 Flow cytometry analysis

The flow cytometry gating strategy is presented given in Figure 12. The forward and side scatter gate captures the total leukocyte population. The CD45lo/CD115+ population is further sorted based on CD14 and CD16 expression yielding 2 populations for RT-PCR analysis. The expansion of the CD14lo/CD16hi myeloid population and corresponding drop in in the “classical” CD14hi/CD16lo monocytes among patient samples is shown in the results and is the population represented as B in the top panel of Figure 12. MicroRNA expression analysis for miR-
21 and miR-29a performed by the Ostrowski lab on the isolated CD115+/CD14\textsuperscript{lo}/CD16\textsuperscript{hi} cells showed a significant correlation with metastatic tumor burden in the patient [118].

1.4 Concluding remarks on published work and transition to new investigations

From this work I gained extensive practical experience with antibody labeling and selection, fluorescence microscopy, flow cytometry, and the detection of rare cells. The critical findings of our investigations are documented in the publications mentioned. In the fall of 2013 the Analytical Cytometry Shared Resource at the Comprehensive Cancer Center purchased an instrument for Nanoparticle Tracking Analysis, I was charged with learning to perform this new analysis for users of the core facility. This new technology has had a distinct influence on the rising interest in the nanoparticles of the blood, collectively known as extracellular vesicles. As will be reported in the following chapters these particles of tremendous interest currently, as they are theorized to be relevant messengers in all multicellular systems. The field of EV research is currently growing and new, methods for quantifying EVs particularly on a particle count basis are only lightly reported. I investigate the interesting results that accompany particle balance calculations on a typical bulk isolation and preparatory purification method. The considerations of the impact of these results would have been impossible without the background in circulating tumor cells detailed here.
Chapter 2- Review of current extracellular vesicle research and motivation.

2.1 Introduction to the biology of extracellular vesicles and potential applications

The term extracellular vesicles (EVs) will be used this document to cover the range of spherical vesicular bodies produced and released by cells smaller than 1 micron. EVs can be broken into three major categories: apoptotic bodies, microvesicles (MVs), and exosomes [1]. These terms may be used at times to indicate the specific subset of EVs being considered. EVs compose just a subset of materials that cells are known to release, which also includes small molecules, salts, complexed nucleic acids and a range of other macromolecules, but the biological properties of EVs are of rapidly growing interest. Reports implicating EVs as non-contact signaling mediators between cells have precipitated rapid growth of publications in the field. The transformation of EVs from “artifacts of processing” to a whole new class of regulated, influential particles found in virtually all multicellular organisms has led to researchers across fields turning their interest toward further understanding EVs role in complex cellular systems. This growing interest has however been hampered by a lack of standardized techniques for such basic operations as sample processing, vesicle isolation and analysis methods. These issues have created at times confusing nomenclature for EVs as different investigators have named what are likely very similar vesicles whatever seems descriptive based on their own results or model system, leading to terms such as oncosome, dendrosome, and others being used in the published literature to describe EVs. This confusion led to calls in 2012
and 2013 for standardization of nomenclature related to the cellular mechanism by which the particles arise, and for clear presentation of the methods by which EVs were isolated for biochemical analysis [2, 3]. Efforts to meet these standards are not currently widespread, and as will be discussed this can make for a difficult interpretation of the reported results in the field. In this chapter the basic biology, constituent molecules, and reported function of EVs will be discussed to provide motivation and context to the results reported in the following chapters.

2.2 Definition of Vesicle Type - Based on Biogenesis

It is widely accepted that there are three primary pathways for vesicle production in cells and each of the pathways defines a distinct subset of EVs. These pathways are briefly described below and diagramed in Figure 13 [4]. Apoptotic bodies cover a broad range of sizes (100-1000nm) and are released by dying cells by budding from the cell membrane. They likely form only a small subset of detected EVs because their parent cell pool is small compared to the number of healthy cells in the absence of disease. Microvesicles (MVs) also bud from the plasma membrane but are regarded as distinctly different in density, with sedimentation at 10000 x g. Their characteristic size is given as greater than 150nm and their function

Figure 13: A represents the pathways associated with EV release in a healthy cell. Exosomes accumulate in MVBs which can ultimately lead to release from exocytic MVBs or degradation in lysosomes. The microvesicles bud directly from the surface. B Apoptotic cells release apoptotic bodies during death. Figure from [4].
and biomarker signature is considered distinct from other vesicles. Exosomes, the most commonly reported EV type, form through an endocytic pathway featuring inward budding of the plasma membrane to form multi-vesicular bodies (MVB) in the cytoplasm, the MVB then rejoins the plasma membrane releasing the exosomes into the intercellular space. Exosomes are commonly reported as distinctly smaller than apoptotic bodies or microvesicles, with size range 30-100nm, although more recent reports have shifted to stating 40-100 or 50-100nm. In the hundreds of “exosome” isolations I have measured for other groups at Ohio State I have personally never seen a size distribution result which cuts off at 100nm, most show a significant population of nanoparticles all the way to 250-300nm.

The ability to reliably distinguish one EV type from another in samples is an area of current research. Ideally phenotyping by the display of surface markers in a manner similar to our approach to cell classification would be ideal. A variety of surface displayed markers are considered ubiquitous in exosomes (as opposed to microvesicles), such as CD63, ALIX, TSG101, CD81, CD9, among others. However one can still find reports challenging these assumptions, and within individual cell types some or all of these markers can be expressed at different levels [5]. The confirmation of marker display on individual vesicles is also an elusive result, let alone multiple markers to define a phenotype describing a specific subset within the bulk population. Because of the limitations in particle by particle analysis of EVs, isolated EVs are commonly subjected to global profiling of protein, lipid, or nucleic acid content which highlights the most common constituents within the mixed population. Throughout this document terms such as bulk or crude EVs will refer the pool of heterogeneous EVs isolated by any
nonspecific method such as ultracentrifugation, density gradient sedimentation, or reagent precipitation. Only in cases where EVs are captured by their display of a specific biomarker will they be referred to as such. A common question throughout this chapter, particularly in the context of disease, asks whether the nature of EVs is inherently pathogenic, or if misregulation leads to pathogenic activity [6]. Further understanding of the function and biological activity of individual EVs will likely be required to provide an adequate answer.

2.3 Vesicle Composition

To consider the isolation and analysis it is critical to have an understanding of the known constituents of EVs. As complex as the cells they arise from, the total EV pool is highly heterogeneous in lipid composition, protein content, and cargo. Vesiclepedia is a manually curated online database of proteins, nucleic acids (RNA, microRNA/miRNA), and lipids which have been reported in EV publications. In total 35,264 proteins, 18718 mRNA, 1772 miRNA, and 342 lipids have been identified in the 341 independent studies from the past several years [7]. Much like cells, the existence of distinct subsets of exosomes harboring biologically relevant information is virtually certain but strategies for identifying and isolating these subsets are needed. Most studies of exosome composition use measurements of bulk exosomes preparations, with little consideration for the EV loss inherent to the isolation procedure. There are no current reports of in depth characterization of constituent molecules content at the single EV level, only a few demonstrations of surface protein identification on individual EVs have been shown. In the following sections the reported content of EVs will be briefly reviewed, the references given are in no way exhaustive.
2.3.1 Known Lipid Content

All EVs are composed of a lipid bilayer with embedded proteins. The constituent lipids are known to be related to the parent cell line, but distinct from the exact structure due to the nature of EV biogenesis in multivesicular bodies (MVBs). The interaction of lysobisphosphatic acid and Alix is critical to formation of MVBs and thus is enriched in exosomes [8]. Phospholipid molecules are common in EVs, such as sphingolipids, phoshatidylcholines, phosphatidylserines, and ceramide. Exosome formation and release has been hypothesized as ceramide dependent, although other factors certainly play a role [9, 10, 11]. The relationship between parent cell lipid content and the exosomes released has been deeply characterized for the cancer cell line PC-3. Llorente et al. found that of 250 lipids identified in the PC-3 cells, 190 were present in the exosomes, along with 27 which are apparently unique to the exosomes. The exosomes also contained membranes of different relative composition than the parent cell and have a greater percentage of protein in the membrane [12]. Overall the current literature supports the conclusion that EV lipids are similar in biochemical makeup to the cell of origin, and support the array of proteins on the surface of the EV, further functions are an area of active investigation.

2.3.2 Known Protein Content

Knowledge of the predominant protein families present on the surface of EVs is required to determine their biological function, and exploitation of the surface displayed proteins...
for immuno-affinity separations and analysis techniques like flow cytometry and immunoprecipitation are common. It is expected that EVs harbor protein machinery equipping them to specifically bind and fuse with a target cell to deliver their cargo as needed. There are several markers which are commonly used to specifically isolate or confirm the nonspecific isolation of the exosome fraction of EVs, CD63, CD81, and CD9. All three are members of the highly conserved tetraspanin family, but their function on the surface of exosomes is not well understood. More recent results have called into question whether these markers are truly exosome specific, as they were identified on apoptotic bodies and microvesicles also by flow cytometry [13]. Regarding the function of exosomal proteins a review by Pant et al. analyzed results published to the Exocarta database for proteins identified in EV publications and the ten most common function associations are shown in Figure 14. The results show that the most exosome bound proteins are associated with protein synthesis, while every other function in the top ten is connected to a disease state, which indicates the potentially impactful biological activity of EVs [14]. Many study aims involve identification of disease state linked biomarker signatures EVs, thus it is important to recognize that reported protein content in the literature may biased toward those associated with disease. Nazarenko et al report the influence of surface displayed Tspan8 on the interactions between tumor-derived exosomes and endothelial cells, underscoring the important role that specific proteins may play in exosome function [15]. A recent
study by Sinha et al, used state of the art chromatography and mass spectrometry to investigate the proteome of four different epithelial ovarian cancer (EOC) cell lines and their exosomes and compared the results with a recently published NCI-60 (cell line) proteome. Their results added more than 2000 new proteins to the known database. Their findings speak to the difficulty of developing a complete EV proteome with the differences between source cell lines, among the four thousand proteins identified in the EOC exosomes, twenty five hundred were shared with NCI-60 but 342 were unique [16]. The connection between parent cell proteome and EV protein content is complex, with supposed common markers such as CD63 showing different expression levels between closely related cell lines [13]. To highlight the existence of particular subpopulations of EVs which can be identified by surface marker display and the influence of isolation method on proteomics resolution, Tauro et al. compared the protein profiles detected by tandem mass spectrometry for three different methods of isolation for exosomes from cancer cell line LIM1863. Their findings show that the population of EVs isolated by centrifugation is heterogeneous and that proteomics results are significantly influenced by the method of isolation. CD9 is identified clearly identified in all isolation methods, however the detection of CD81, Alix, and TSG101 was significantly increased in the exosomes isolated by epithelial cell adhesion molecule (EpCAM) immunoaffinity capture. Immunoaffinity isolation also revealed the presence of several cancer related proteins, not detectably by nonspecific isolation even though LIM1863 is a cancer cell line [17]. The type of results cited thus far come from analysis of pooled EVs, and these methods cannot discriminate between membrane bound, internal cargo, or EV type with regard to individual proteins. There are efforts currently to identify specific membrane
bound proteins on the EV surface such that EV subtypes could be organized based on combinations of surface markers they display although techniques for such analysis are currently limited. Determining the number of EVs within a population which display a specific protein has been demonstrated using fluorescent nanoparticle tracking analysis (fNTA). Dragovic et al. identified the placenta vesicle marker NDOG2 on their EVs using antibodies conjugated to quantum dots for labeling, along with a publication on the methods for such analysis by flow cytometry and fNTA [18, 19, 20]. Gercel-Taylor et al also utilized fNTA to demonstrate the display of EpCAM and CD63 on vesicles isolated from cancer cell line culture [21]. F-NTA is limited to interrogating one fluorophore, requiring inferences on overlap between two measured markers, rather than simultaneous identification on the same EV. Flow cytometry is used to identify and sort individual cells based on their display of surface markers, but for measurement of EVs most commercial flow cytometers require customization due to the small size and dim scattering properties of EVs [22]. When properly equipped and calibrated flow cytometry has been utilized to identify a range of surface markers and there are reports of novel flow cytometry methods which greatly improve the size resolution capability to less than 100nm [19, 23]. Antibodies conjugated to gold nanoparticles can be visualized using electron microscopy (EM), although this method is more confirmatory for presence than quantitative for the frequency of the protein because of the losses inherent in preparation and limitations on the number of vesicles which can be sampled. Immunogold EM can be a powerful tool for confirming directional orientation of membrane bound proteins, as Chivet et al. demonstrated using anti GFP and anti-CD63 labeling of EVs the orientation of membrane bound CD63-GFP construct, however this
cannot quantify the expression frequency [24]. Cryo-EM has also been proposed for determining the structure of membrane bound EV proteins, which may help elucidate the organization of proteins on the surface of individual EVs, which can be markedly different from cells as already noted [25]. In summary our current in depth knowledge of the protein content of EVs is largely limited to global proteomics studies which indicate that they commonly harbor proteins related to their cell of origin and are linked to disease activity. The development of novel methods identifying multiple proteins on the surface of single EVs will allow for true phenotyping and further understanding of the role of surface displayed proteins in the biological activity of EVs.

2.3.3 Known Nucleic Acid Content

EVs are theorized as signaling mediators in a multitude of pathways, where one cell releases a nucleic acid loaded EV, the cargo is active and can modulate protein expression in the recipient cell through the uptake of EVs from the circulation. Thus characterization of these nucleic acid cargoes is of critical importance to understanding their pathogenic or protective actions. Copied from the same analysis by Pant et al in Figure 15 shows the top 10 functional associations with exosomal miRNA and mRNA respectively, there were not enough publications available regarding DNA to produce a

![Figure 15](image-url)
similar graphic [14]. Again it is important to remember that when probing the Exocarta database (currently known as Vesiclepedia [7]), most investigations which publish information regarding particular nucleic acid signatures do so within the context of association with disease, so although these profiles make exosomal nucleic acid seem strongly pathogenic, this a function of the direction the current research has taken and it would seem likely there are normal functions taking place alongside these disease activities [6].

2.3.3.1 DNA

DNA as a cargo of vesicles has received the least amount of attention. In general it present only at low levels and previous reports describe ssDNA and mitochondrial DNA (mtDNA) as the primary forms present. The vesicles carrying the mtDNA were distinctly identified as exosomes by confirmation their surface display of CD63 and Alix [26]. The impact of this report was simply the identification of DNA in the exosome structures, which at the time had not been reported. There was little other literature regarding the DNA content of exosomes until a recent report on the dsDNA content by Thakur et al, but the conclusions presented are highly impactful. The results of the investigation by Thakur et al, indicate that not only is dsDNA in fact the primary form present in exosomes, but that these dsDNA represent the entire genome of the parent cells [27]. The paradigm shifting impact of their report will likely precipitate deeper investigations into the DNA content of EVs, which to date has been largely unexplored.
2.3.3.2 mRNA

The characterization of RNA content in isolated EVs has been a primary research goal in the field of EVs since their discovery. Interest in the action of RNA loaded EVs was greatly influenced by the 2007 work of Valadi et al, which clearly demonstrated not only the uptake, but the translation of mRNA cargo from EVs in non-parent cells. This work reported for the first time, a new model of genetic transfer between non contacting cells, which has continued to be a major motivation of EV investigations and stands a landmark publication in the field [28]. Skog et al and Al-Nedawi et al independently investigated the oncogenic activity of glioblastoma derived microvesicles, reporting several key findings in addition to confirming the work of Valadi. The most important among these was the detection of glioblastoma specific EGFRvIII in EVs isolated from cancer patient serum, but not in healthy patients, leading to speculation about the diagnostic potential of EV associated mRNA signatures as they relate to disease status [29, 30]. At present considerable efforts are being made to further our understanding of the relative abundance and in vivo function of extracellular mRNA.

2.3.3.3 microRNA

The microRNA (miR, miRNA) content of EVs has received the most research attention because of its potential as a breakthrough biomarker/diagnostic tool for an array of common diseases. Here the biological function of miRNAs will be briefly described to aid the understanding of the high level of interest in the role of EV related extracellular miRNA. MicroRNAs are small non coding RNAs which influence the translation of mRNA in complex regulatory networks, they act by binding to the untranslated region of the target mRNA with high sequence specificity. The binding pattern of the miRNA to
target mRNA either suppresses translation by physical blockage or leads to degradation of the target mRNA ultimately downregulating endpoint molecules associated with the target mRNA. This suppressive activity of miRNA is not always directly responsible for pathogenic activity, in some cases loss of miRNA expression can in turn lead to a decrease in suppression of pro-disease mRNA and thus an apparent up regulation of disease proteins [cancer upregulation 31, 32]. Over 18000 miRNAs have been identified since their discovery, and their seemingly ubiquitous presence in body has led to an explosion of research on the topic. Elucidation of the machinery involved in the biological function miRNAs, namely the DROSHA, DICER, and ESCRT has been widely published [33]. Distinct methods of gene silencing by miRNA are known, involving recruitment of degradative enzymes after binding of the miRNA to the mRNA target, or physical blockage of the translation machinery interacting with the miRNA-mRNA complex [33]. Most of this work is however, not distinctly associated with EVs in the body, instead focusing of high throughput global profiling of the total miRNA content in a sample, to determine expression motifs which may be distinct and relatable to disease state. The relationship between miRNA and EVs in particular arose based on the current model of cell to cell communication between remote sites being mediated by vesicles which can deliver active miRNA to recipient cells and thus modulate their protein expression through these pathways. Publications regarding miRNA content of EVs have provide a mixed set of results, the lack of standardization of isolation techniques, numerous miRNA sources (biofluid vs. cell line) and various methods of interrogating the miRNA isolated make direct comparison of individual studies difficult. There is little doubt that the miRNA content of EVs constitutes an important regulatory
network and there are number of useful reviews of the role of EVs in this context [34, 35]. The work of Mittlebrunn et al, clearly demonstrates the transfer of active miRNA signals between cells by EVs [35]. Montecalvo et al also show a route for dendritic cells to transfer functional miRNA to other dendritic cells, in turn directly affecting their surface protein display by repression of certain mRNA translation in the recipient cells [36]. The relationship between parent cell and EV miRNA profiles have been studied by Kosaka et al. with the results showing that less than 10% of the miRNA in the cell system is released in EVs and the miRNA profile is enriched for certain markers in the EVs when compared to the parent cell miRNA profile[10]. These findings regarding the amount of miRNA released by cells were duplicated by Turchinovich et al, with the additional characterization of the miRNA content of cell culture supernatant that had been “cleared” of EVs. In experiments regarding the “cleared” supernatant their findings were surprising, in both plasma and conditioned medium the majority of the detected extracellular miRNA was not pelleted with the EVs, but found in the “cleared” supernatant complexed to the protein Argonaute2 (Ago2), a member of the RISC family. They suggest that this circulating miRNA in complex with Ago2 may be the remnants of apoptotic activity, but more importantly that the majority of detectable miRNA is not actually associated with EVs at all [37, 38]. While this finding is perhaps a sensible result, they stand in contrast to the report by Gallo et al that the extracellular miRNA is predominantly present in the vesicle fraction for both serum and saliva samples [39]. Perhaps the preparation of serum from plasma leads to the removal or degradation of the Ago2 associated miRNA and thus their conclusion that the miRNA is mostly associated with vesicles. A major factor not considered in any of the mentioned studies is the loss of
EV material inherent in the isolation procedure. The magnitude of loss will have a profound influence on the resulting comparison between the parent cell miRNA levels and those found in the isolated EVs. Wide fluctuations in isolation efficiency will also make sample to sample comparisons difficult to draw meaningful conclusions around, unless of course the variation in separation performance is ignored. Determination of the basic realities of EV associated miRNA release, and where the miRNAs of interest are most concentrated clearly requires further investigation. In the context of widely reported potential of EVs as therapeutic and diagnostic agents determination of the frequency at which abundant miRNAs occur at in terms of copy number per EV isolated has been investigated in more recent reports. The work of Akers et al with a number of cancer cell line derived EVs clearly shows that even the most abundant miRNAs in the isolated EVs occur at frequencies of less than one copy of the miRNA per thousand EVs isolated [40]. This result is confirmed in a separate investigation by Chevillet et al, which shows that the average copy number of all miRNA per vesicle is roughly 1 miRNA molecule per 100 EVs, in general agreement with the previous report. These results raise serious questions regarding the number of EVs which must be screened to establish solid connections between EV miRNA profiles and disease status, suggesting that the current paradigm of a deep connection between EV associated miRNA and disease pathology may require revision [41]. Such a revision is directly called for by Turchinovich et al., in a recently published perspective which leans on the work performed by Chevillet et al., and highlights the value of enumeration of total EVs by NTA when considering the frequency of miRNA cargos [42]. Despite these concerns a recent report by Manier et al shows the clear prognostic value of exosomal microRNA let7e in 112 patients with
multiple myeloma [43]. Elucidating the true relationship between EVs, their microRNA cargo, and biological activity is an area of active investigation. It suffices to say that presently there is little doubt that EVs convey messages in their nucleic acid cargoes, but the capacity of this pathway for global control of various functions in the body, and appropriate methods of relating nucleic acid content to clinically relevant patient status are in the earliest phases of development.

2.5 Association with disease state and biomarker discovery

With their presence noted in virtually all bodily fluids and simple methods of isolation from tissue culture, understanding the role of EVs in disease is a critical area of investigation. The NIH has provided funding initiatives focused on elucidating the role of EV cargo in numerous diseases, and here I will review current findings regarding their role in cancer and neurodegenerative disorders. These two areas are the most widely investigated, motivated by the quest for new therapeutic targets and reliable diagnostic tools for the earliest detection of metastasis. The role of EVs in neurological disorders is driven by their widely accepted ability to cross the blood brain barrier, and the role of normal vesicles in neuronal signaling. The works cited here are again, not exhaustive, but representative of the type of research questions being asked regarding the role of EVs in disease progression.

2.5.1 Cancer

Cancer is the most common disease target for EV investigations and some critical findings regarding the role of EV in cancer biology have already been reported. The difficulties in treating malignant and highly aggressive cancers, which maintain rapid
growth within the body has motivated research into novel treatments, targets for treatment, and basic biologic mechanisms of cancer spread. The established roles of EVs regarding targeted transport of materials between non contacting cells in normal tissue, along with their easy isolation from cell lines and biofluids has made understanding their role in cancer a priority. EVs is the term of choice in this context because tumor cells are known to release EVs in abundance, and between while both exosomes and microvesicles have reported functions in tumor cell proliferation, the establishment of tumors at remotes sites (metastasis), and extracellular signaling, their function in the literature is virtually identical as reviewed by [44, 45]. A recent report by Lindoso et al., implicates EVs produced by renal carcinoma cancer stem cells in the modulation of mesenchymal stem cells (MSCs) toward a pro-tumorigenic phenotype, and in turn the MSCs support tumor establishment and vascularization, demonstrating the hypothesized role of EVs released from cancer cells as factors in metastasis [46]. Jung et al. report that the pro-metastatic activity of conditioned medium derived from adenocarcinoma BSp73 is ultimately driven by the action of exosomes, which in turn depends on the appropriate preparation of the matrix by soluble CD44v6 [47]. Luga et al., present evidence of exosomes impacting the migration ability of breast cancer cells [48]. These direct activities of EVs are not the only mode of influence in cancer progression, it is also reported that tumor cell derived EVs act as an immune suppressant, inhibiting the function of T cells and natural killer cells while increasing the number of immune suppressive cells in the circulation, thus increasing the likelihood of successful tumor establishment [49]. Biomarkers with prognostic and diagnostic potential are also a key research area, a recent report from Huang et al. details the prognostic significance of
exosomal miR-1290 and miR-375 in castration resistant prostate cancer. The levels of these two miRs were significantly correlated with overall survival [50]. There are additional reports of the prognostic and diagnostic potential of other EV microRNA expression patterns in melanoma, colon, lung, and prostate cancer among numerous other diseases [51-54]. Continued investigation of these cancer microRNA signatures should precipitate meaningful advances in therapy monitoring and clinical decision making, as changes in the miRNA expression are theoretically detectable prior to development of identifiable symptoms. As the resolution of EV separation techniques are improved to the point that EVs directly expelled by tumors can be captured distinctly from those of normal function in patient and animal tissue samples, the true influence of EVs on cancer initiation, maintenance and metastasis will be clarified and more importantly the identification of treatable EV targets may provide a new mode of therapy which disrupts cancer progression at the earliest stages.

2.5.2 Neurological Disorders

Demonstrations of EVs crossing the blood brain barrier, their detection in cerebral spinal fluid, and the identification of vesicle like particles passing between neurons are the key drivers of interest in the role of EVs in neurological disorders. The role EV in brain cancers has been reported, with identification of specific miRNA and protein signatures in the EVs from glioblastoma having been identified in patient CSF and cell lines [40, 29]. Other noncancerous neurological disorders such as Parkinson’s and Alzheimer’s disease have also received significant attention with reports implicating exosomes as active players in the basic biology of both diseases [55, 56]. Alpha-Synuclein aggregation is characteristic of disease spread in Parkinson’s and reports have identified
exosome mediated transfer of alpha-synuclein between neurons, and the exploitation of EVs for export of alpha-synuclein. Whether the release of exosomes by neurons is protective of the parent cell or pathogenic to the cells which take up the exosomes is a subject of debate [57, 58]. A more recent report characterizes the biological activity of EVs derived from the sera of Parkinson’s patients, concluding that they do carry bioactive signals and that appropriate phenotyping of individual EVs will help reveal the fraction of vesicles that can be targeted for therapeutic intervention [59]. In Alzheimer’s disease exosomes have been reported to have a role in insulin resistance and amyloid-beta trafficking, and are significantly lower in cellular survival factor cargoes when isolated from patients with Alzheimer’s disease compared to healthy controls [56, 60, 61]. Yuyama et al., reported increasing levels of circulating amyloid-B, and found the addition of exosomes to provide a protective effect although the mechanism of this activity is not understood [60]. The debate on whether the nature of exosome activity is misregulated or pathogenic by nature in Alzheimer’s is noted by Joshi et al [62]. In summary there are a number of potential functions for EVs in neurological disorders but understanding their pathogenic nature requires further investigation.

2.5.3 Interest in exosome function in other diseases

The connection between EVs as mediators of cell-cell communication and the myriad of functions which are attributed to their activity in disease states make a complete review impossible. The publication of meaningful results derived from EV interrogation in completely disconnected diseases and patient populations speaks to their ubiquitous nature. It will suffice to say that the same research themes presented in the review of cancer and neurological disorders can be found in the literature for any other unrelated
diseases. The questions on the true function of EV activity, and whether it is inherently pathogenic, or if disease activity is caused by disruption of normal EV pathways remain open and debated. There is little doubt that a wealth of clinically relevant information and potential therapeutic targets exist within the pool of EVs constantly in circulation, which can only be understood with time and further investigation.

2.6 Beyond Biology- Potential EV applications in pinprick diagnostics, RNAi vehicles and future medicines

Most current research questions center on EVs role in signaling within biological systems, and far too little is currently understood to develop interventions aimed at interfering directly with EV mediated cellular events. However our current knowledge of EVs has made for a popular proposal that they make ideal drug delivery vehicles. Their apparent lack of immunogenicity, combined with the cells ability to prepackage a desired cargo and display a targeting molecule to direct the EV to a specific site for action is conceptually intriguing. This idea is proposed in greater detail with consideration of mesenchymal stem cells (MSC) as the exosome source by Wee Yeh Yeo et al. in their 2013 review [63]. They claim that immortalized MSCs are the most productive cell line for exosomes based on affinity isolation from conditioned media and CD81 ELISA to quantify the productivity. The exosomes released by MSCs are therapeutic in animal models and immunosuppressive. Activity which the authors link to the efficacy of direct MSC transplantation proposing that secreted vesicles are the mediators of the therapeutic effect [63]. The combination of an EV delivery vehicle combined with an RNA interference (RNAi) molecule packaged inside, whether it is microRNA, silencing RNA, or others is a an intriguing possibility that has not been fully explored. The proposed
connections between circulating miRNA levels their association with EVs makes it possible in theory to interrupt what may the root cause of many disease. RNAi therapeutics have already been demonstrated using artificial drug delivery vehicles for both and up and down regulation of specific targets in cell line and animal models, but the global effect of RNAi treatments is not well characterized. A single miRNA may have unknown effects on pathways other than the one intended, complicating their development as viable treatments [64-67]. The most commonly cited motivation for therapeutic EVs is the work of Alvarez-Erveti et al. Which convincingly demonstrates the isolation of EVs from dendritic cells engineered to display a targeting peptide on the exosome surface, subsequent loading of isolated EVs with siRNA by electroporation, and most importantly the expected RNA silencing response in the brain tissue of mice injected with the loaded EVs [68]. The number of novel demonstrations with in this work alone is tremendous, at once showing not only the ability to cross the blood/brain barrier from an intravenous injection, but an effective silencing of the target mRNA, in a specific cell population based on the targeting moiety engineered onto the EV surface. In their small scale animal model study the isolation by ultracentrifugation and loading of RNAi molecule by electroportation is a reasonable strategy for preparing the therapeutic EVs, but these processing techniques do not scale well, and at the size required for a widely employed therapeutic the costs become prohibitive if not insurmountable. Investigation of cell line transfection methods which will lead to the cell producing a desired RNAi molecule in culture, preferentially excreting the desired molecule into the culture medium packaged in an EV which displays a certain targeting moiety on its surface have been proposed. These EVs could then be purified from the conditioned
medium by an affinity based method, at once screening for both the targeting molecule on the surface, and ideally the cargo inside. EV packaged therapeutics are one of the potential breakthrough treatment methods of the coming years, with potential to treat virtually any disease. This report and proposals from it have motivated questions regarding the feasibility of producing EVs in cell culture, and the specificity of separations at the bench scale will be addressed in the original reports contained in later chapters.

In the realm of diagnostic and prognostic EV assays many investigations aim to correlate some measureable biomarker expression profile in an isolated EV population with patient disease status. EVs are easily isolated from small volumes of biofluids, making for a far less invasive method than a tissue biopsy, and less costly than a CT scan, however the isolated EVs may represent only a small fraction of the total population, and losses associated the purification process are not currently well reported. Taylor et al. have published investigations in both lung and ovarian cancer regarding the exosomal miRNA profiles, but neither study establishes conclusive results between the measured miRNA and patient status [21, 54]. As presented above more recent reports of meaningful links between EV biomarkers and cancer status are spread across a wide range of unrelated cancer types. Establishing meaningful relationships between the biomarker signatures will require a thorough understanding of method of isolation, the true distinctions between extracellular and EV associated nucleic acids, and large numbers of both healthy and sick patients to provide adequate statistical strength in the results. There is a term which describes these obstacles succinctly, heterogeneity, which in this context is exemplified by the variations in experimental design, isolation methods, and interrogation
techniques across different investigations. Heterogeneity can stop a promising technique in its tracks, to illustrate this point, we can consider circulating tumor cells as there are many parallels between CTC history and the current arc of EV investigations. CTCs are exceedingly infrequent cells in the blood stream which are theorized to be shed by tumors into the blood and impact themselves at remote sites in the body, leading to metastasis. Significant efforts have been made to understand their biogenesis, metastatic potential, and heterogeneity [69, 70]. Early entrants in the field of CTCs focused on utilizing EpCAM as an ubiquitous CTC marker (it is in fact not ubiquitous [71]), and developing methods of isolating EpCAM positive cells from patient samples, then labeling the captured cells with additional markers such as cytokeratin to confirm their oncogenic origin, and finally determining the total concentration of CTCs in the body. This has led to an FDA-cleared screening device, CellSearch, which has identified levels of CTCs/ml determined by this assay which are prognostic for metastatic breast, prostate, and colon cancer patient outcomes [72]. However the cells isolated by CellSearch represent only a fraction of the CTCs owing to their requirement that a cell display EpCAM in order to be captured. Any valuable biological data harbored in cells, among them CTCs which do not display EpCAM is lost. This leads to a classic example of screening bias, and our own work in the field has shown that there are a variety of CTCs present in the blood beyond the traditional definition, and that only a subset of the total detectable population is likely to have significant metastatic potential. The heterogeneity of the CTCs can only be realized by removing the normal cells, rather than capturing the supposed cancer cells [73, 74]. The parallel to draw between CTCs and EVs is that in attempting to connect a measureable biomarker signature from EVs to a disease state in either a prognostic or
diagnostic manner, it is important not to lose sight of the significant heterogeneity present in the populations, and that our methods of identifying and isolating, let alone understanding the biological function of subsets of EVs are currently poorly developed. This motivates the following report in which we ask basic questions about the process of EV isolation and comment on the impact the findings have on the current modes of EV research.
Chapter 3- A review of common techniques for the isolation and detection of EVs

3.1 Introduction

The production of EVs by cellular systems is seemingly ubiquitous, with EVs having been isolated from the conditioned medium of any cell line of interest, and every bodily fluid. However there is still little agreement or standardization regarding the methods used in isolation and detection of EVs, with several completely different isolation methods being widely reported in the literature and a myriad of suitable methods of detecting isolated EVs reported in the literature [75]. The most common isolation methods are sequential ultracentrifugation (UC), where the EVs are pelleted by being subjected to increasing high speed spins with each step in the process subjecting the mixture of particles to increasing sedimentation force and thus removing different contaminants until the pellet of the final spin (>100,000 x g) is greatly enriched for EVs (in particular exosomes but that will be a point of discussion). The purity of EVs can be increased by incorporating a density gradient for ultracentrifugation. Isolation kit reagents such as Exoquik (Systems Bioscience) and Total Isolation Reagent (Invitrogen) are thought to tie up the water in a sample allowing collection of the less soluble components such as EVs in a low speed spin after mixing. Lastly affinity isolation where micron size beads are functionalized with antibodies against a desired EV surface biomarker allowing separation of those EVs which display the marker of interest. Each of these techniques has its own pros and cons and selection for a specific study is often
based on the availability of equipment, need for purity, and past experience with the same technique. Methods of detection for isolated EVs are also highly varied and common techniques include nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), electron microscopy (EM), and flow cytometry among others. No single technique is perfectly suited to the task of EV detection, in this chapter the first principles of each method and representative applications will be discussed. Based on the ongoing development of therapeutics in EVs it is important to understand the impact of separations procedures on the recovery of EVs, and in turn strengthen conclusions about the productivity of EVs per cell in culture, and the relative frequency of biomarker signals within the large, heterogeneous population of crude EV preparations.

3.2 Methods of Isolation

There is no standard in the method of isolating EVs in peer reviewed studies, with all of the common methods represented in the literature. To understand the methods typically employed to isolate EVs I present a basic review of the techniques mentioned briefly in the introduction. The goal of any isolation method is to sufficiently purify the EVs from potential contaminants for downstream analysis, however the composition of the EV source material can have significant influence on the purity of isolation. The loss of EVs during processing in each isolation protocol is also poorly understood and often not reported, despite the significant impact the losses will have on the results of endpoint biomarker assays [76, 77].
3.2.1 Ultracentrifugation and Density Gradient Sedimentation

During ultracentrifugation samples are subjected to a period of high gravitational force (100,000+ x g) by rapid spinning in specially designed ultracentrifuges. This technique is widely applied to characterize a number of physical properties of polymers and nanoparticles, including molecular weight, partial specific volume, and others based on their movement through a suspending medium [78]. In the field of EVs, UC is typically applied not as a characterization tool, but as a separation, with sequential spins at higher speeds removing particular components of the sample. Thery and colleagues have published studies which address the issues involved with isolation and characterization of EVs, and their protocol published in 2006 for sequential UC is still widely used today, with minor modifications for the isolation of EVs from conditioned medium and biofluids. The protocol is shown in Figure 16 [79].

The purity of EVs isolated by UC is a subject of debate, particularly from complex systems biofluids like blood components when using a pelleting strategy. Momen-Hevari et al., note that the viscosity of biofluids (serum, plasma, saliva) is a critical factor in

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**Figure 16: Flowchart for the isolation of exosomes by Thery et al. Note that cell debris is discarded after the 10000 x g spin, this is today considered to be the microvesicle fraction. Image from [79]**
efficient sedimentation of EVs, recommending a level of dilution in order to lower the viscosity and pellet more of the EV content [80]. EVs isolated by UC from biofluids are likely contaminated with HDL, LDL, and extracellular proteins not associated with EVs. HDL and LDL have been noted to coisolate with EVs even when a density gradient is used during the UC protocol. This is an important observation because lipoproteins have their own subset of associated miRNAs [81-83]. The soluble protein Argonaute2 (Ago2) is another common source of contamination as it is an important part of the miRNA processing machinery. Ago2 is often found in complex with miRNA in the extracellular space and can co-isolate with EVs in a typical UC protocol [38]. These sources of contamination make it difficult to distinguish which biomarkers signals can be distinctly attributed to EVs. In order to more clearly separate populations of EVs, density gradient sedimentation (DGS) utilizes layers of suspending medium with increasing density, typically sucrose or iodixonal at increasing percent weight, the sample of interest is added on top of the layers. After ultracentrifugation for some time all of the particles in the sample will have moved to their isopyntic point within the density gradient, and can be extracted for analysis by careful collection of each fraction, and data from DGS has indicated that many EVs (specifically the exosomal portion) have density 1.12-1.15 g/ml, although many fall outside that range in the broader 1.05-1.20 g/ml [17, 84]. While EVs collected from density gradient preparations are often of higher purity than dead end UC, the recovery is markedly lower which must be taken into account when analyzing results. Despite the deep characterization of the performance of the UC and DGS isolation methods by some research groups, there remains little consensus on whether any particular protocol is most appropriate Cjvetkovic et al., have reported the influence of
rotor type, g force, and centrifugation on recovery as measured by RNA and protein content of the pellet, but without enumeration of the vesicles. They conclude that most typical protocols are insufficient or too poorly developed to capture all vesicles in a sample, and all of the investigated variables are of significant influence [85]. Jeppesen et al., report markedly different characteristic size distributions of recovered EVs and broad differences in apparent pelleting times when comparing isolation by UC from two unrelated cell lines [86]. Within the EV research community there have been formal positions taken on the need to establish a standard, but little headway has been made in the implementation of such. In surveying the literature the only distinct commonality between different groups in terms of UC isolation protocol was noted when the protocol shown in Figure 16 from Thery et al. was cited. Those who did not cite a source for the protocol showed no agreement on spin rates and times for each step in the process.

The method of EV isolation we used in the work presented in the following chapters is primarily ultracentrifugation. The protocol employed is not extraordinary or optimized. It is rather intended to represent what is a “typical” method of isolation in the field and from there, determine the characteristic size and concentration of particles isolated at each step in the process to consider the inherent losses.

3.2.2 Proprietary Reagents

With rapidly growing interest in EVs methods for avoiding the expensive equipment and labor intensive techniques involved in ultracentrifugation have given rise to several commercial reagents for the isolation of EVs. Examples include Exoquik (Systems Bioscience) and Total Isolation Reagent (Invitrogen). These reagents are sold in slightly different formulations (although the exact composition is not revealed) for isolation of
EVs from conditioned media, plasma, or urine. They are thought to work on a solubility exclusion principle, by using polymers to tie up the water in the system they force the less soluble vesicles out of the water suspension, where they can be easily pelleted by a short low speed spin. Manufacturer provided data suggests that these reagents purify EVs based on the western blot detection of common EV markers such as CD63 and TSG101, and claim improved recovery compared to an ultracentrifugation isolation. Rekker et al performed a direct comparison of the miRNA profile of EVs isolated by ultracentrifugation compared to Exoquik precipitation, finding the results comparable but the Exoquik method precipitating particles other than EVs [77]. The coprecipitation of contaminants by these reagents make them unattractive choices for purity, but as a primary isolation step they may provide enhanced recovery of vesicles which can be subjected to more stringent purification techniques.

3.2.3 Immunoaffinity Isolation

Immunoaffinity isolation is the separation of a specific subset of EVs from a bulk population (often pre concentrated or isolated nonspecifically) based on their expression of certain surface displayed biomarkers. Large (>2 µm) beads are functionalized with antibodies against a biomarker expected to be displayed within the population, but at an unknown level. By incubating the beads with the heterogeneous EVs those which display the biomarker desired will be captured on the bead and can be easily spun down in a low speed centrifugation attached to the large bead, and the uncaptured EVs are discarded thus creating a supposedly more homogenous population in the bead associated EV pool. Separation of subsets of EVs is desired to reveal whether their cargo or function can be differentiated based on their display of the target marker. This isolation strategy has been
compared to ultracentrifugation and density gradient sedimentation by Tauro et al. In their study EVs from the colon cancer cell line LIM1643 were isolated by each method and then interrogated for specific protein content by tandem mass spectrometry. Not surprisingly immunoaffinity capture based on display of EpCAM, a widely used marker for cancer associated cells, led to the identification of more oncoproteins than in those samples which were not enriched by affinity isolation. However the immunoaffinity approach isolated more EVs by total protein analysis than a DGS method, which is difficult to understand [17]. Mathivanan et al purified exosomes displaying the marker A33 for proteomic analysis, and comments on the importance of using immunoaffinity purified EVs to eliminate confounding signals [87]. When properly developed immunoaffinity isolation can enhance sensitivity to infrequent or disease related markers critical to the investigation. In Chapter 4 I will probe the particle balance surrounding immunoaffinity isolation of EVs on magnetic microbeads with surprising results.

3.2.4 Chromatography Based Methods

Antibody affinity isolation using microbeads on small samples is a useful analytical tool for isolation of specific fractions of EVs. However it cannot meet the needs of large scale separation applications. However if the antibody were immobilized on a chromatography matrix it might be possible to separate a large number of vesicles from complex mixtures like conditioned media in a typical chromatography approach. A recent report by Boing et al., details the application of size exclusion chromatography for the separation of EVs [88]. Because the throughput available with chromatography can work at much larger scales than the other isolation methods it presents significant opportunities in the purification of therapeutic EVs at scale.
3.3 Methods of Detection

Perhaps the most confounding part of EV investigations is definitive determination of the amount of “true” material present. More distinctly that is, the amount of protein or nucleic acid that is truly entrained on the surface and interior of the isolated EVs is difficult to measure in “total” assays as it fails to screen for contaminants and provides no confirmation of EV association [89]. Thus accurate determination of the size distribution and number count of the nanoparticles present in EV isolates is a critical measurement for understanding their function and validating isolation protocols. Much like the variation in isolation methods there are a number of options for assessing the characteristic size and concentration of EVs, each with their own pros and cons. For many years the only useful technique for sizing EVs was electron microscopy (EM), requiring expensive equipment, deep user knowledge and laborious preparation techniques which can influence the outcome of the measurement. While still a standard for absolute sizing, EM as a complementary technique to biological studies it is often too demanding on time and resources to work as a confirmatory technique for isolation. Traditional ensemble light scattering techniques such as dynamic light scattering (DLS) and laser diffraction are often not suitable for determination of particle size distributions (PSD) in EV preparations because of the innate heterogeneity in size. EV isolates typically range from 50-350nm in diameter, and the effect of the presence of a small number of larger particles in measurements by DLS is clearly described by Filipe et al in comparison to nanoparticle tracking analysis [90]. The disparity in determined PSDs is because Raleigh scattering is a sixth order function of diameter, and thus small numbers of large particles will overwhelm the signal from many smaller particles leading to PSD results by
ensemble measurement techniques which are difficult to repeat and not representative of the true PSD. In addition to PSD measurement, quantifying the number of vesicles which are present in a sample is a major obstacle as nanoparticle counting techniques were not well developed for polydisperse samples, and approximations of EV concentration based on DLS results can be essentially meaningless for broad PSDs. The issues with determining the size and quantity of EVs in a sample were a limiting factor in the research interest in the field for many years. At present several techniques have entered the field with the capability to analyze EVs on a particle by particle basis and will be discussed briefly in the following sections. For the interested reader deep comparisons have been reported by Van Der Pol et al, and Mass et al respectively [100,101].

3.3.2 Measurement of EVs by Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) is a method of determining the size distribution and concentration of nanoparticles in suspension. Using a laser shaped such that the illumination depth is very similar to the focal depth of a typical microscope objective lens, nanoparticles can be visualized as point scatterers when measured at appropriate concentrations. The particles in suspension move under Brownian motion and their size is directly related to the velocity of the Brownian motion. By recording videos of the particles moving and then determining the mean squared displacement between each frame of the recording for each particle visible on the screen using the associated software. By monitoring the sample temperature in the measurement region and requiring user input for the viscosity, the size of each particle can be calculated from the relationship between diameter and mean squared displacement presented by the Stokes-Einstein equation [91]. Thus the resulting size is equivalent to the hard sphere
hydrodynamic diameter for each particle, and includes the hydration shell around the particle. In this way NTA results should be used as an approximation of absolute size with a tendency to be shifted to larger sizes compared to EM distributions. The interrogation of each particle builds a distribution of the sizes that is more sensitive, and provides higher resolution than ensemble techniques like DLS, particularly in the case of polydisperse samples as is demonstrated clearly by Filipe et al [90]. The particle by particle analysis approach of NTA also makes it possible to estimate the number concentration of EVs in a sample, a measurement that was previously inaccessible or at least exceedingly difficult to determine. However NTA is typically reported to have a lower detection limit of 30nm for organic particles, so vesicles near the detection limit in the may not be realized as an absolute number count. Because the dynamic range of the concentration measurement is effectively $5 \times 10^7 - 2 \times 10^9$ particles/ml most samples require dilution to be properly measured, sometimes more than 1000 fold. The resulting PSD after dilution is representative of the most frequent particle sizes in the sample, but lacks sensitivity to less prevalent populations. There are numerous publications which have assessed the measurement of EVs by NTA, studying their size, count, and stability, and NTA is often used as a quick characterization tool when research questions focus on the biology of EVs [92, 18]. With the ability to quickly analyze isolated EVs size and concentration using NTA, research interest in the function of these EVs has increased tremendously in the past ten years and led to evaluation of a number of other techniques for measuring the absolute size distribution and concentration of EVs. In the following chapters I will use the concentration measurement of EVs by NTA extensively in the comparative analysis of separations techniques.
3.3.3 Measurement of EVs by Tunable Resistive Pulse Sensing

Tunable resistive pulse sensing is a technique which allows for determination of the size distribution and number concentration of nanoparticles. The particle suspension is diluted to an appropriate concentration and driven across a non-conducting membrane with a single pore of diameter slightly larger than the particles of interest. Size and count of the particles are detected by a measurable change in the resistivity across the pore as a single nanoparticle moves through. The magnitude of the resistive pulse event is a third power function of particle diameter, and a fourth power function of pore diameter \[93\]. TRPS systems can be equipped with different sized nanopores to provide detection capability across a range of sizes. In general larger pores will prove insensitive to the smallest particles in a measurement, while smaller pores can be prone to blockages disrupting the flow of particles through the pore and requiring user intervention to continue the measurement \[94\]. Each measurement uses a standard calibration nanoparticle solution to establish the resistive response to particles under the particular instrument configuration because several variables can impact the results and must be kept constant throughout a measurement including the pore strain, pore rating, transmembrane voltage, and particle driving pressure \[93\]. Direct comparisons of TRPS with other techniques have been made, with the dependence of the results on the size of pore chosen being clearly evident when compared to other techniques \[95\]. However when properly performed TRPS measurements can provide comparable size and concentration reports to other EV analysis techniques.
3.3.4 Measurement of EVs by Electron Microscopy

Electron microscopy (EM) techniques allow for detailed determination of the size and morphology of EVs. It was the first widely used method of confirming EV presence after isolation. EM studies of the sub-micron particles released by reticulocytes are often cited as the first reports of EVs [96]. The study of exosomes in particular by EM gave rise to their popular description of 30-100nm spherical vesicles which display a cup shaped morphology. Advanced applications of EM are covered in [24, 25]. EM techniques remain the gold standard of determining the edge to edge size of nanoparticles, including EVs. Some of the limitations of EM techniques are the requirement of access to expensive equipment, laborious sample preparation with poorly understood effects, and time consuming hand interpretation of results. Overall the method remains the most absolute method of sizing, with added benefits in the hands of experienced users.

3.3.5 Measurement of EVs by Flow Cytometry

Although it is not traditionally a nanoscale analysis technique flow cytometry is one of the most commonly reported methods of analysis for EVs, particularly for platelet derived microparticles [97]. There are a large number of considerations that must be made for effective analysis of EVs using flow, in particular the wide variation in instrument capabilities and equipment. Most commercial flow cytometers cannot visualize polystyrene particles smaller than 300nm, making EVs, which have lower refractive index than polystyrene beads often used for size calibration exceeding difficult to detect as single particles [98]. Because a typical flow cytometer relies on sufficient signal on the forward and side scattering detectors to determine a particle of interest has been measured the lack of scattering signal from vesicles leaves them lost to the
instrument noise. Van der Pol et al have produced detailed publications regarding the
analysis of EVs with flow, concluding that most vesicle detection can be attributed to
large single vesicles or what they term swarm detection, multiple smaller vesicles passing
through the detector at the same time and triggering an event as if they were a single
vesicle [99]. This makes enumeration of vesicles with flow cytometry exceedingly
difficult, although the confirmation of biomarker presence by the fluorescent signal is
still measured in a semi-quantitative result. The widespread access to equipment and
common knowledge of the technique make flow cytometry an oft used method of
biomarker identification on EVs, although some reports push the technique to the limit of
detection.

3.3.5 Additional Methods

There are methods of determining the size distribution and concentration of nanoparticles
which can be used to EVs. These include asymmetric field flow fractionation, various
chromatography techniques beyond size exclusion, and dynamic light scattering. Their
application is less frequently reported in the literature than the methods described above,
although each technique can provide insightful data on the EVs of interest. A thorough
comparison of these less often used methods has been presented in 2013 Van Der Pol et
al, and 2014 by Petersen et al, respectively [102, 103].

3.4 Concluding comments on detection and isolation

An important observation to take away from the cited literature is the variation in
performance measurement for isolation techniques. If the scientific question asked needs
only to confirm the presence of EVs and we screen for whether the EVs are present, the
answer is often yes, the EVs are present. The question that has often gone unasked is quantitative, what is the recovery in terms of particle number and morphology. How many particles of typical EV size are present in measurement, for a functional assay regarding the activity of EVs measured by exact amount of active “reagent” being used is not simple to determine. With the acceptance of EVs as signaling mediators, particularly in RNAi, the need for clear understanding of their prevalence within an experiment is critical. Questions of scalability for these types of processes must be addressed for realization as therapeutics. In the following chapters I will directly compare the recovery of particles by the kit reagent to that by ultracentrifugation and discuss the implications of the findings within this context using NTA detection for sizing and enumeration of the EVs in each sample.
Chapter 4- Closing the particle balance on EV isolation, determining the productivity of culture systems for therapeutic EV applications.

4.1 Introduction

Based on their demonstrated biological function, natural display of targeting molecules, and lack of immunogenicity EVs have been proposed as ideal drug delivery vehicles [63, 104-107]. As natural mediators of nucleic acid signaling their development as potential RNA interference (RNAi) treatments is a real and current topic of investigation. Many publications demonstrate the variety of effects these treatments can elicit, focusing on the knockdown of specific protein targets in particular organs as expanded on in Chapter 2.6.

There are obstacles which must be overcome in order to realize RNAi therapeutics. The large scale synthesis of therapeutic oligonucleotides, and development of a suitable delivery vehicle make investigations larger than laboratory scale prohibitively expensive. Thus strategies for programming cells to load their naturally produced EVs with a desired nucleic acid (which they already have the machinery to accomplish) is an ideal exploitation of cell culture produced therapeutics. What is often given little attention in the current literature is the capacity of cellular systems to produce EVs for these applications. Studies of the effect of EVs in cellular or animal systems typically call for doses on the order of µg, but it has been questioned what exactly constitutes 1 µg of exosomes [108]. Despite the lack of characterization to determine what molecule within the pool of EVs is responsible for therapeutic effects the promising results in terms of
efficacy demand investigations into the productivity of the cells to better define the issues with scale up that must be addressed. In collaboration with Dr. Schmittgen’s group who are investigating exosome based RNAi treatments we conducted a study to determine the EV productivity in spinner flask cultures, comparing the recovery of EVs by ultracentrifugation and precipitation reagent (Total Isolation Reagent from Invitrogen) on a number count basis. By measuring the size distribution and concentration of the nanoparticles present at different steps in the isolation process we identify losses and can project the scalability of production. The nomenclature for samples in this chapter is as follows, 10K refers to the supernatant drawn off after a 30 minute centrifugation of the conditioned media at 10000x g, which should be free of cellular debris and dense microvesicles. Clear refers to the supernatant after the 1.5 hour spin at 100000x g which should be free of virtually all particles. Extracellular vesicle pellet (EVP) refers to the resuspended extracellular vesicle pellet from the 100000x g spin. Throughout this chapter the term nanoparticles will be used to describe the concentration and size distribution results, this is a prudent discussion on the topic and it would be too bold to claim that every particle measured is actually an extracellular vesicle in light of the known contaminants.
4.2 Methods and Materials

Isolation of EVs from HEK293 Conditioned Media

EVs are harvested from the conditioned culture medium of human embryonic kidney 293 T (HEK 293T), adapted for growth in suspension and exosome free media. Fetal bovine serum (FBS) is a common supplement to culture media but is known to contain large numbers of bovine EVs, these are removed by ultracentrifugation before the media is prepared. Thus when evaluating the EV content of the conditioned media we can infer that EVs are all from the cells of interest and not contaminants from the media. Cells were grown in 400ml spinner flasks with ATCC media supplemented with 8 mM L-Glu and exosome depleted 2.5% FBS. Cells were passaged to fresh media after 72 hours of growth with confluence greater than 80%. The conditioned media was subjected to the EV isolation protocol in Figure 17. Isolated EVs were either used immediately or stored at -80 C. We defend the ultracentrifugation protocol only as being representative of those used in many other studies (see Chapter 2.2.1). The addition of a density gradient, or additional filtration is known to reduce the recovery of the isolation and was thus left out of this protocol, as the goal is to understand the maximum productivity of the culture system, not optimization of the separation technique.
Isolation of EVs using Invitrogen Total Isolation Reagent

From the 10K supernatant volume, one ml was set aside for isolation with Total Isolation Reagent (Invitrogen). Isolation of vesicles by reagent precipitation was conducted according to the manufacturer’s instructions. Briefly 1 ml of conditioned media was mixed with 500 ul of the reagent, pipetted vigorously, vortexed and stored at 4 C overnight. EVs were isolated the following day by centrifugation at 10000 x g for 3 h. The pellet was resuspended in 1ml PBS for measurement by NTA.

Measurement of EVs by Nanoparticle Tracking Analysis

The size distribution and number concentration of the EVs in each sample was measured using a NanoSight NS300 (Malvern Instruments) with 532nm laser source and high sensitivity Hamamatsu sCMOS camera. All samples within each set (10K, Clear, EVP) were measured at two dilutions to verify the concentration result. The settings for camera level and detection threshold were held constant for all samples. Three captures of 60 seconds were taken under syringe pump flow. Further consideration of this method of particle counting is given in later sections of this chapter.

4.3 Closing the particle balance

There are a number of approaches for considering the apparent recovery of EVs in isolation procedures. One often used method is tracking the total protein isolated and assuming the result is representative of the EV concentration in each step, although the correlation between EVs and total protein can be highly influenced by contamination [89]. A more refined approach based on protein content is measuring specific proteins such as MHC II, which are known to be associated with EVs [79]. Using this method
Lamparksi et al., probed the isolation of exosomes by classical ultracentrifugation, determining that 5-25% of vesicles are recovered [104]. A drawback to this method is the unknown level of specificity for the intact EV particles, and the requirement to develop a correlation between the measured protein content and EV particle count. The variation in protein level between different preparations is driven by the co-isolation of other protein bearing complexes in a nonspecific centrifugation, along with soluble proteins when the spin time is long, as has been discussed previously. Using NTA it is possible to enumerate the total particles present in each step of the process on a particles per milliliter basis. This begs the question of whether a particle balance around the EV isolation protocol can be closed. By determining the number of EV sized particles in each step of the isolation protocol it is possible to make assessments of the recovery and more importantly the theoretical maximum productivity of the culture system for EVs. With respect to the specific application of EVs discussed in this chapter, the “theoretical maximum” must be reported because it is certain that not all the EVs measured after isolation contain the specific cargo; distinguishing those EVs which certainly hold the cargo of interest is currently not possible. The size distribution of isolated particles, which should change with each step in the ultracentrifugation procedure according to published literature is also clearly measured, such that comparisons between pelleted particles and those still in suspension can provide valuable insights into the strength of the separation. The results presented here explore the total nanoparticle productivity of the culture system and the losses owed to the isolation procedure. We explore the relationship between cell density (cells/volume) and apparent EV productivity, and the
effect of centrifugation on the size distribution and concentration of the isolated nanoparticles.

4.3.1 Method of Concentration Determination by NTA

Here we discuss more critically the measurement of concentration with NTA and develop an understanding of how the concentration result is determined. The values reported by NTA for concentration are number weighted, where the size distribution is based on the frequency of observation of individual particles of a particular size over the course of the measurement. The sizing of each particle is subject to a statistical test for validity based on the length of time the particle was tracked: upon passing this test the track is “valid”. The size distribution based on “valid tracks” is fit to the measured total nanoparticle concentration, which is the average number of particles observed in a single frame of video, without regard for their size. This fit leads to a size distribution where the area under the curve between any two sizes is equal to the total concentration of particles in that size range. The method also requires that the user be conscience of sources of bias in their measurement, and make strong qualitative assessments of the data in the image on screen while making NTA measurements. It is imperative for quantitative inter-sample comparisons based on light scattering that results be taken under the same instrument settings.

4.3.2 Method of Dilution and its Influence on Concentration and Size Results

NTA is a visual method and the size distribution determination is predicated on measuring the nanoparticles at an appropriate concentration where individual particles do not cross paths on screen with others frequently within the viewing volume, which
corresponds to an optimal total concentration in the $10^8 - 10^9$ particles per milliliter range. Below this concentration long (>60 sec) videos become necessary to complete the 200 valid tracks per measurement required for confidence in the size distribution result (the agreement between repeat measurements is also indicative of sufficient capture length), and above which it becomes impossible to identify all the particles in an image because of their density in the viewing volume. I have verified the linear response in concentration result for both 100nm and 56nm polystyrene standard at different dilutions over the working range as shown in Figure 18. It would be known that above the range measured the response shows a flattening of the linear trend as the maximum number of particles which can be tracked is reached.

Preparations of EVs (from any source) are typically several orders of magnitude more concentrated than is suitable for NTA measurement thus requiring serial dilution for an accurate measurement of the size and concentration. For proper results from serial dilution it is important to maintain correct and consistent pipetting technique. I personally recommend diluting no more than 100x in any one step, and avoiding very small volumes (<5 % of total), it is most common to dilute samples to 1ml total volume for analysis, using the first 600ul to prime the instrument, and taking the measurement

Figure 18: Serial dilutions of 100nm (squares), or 57nm (triangles) monodisperse latex beads to verify linear response of NTA concentration measurement.
within the remaining 400ul. Measuring the same sample at two dilutions a factor of two apart, and verifying the linear response of the concentration reported is a method of verification for the measurement results, and the slope of the linear fit to all dilutions within each sample pool is given in Table 6. Table 6 shows two important results, first the average column indicates that the expected concentration response was observed between dilution levels. Second the standard deviation of the response is near 10%, which is in line with the run to run standard deviation of the measurement technique. These two results give confidence that the nanoparticle count for each sample is accurate.

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Avg. Slope</th>
<th>St Dev</th>
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<tbody>
<tr>
<td>10k</td>
<td>1.06</td>
<td>.10</td>
</tr>
<tr>
<td>Clear</td>
<td>.97</td>
<td>.09</td>
</tr>
<tr>
<td>EVP</td>
<td>.96</td>
<td>.09</td>
</tr>
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Table 6: Verification of concentration measurement for each sample pool. A perfectly linear response would yield and average slope of 1. The standard deviation of the slope calculation is in agreement with the typical variation within each measurement.

At the upper limit of the concentration measurement the NTA instrument will report 3-4x10⁹ particles/ml, but at that point the particles are too densely packed and the number of tracks is at a maximum, which means that the concentration can be underrepresented. Although these upper values can be measured, from experience reliable results are less than 2x10⁹ particles/ml, based on Figure 18, where the result is linear with dilution. As the EV density in a measurement increases it might be supposed that with higher concentration there will be an inherent tendency to report larger modal size for the distribution, as smaller particles are occluded by the larger particles. However plotting the results from every measurement used in this investigation as modal size against concentration we see that the measured concentration is not related to the determined modal size when particles are diluted appropriately, as clearly show in Figure 19. The noted lack of association
between determined concentration and size provides additional support that the total EV content has been accurately measured for every sample. If instead the linear fit showed a strong upward or downward trend in modal size with concentration changes both the size distribution and concentration result would be unreliable.

To compare directly the result of two different measurements by NTA it is critical that the settings used for capture and analysis be the same. There are only two user adjusted settings which influence the result by NTA, 1) camera level, which is a representation of a certain gain, exposure, and intensity histogram set before capture and 2) detection threshold, which is the sensitivity of the processing algorithm to a potential particle during analysis. With the broad size distribution characteristic of EVs the best approach to NTA measurements are selection of settings which reveal the maximum number of particles. This approach can be summarized as, camera level as high as possible (to reveal the smallest particles in the measurement), and detection threshold as low as possible (to track the dimmest particles). When appropriately diluted EVs should be clearly separated on screen as they move, and not densely packed, with an sCMOS camera set up the EVs should typically allow for camera level 13-16 without saturation of
many particles (indicated by false coloring of particles on the screen during measurement). Careful consideration should be given by the user when viewing the live images of the particles during NTA measurement, examination of the areas between clearly defined particles for signal from additional particles which may be hidden is important. For a sample of unknown concentration sufficient dilution is achieved when no further particles appear to be revealed by increasing the camera level. The detection threshold should be set as low as possible without triggering noise warnings, for well diluted samples recorded at appropriate camera level this is typically a value of 3-5 based on my experience running hundreds of samples for other researchers in the Analytical Cytometry Shared Resource at Ohio State. When the detection threshold is set too low the user will notice a jump in concentration without a shift in the size distribution. This effect arises from tracking of noise in the videos of the EVs, which is eliminated with a higher detection threshold, and does not have an effect on the measured distribution. Selection of an appropriate detection threshold is critical to producing quality data. Monitoring of the particles engaged for tracking by the software is an important qualitative visual cue during the course measurement that can be critical to producing high quality results from NTA. All samples in this chapter were measured at camera level 14, and detection threshold 4 with syringe pump flow of the sample to increase the number of particles sampled per measurement. Dilution for each sample pool was kept constant such that an appropriate number of particles were measured for each sample, 1K measured at 25 and 50 times dilution, Clear measured at 2 and 5 times dilution, and EVP measured at 100 and 200 times dilution. The dilution level is chosen so that results from the measurement fall in the range of $1 \times 10^8$ to $1 \times 10^9$ particles/ml where both the
concentration and size distribution are reliable. From the different dilutions of each sample pool we can verified the concentration measure by the expected factor of two between each dilution result (Table 6), and also whether the mode of the size distribution is influenced by the measured particle concentration as illustrated in Figure 19.

The details provided here regarding the verification of nanoparticle size and concentration measurements can be used as a guide for designing and implementing useful NTA methods. Despite frequent publications presenting NTA data on EVs there is little discussion of how these measurements are accomplished, or adequate representation of the variation between measurements of different samples. The need for standardization of measurement techniques in the field demands consideration be given to the settings used for both capture and processing with the goal always being able to accurately track and count every particle in the mixture for studies where quantitation of the EVs is required. Reporting of both instrument settings and sample preparation methods for the measurements taken will help to establish optimal methods of sharing results between laboratories.

4.4 Results

4.4.1 Regarding EV recovery by ultracentrifugation and precipitation reagent

To determine the average recovery of EVs by each method nine 400 ml batches of conditioned media were subjected to isolation by both ultracentrifugation and precipitation reagent (using an aliquot of supernatant after the 10000 x g spin). The concentration of nanoparticles reported in the 10K samples is taken to represent the total possible EVs in the system. There is no doubt that some portion of the observed particles
can be attributed to protein aggregates or other contamination which falls into the same size regime as EVs, but conceptually this does not impact the message of the results in this chapter. Using the 10K concentration as a baseline, and the EVP concentration as the final recovered particles a straightforward calculation of the nanoparticle recovery can be made. The results from all batches give an average recovery of 31 +/- 18% by ultracentrifugation, and 80 +/- 57% by the reagent method indicating an obvious lack of consistent sedimentation in both cases. Removing the lowest and highest values from each set of results gives 30 +/- 14% and 67 +/- 27% respectively. Within the nine batches tested the isolation reagent had one result greater than 200% recovery, which leads to the sharp decrease in standard deviation when removed.

As stated it is known that not every particle in the size range measured is an EV. This fact aside, the total particle balance is a sensible route to understanding the recovery of the process, with no complete losses of particles, and no recoveries greater than 100% for any of the batches measured by ultracentrifugation. The reagent kit provided a higher recovery of particles for every batch, which would seem advantageous depending upon the goals of the experiment. In a situation where the vesicles will be interrogated in bulk for protein or nucleic acid content the superior recovery may be a beneficial for isolating more vesicles but in two batches the recovery was calculated as greater than 100%, indicating that the reagent adds a measureable level of particles to the isolated EVs, so the true improvement in isolation is difficult to quantify by particle balance alone. The coprecipitation of contaminating species with the reagent method is not well understood in the current literature and sentiment within the field weighs against the use of reagents as an isolation technique although the data to support such is not widely disseminated.
The overall low recovery by ultracentrifugation demands answers regarding the fate of the missing particles. If all EVs fall into the reported density range of 1.10-1.20 g/ml, there is no explanation for their disappearance from the EVP samples.

Measurement of the Clear supernatant remaining after the high speed spin (see Figure 17) from each batch found that 3-15% of the particles from the 10K were still in suspension after the 100,000 x g spin. The exact percentage of particles in the clear sample for each batch is presented in Figure 20. In order to compare the size distributions in the samples with different total concentrations and dilution levels, the size distribution from each measurement is normalized to the measured concentration to give a number fraction distribution.

The average number fraction distribution of all samples from the Clear, 10K and EVP is compared in Figure 21, which shows the Clear samples have decreased but still measurable percentages of particles greater than 100 nm when compared to the 10K and EVP samples. The shift in the mode of the number
fraction distribution to smaller sizes in the Clear samples is explained by the removal of the larger particles during the ultracentrifugation process, this action leaves the remaining smaller particles representing a larger fraction of the distribution. In this way Figure 21 does not indicate that the total number of smaller particles in the Clear samples was greater than the 10K and EVP because the fractions are normalized to the total concentration in each sample. It is curious why these particles do not pellet during the high speed spin. We must attribute this observation to one of two phenomena. Either the nanoparticles observed in the Clear samples are less dense than typical EVs and thus not pelletted in the same amount of time under constant centrifugal force, or they have diffused away from the pellet and back into the supernatant after the centrifuge has stopped if the pellet is not collected in an appropriate amount of time, although this effect is difficult to quantify in hindsight (the liquid handling method used for resuspending the pellet could also influence these results). The more critical result is that the nanoparticles in the Clear fraction still account for less than 15% of the missing particles. Where exactly the other nanoparticles have been lost is difficult to understand. There is no shift toward larger particles in the EVP size distribution, making it seem unlikely that the ultracentrifugation method fuses multiple EVs into a single particle. Perhaps some fraction of the EVs are lysed due to osmotic effects of washing after the removal of the medium but the consistency of count in different buffers would seem to exclude such (see Figure 25).

Based on the modal values of all measurements we can approximate the most frequent EV diameter as being near 80 nanometers. It could be suggested that the force of the ultracentrifugation causes fusion. As EVs are composed of lipid membranes it is easy
to model the fusion based on the total lipid surface area that will compose the fused particle and thus its diameter. Thus it is straightforward that the number of EVs required to make an aggregate of diameter D, is the ratio of D to the nominal EV size, squared. This second order function of number aggregation also brings up another point, that the concentration of aggregates will be significantly lower than the original particles.

Because there is no appreciable difference in the number fraction distribution of the EVs before and after ultracentrifugation as shown in Figure 21, we must assume that if most of the missing particles have formed aggregates they are larger than 300nm. If such is the case their concentration will be 1-2 orders of magnitude lower and thus difficult to detect after dilution of the sample. This is confirmed by comparing the percentage of particles between 0 and 300 nanometers from all samples in the 10K (96 +/- 3%) and EVP (95 +/- 6%), with no apparent increase in the number of larger particles and no shift in the number fraction distribution it would seem that either the fusion is infrequent, or impossible to detect under the measurement conditions.

Beyond these points the fate of the missing nanoparticles degrades into pure speculation. Ultimately the conclusion from these measurements is that both isolation methods produce wide variations in particle isolation efficiency from sample to sample and results of biomarker assays which show less than 20% difference may be purely a function of the variation in isolation protocol performance.
4.4.2 **Regarding relationship between cell density, total nanoparticle concentration and EV production**

Determination of the quantity of EVs produced per cell based on the final isolated EVs and culture density in terms of cells per milliliter of culture volume is the critical finding of this investigation. Knowledge of this parameter is necessary for considering the scale up, total cargo capacity, and separations obstacles that will be necessary for realization of therapeutic EVs at scale. To date little consideration has been given in the literature to this value, perhaps because many biological questions regarding the function of EVs don’t center on the number of particles present only whether EVs in a system ultimately promote or suppress some cellular function. As mentioned previously the calculations here pertaining to the productivity must make the assumption that all detected particles are potential cargo bearing EVs, in this way the results here can be taken as theoretical maximum for production in our specific cell line and culture system. For a different cell line or culture strategy a similar set of measurements should be made, as the EVs produced by different cell lines can be variable in the quantity and biomarker display. To calculate the EV/cell productivity we simply used either the total particles in the 10K, or the total particles in the EVP normalized to the number of cells in the culture at the time of harvest. Intuitively one might think that more cells present would lead to more EVs in the conditioned medium, however the results here are surprising.

A comparison of the average productivity for the EVs/cell based on the 10K and EVP is shown in Figure 22, with the difference between the two representing the processing loss
of nanoparticles as discussed previously. There are a number of noteworthy findings presented in the Figure 22. At a glance the results show that all of the cultures produced EV/per cell results within the same order of magnitude and this does not depend on whether the assessment is made using the 10K result, or EVP result, a positive result regarding repeatability of the process and productivity of the culture system. The percent recovery by each isolation method (UC vs TIR) shows the recovery is clearly enhanced by using the reagent kit, but two of the data points indicate greater than 100% recovery, with no obvious reason for the enhancement based on the UC results. Looking more closely at the 10K measurements (green bars), we note that three of the cultures (1, 2, and 9) exhibited markedly greater concentrations of nanoparticles per cell after the 10K spin, but this did not translate into greater recovery or total EVs, and in fact these three batches have the lowest recovery at 7%, 12% and 16% respectively. To examine this phenomena we show both the 10K and EVP concentration plotted against culture density in Figure 23 where the three batches with the lowest total cells, show apparent productivities after the 10K spin approximately a factor of four greater than any other samples, despite the fact that the cell density is lower. However the nanoparticle content in the EVP is more consistent between samples, and the three samples with high loss of particles contribute

Figure 22: A complete depiction of the productivity and recovery of EVs in culture. The bars are plotted to the unitless left axis, the values are meaningful based on the legend. Cell density is plotted as 1000 cells/ml to fit the scale. Red and Yellow dots are plotted to the right axis as percentage recovery of the nanoparticles measured in the 10K samples, green bar. The bottom axis is the batch number.
to the high standard deviation in the recovery. The fact that three of the samples showed such behavior is also why removing the highest and lowest values from the UC based recovery calculation had little influence on the result, while removing the major outlier in the reagent recovery (batch 6) reduced the standard deviation by 30%. The true nature of the increased particles in the 10K samples is difficult to understand and should prompt further investigation into the dynamics of nanoparticles in culture systems.

4.5 Discussion

4.5.1 Implications for at scale realization of cell produced RNAi therapeutics

The investigation by Alvarez-Erveti et al. electroporated 150ug of siRNA into 150ug of exosomes [68] for each mouse they dosed. Using a molecular weight of 7000 g/mol for a 22 NT siRNA sequence, and an average exosome density of 1.15 g/ml, this corresponds to approximately 200:1 ratio of
siRNA to exosomes. If we guess that 2 siRNA are loaded into each exosome before injection, and that all loaded exosomes are recovered, this corresponds to a dose of roughly 70 mg/kg to achieve the demonstrated gene knockdown. Increasing the estimate of siRNA copies loaded in each exosome only results in an increased dosage requirement. For a 60 kg human this would potentially require 4 g or more of the active miR assuming the same dose is appropriate in humans. Of course this estimation is based on a complete loading of the desire siRNA into the vesicles, which may or may not be achievable by electroporation as has been investigated by [109]. Our current estimate of the total exosome production per HEK-293T cell is 1500 +/- 700 EVs/cell, based on Figure 22, the high variability is reflective of the variability in the isolation process. Using the number of EVs produced per cell Figure 24 relates the culture volume which would be required to produce 4 grams at current EV productivity (one copy of desired miRNA per EV), as a function of the percentage of EVs which are loaded with the desired cargo. It will be of critical importance to develop cell lines which are either more productive in terms of EVs/cell or are quite efficient at producing EVs with the desired cargo. It is possible that at maximum cell density the EVs in the culture reach an equilibrium concentration, where they are being released and taken up at the same rate on average in the total volume, while the lower cell densities are in a regime where the cells are still growing and thus flooding the media with their growth signaling EVs. This phenomena is the motivation for attempts to correlate overall EV counts (in serum, urine, etc.) with disease status, with the idea that rapidly growing tumor cells (in the case of cancer) would be releasing abnormally high numbers of EVs. In the context of disease I do not think this parameter is likely to be a revealing measure because the tumor cells make up a
small fraction of body mass, and the variation in performance of the isolation method across many samples makes detection of subtle changes in total EV concentration difficult to validate. Without a continuous method of screening the cargo of individual EVs it becomes difficult to understand at what point in the growth the maximum number of EVs loaded with the cargo of interest are available for collection, making the most useful separation approach some type of continuous affinity isolation which would constantly remove the cargo filled EVs without starving the cells of their normal signaling EVs. This can be related to the wildly successful method of fed batch growth for production of other therapeutics.

4.5.2 Ultracentrifugation does not give size specific isolation

An additional finding worth discussion here is that despite the ultracentrifugation protocol being quite similar to those which were associated with isolation of 30-100nm exosomes, some publications would even claim it is “optimized” to pellet a specific fraction of EVs, nothing in the results of our investigation suggests that this protocol has specificity toward smaller sizes or that the isolated EVs bear the size distribution commonly reported in the literature as 30-100nm for exosomes. Referring again to Figure 21, we can see that a large portion of the vesicles measured in the EVP samples even at 100x dilution are in the 100-200nm size range. While this result is in opposition to the widely reported size distribution of exosomes which should be prevalent in the EVP samples it is not uncommon. In the hundreds of EV samples I have measured for various groups at Ohio State, no matter the isolation method, I have never seen a size distribution that cuts off at 100nm in exosome preparations. However this result does make physical sense, if a fraction of the EVs are largely more dense than the suspending medium and
the suspension is spun for a sufficient amount of time to move all of the particles to the bottom of the tube there is no reason that larger or smaller particles would be preferentially pelleted in the time of the spin. The influence of vesicle density is a much greater determining factor as shown by Ettelaie et al, in their study of tissue factor bearing microvesicles. Microvesicles (MVs) are often reported to pellet at 10000xg, however the majority of tissue factor bearing vesicles were found in low density (1.03-1.08 g/ml) vesicles with diameter 200-350nm as measured by NTA [84]. The measured size is in agreement with the literature of microvesicles being generally larger bodies than exosomes, but the low density is difficult to reconcile with the claim of MVs pelleting at lower speeds, and these low density bodies would be completely missing from an isolation at 10000 x g [110, 111]. As more and more new investigators join the field of EV research we hope that clarity will be brought to the subject of exactly which particles are isolated by each technique.

4.6 Conclusion

In conclusion this chapter highlights the valuable insights the particle balance approach lends to repeated isolation of the complex, difficult to characterize pool of EVs. By tracking the particle size distribution and concentration in each step of the isolation it is possible to understand the loss of EVs inherent to the isolation protocol which can be taken into account when analyzing biomarker assay results. The isolation of vesicles by ultracentrifugation either method had a high variability from sample to sample but was within the same order of magnitude for all samples, giving confidence that these results are representative of the type of production and recovery that can be expected in EV directed culture systems.
Chapter 5- Bench scale particle balances

5.1 Introduction

Having studied the large scale particle balance in chapter 5 we will consider here the particle balance at the experimental scale using anti-FLAG functionalized micron size beads and EVs engineered to display FLAG at their surface. The FLAG tag is a 22 amino acid sequence which can be expressed on either the N or C terminus of a desired protein, the protein (or in this case EV) of interest can be purified by using an anti-FLAG antibody [118]. The separation of EVs displaying a specific surface marker on micron size beads is widely reported in the literature for collecting purified fractions from EVs [17, 68, 87]. The EVs on the bead can then be eluted from the bead for other assays [68] or analyzed in place on the bead as described by [17, 87]. The methods for these isolations are often quickly noted, and validation of the number of EVs captured is not provided. In this chapter we will probe the specificity and capacity of micron bead immunoaffinity capture of EVs from a heterogeneous pool of EVs isolated by ultracentrifugation on a number count basis, and ask if the particle balance around this type of separation is meaningful.
5.2 Methods and Materials

Extracellular Vesicle Isolation

EVs used for these experiments were harvested from the conditioned media of HEK-293T cells using the ultracentrifugation protocol described in Chapter 3. EVs from a modified HEK-293T line engineered to display a FLAG peptide at the surface exposed end of the membrane bound protein Lamp2b were created by Dhruvit Sutaria of Schmittgen lab.

Preparation of functionalized beads and estimation of capture area

2.8 um Protein G functionalized Dynabeads (Invitrogen) were prepared according to the manufacturer instructions with anti-FLAG (mouse IgG1). Protein G is superior to Protein A for immobilization of mouse IgG1, and the stated capacity for Ab is 8ug/mg beads, beads are incubated in binding buffer with Antibody (Either anti-FLAG or anti-CD45 (mouse IgG1) corresponding to 16ug/mg total in binding buffer for 15 min, washed with 1 ml washing buffer and resuspended to an appropriate volume in PBS for the experiment (dependent on the number of incubations which will be performed). Antibody capture on the bead was verified by flow cytometry.

Incubation of EVs and Beads

EVs were counted prior to each incubation experiment and stored at -80 C between experiments. An aliquot of $10^9$ total EVs were used for each incubation with an experiment dependent aliquot of anti-FLAG functionalized Dynabeads in a 1.5 ml Eppendorf tube. The EV-Dynabead mixture was rotated at 750 rpm on a shaker to prevent the Dynabeads from settling during the incubation for 15 min. PBS was added to
bring the total volume to 1ml, the Dynabeads were gathered near the bottom of the tube and 800ul of the supernatant was carefully removed for measurement by NTA.

*Measurement of particle concentration by NTA*

To minimize the dilution and particle handling steps, supernatants were measured as drawn from the tube in the manner described in Chapter 3.2.2. All measurements were taken at camera level 14 and processed at detection threshold 3. 3 captures of 60 seconds each were recorded with syringe pump flow, all size and concentration values reported are the mean +/- the standard error between the three captures.

5.3 *Experimental Design and Motivation*

Imunoaffinity isolation techniques are commonly used to purify EVs as has been discussed previously, but no measurement of the capture efficiency of this method or quantification of the number of EVs captured has been reported. With this investigation I look to probe the fraction of EVs collected on the surface of micron scale beads using NTA. To determine the fraction of EVs trapped on the surface of the bead, EV concentration measurements are taken of the suspension before incubation, and from the supernatant after the larger beads have been trapped on a magnet. The FLAG antibody was immobilized on the bead surface by interaction with Protein G. Variables of influence included incubation time of the EVs with the beads, equilibrium position of bound FLAG in solution. Using a typical particle size distribution for EVs and the nominal diameter of the microbeads it is possible to estimate the surface area available for capture. The Dynabead solution is provided at 30mg/ml which equates to approximately 5 x 10^8 beads/ml based on the density, the manufacturer does not provide
measurement of the exact count. Approximately 2000 closely packed EVs, based on size
distribution as measured by NTA, would fit on the surface on one Dynabead, assuming a
packing efficiency of 10-20% approximately 6 x 10^6 Dynabeads should be incubated with
10^9 EVs to provide sufficient surface area for pulldown.

5.4 Results

5.4.1 Buffer Exposure Does Not Impact the EV Particle Count

Figure 25 confirms that under short term exposure the composition of the suspending
medium between PBS, .1% Tween-20, and 10mM EDTA did not have an impact on the
particle count for identical volume aliquots of EVs (4ul from concentrated stock) diluted 200x to 1ml
total volume in each of the suspensions. This confirms that incomplete
buffer removal from the preparation of Dynabead will not confound the results of the
incubation experiments, and that the buffers do not contribute particles to the
measurement. Some suspending mediums such as sucrose and BSA solutions can
contribute a measureable number of particles to the total count and the background
particle level must be quantified to determine its influence on the results.

Figure 25: Comparison of the nanoparticle count result for 4ul of crude EVs dispersed in different buffers. 5mM EDTA, .1% Tween20, and 1x PBS. The buffer does not have a significant influence on the number of particles realized.
5.4.2 Particle Balance Measurements Reveal Details of the Interaction between EVs and Functionalized Microbeads

As a measurement of the performance of the separation the particle balance for immunoaffinity isolation in theory offers insights into the pulldown marker expression frequency, the percentage of vesicles isolated, and the influence of conditions on the capture of EVs. The number of particles captured as measured by the decrease in particle count in the supernatant after incubation is ideally indicative of the number of vesicles which display the FLAG tag on their surface. This measurement is beneficial because although the FLAG display in isolated EVs is confirmed by western blot, there is no information on the frequency of FLAG display within in the population. It should be noted that there is little data on the frequency of any other marker reported in the literature either. Where we seek to exploit the FLAG displaying EVs as potentially therapeutic cargo bearing a particle balance using the FLAG+ DynaBeads and FLAG- Dynabeads (CD45 functionalized) revealed apparent capture for all incubations, but with little apparent specificity for the antibody utilized for capture as shown in Figure 26. Note that within the error of repeated incubations the FLAG- Dynabeads capture an approximately equal percentage of EVs 38 +/- 14%, and 52 +/- 4% respectively, paired T-Test p-value = .28 indicating these results are not significantly different. Figure 26 is representative of results across
various binding buffer conditions and incubation times (short and long), in all cases the isolated fraction of EVs was nonspecific with respect to the functionalization of the Dynabead. Considering the approximation of the bead count as solid spheres of specific density, the calculation of the count of the beads may be grossly misrepresented by this method, the bead manufacturer does not directly state the count but in phone conversations estimated it to be closer to 2 x 10^9 Dynabeads/ml, nearly an order of magnitude higher than estimated. To that point incubations were prepared such that the total Dynabead surface area would be one order magnitude greater than required for close packing. Perhaps the extraordinary amount of free bead surface area in the mixture resulting in the nonspecific activity. To investigate this I incubated the same amount of EVs (10^9 total particles) with four different levels of FLAG+ and FLAG- Dynabeads such that the concentration of Dynabeads was reduced an order of magnitude between the highest and lowest levels. The capture results are shown in Figure 27. Note that the FLAG+/- 10 loading levels are equivalent to the level used in Figure 26, and show a similar profile of nonspecificity. Reduction of the Dynabead concentration eliminates the nonspecific capture in the FLAG- trials, while the lowest concentration levels,

![Supernatant Particle Count for Dynabead Titration](image)

*Figure 27: Titration of Dynabead concentration with constant EV count for isolation of FLAG bearing EVs. The counts are the supernatant or uncaptured EVs, thus FLAG- values equal to the EV only (far left) are indicative of no nonspecific binding. The decreasing ratio of Dynabeads to EVs eliminates the nonspecific binding.*
corresponding to the intended total Dynabead surface area of previous incubation show that 35% of the EVs are captured. (FLAG+3, FLAG+1). The argument for specificity of exosome isolation is strengthened by comparing the reduction in particle count in fractions of the EV size distribution representing exosomes (50-100nm) Table 7. Continued random nonspecific capture is noted for larger vesicles.

The reduction in specific size ranges is well represented graphically in Figure 28. The results indicate that among all variables the specific isolation of FLAG bearing EVs is critically sensitive to the relative concentration of the functionalized Dynabeads compared to the total EVs in the suspension. Under appropriate conditions the FLAG+ EVs can be isolated distinctly from a heterogeneous pool of EVs. The results indicate that approximately 35% of the EVs appear to display FLAG. If the FLAG is also confirmatory for the desired cargo (not evaluated in these experiments), this result is

| Table 7: Percent Reduction in Fractions of EV Size Distribution |
|-----------------|---------|--------|--------|--------|
|                 | Flag +3 | Flag +1 | Flag -3 | Flag -1 | P value |
| 50-100nm        | 34%     | 36%     | 1%      | 3%      | 0.0007  |
| 100-250nm       | 28%     | 2%      | -11%    | 18%     | 0.74    |
| Mode (nm)       | 87      | 105     | 93      | 90      |         |

*Figure 28: The size distribution of the supernatant after incubation, the area under the curve between any two sizes is representative of the particle concentration. We note the decrease in the 50-100nm for FLAG+ incubations.*
promising based on Figure 24, as greater than 20% transformation of EVs puts the culture volume into the region of diminishing returns for EV loading, and other methods of productivity increase will need to be investigated.

5.5 Discussion and Implications

It seems difficult to understand why there is a seemingly consistent capture of particles when the EVs are exposed to the microbeads, but confounding that this shows very little specificity to give confidence that the capture is mediated by the antibody on the bead surface and not some other mechanism. The control system using Dynabeads functionalized with a CD45 antibody, a pan-haemopoetic marker which is not displayed on the HEK-293T derived EVs indicate that a measurable percentage of the particles have been captured in virtually all measurements regardless of marker display. Zeta potential measurements of both the EVs and functionalized microbeads exclude the possibility of electrostatic attraction (-7mV and -35mV respectively). A deeper reading of the reported results by Tauro et al, and Alvarez-Erve raise further questions. From Tauro, anti-EpCAM functionalized microsphere isolation of EVs from a 500ul crude preparation yields 195ug of protein, yet the same 500ul of crude preparation spun through a density gradient only yields 150ug of total protein in the “exosomal” fraction, how this is possible is difficult to understand. The interaction of EVs and microbeads is quite sensitive to their surface area ratios as shown in the results here, under appropriate conditions the particle balance method is indicative of specific pulldown, but even five fold changes in the bead concentration result in a complete disappearance of specificity. Confirmatory testing becomes difficult without specificity in the isolation, Alvarez-Erve screen for Lampbl (a common EV marker) to confirm the FLAG mediated capture on
anti-FLAG microbeads. However this method of confirmation would have returned a positive result for EV isolation in every isolation presented here as all tests apparently trapped EVs on the Dynabeads. By asking the question of how many EVs are trapped on the bead and comparing to a nonspecific isolation we realize that the subtleties of the isolation are not lost to the screening technique. The more important consideration in the context of therapeutics is for purification of loaded vesicles which display an affinity tag such as FLAG. In the event that the display of the affinity marker is also confirmatory of the intended cargo in the EV (which is an entirely separate and exceedingly difficult cell engineering investigation), the purification strategy must maximize the capture of all cargo bearing EVs, while minimizing nonspecific capture.
Chapter 6: A method of determining the limit of sensitivity for fluorescence-NTA.

6.1 Introduction and Motivation

NTA is an established research technique for determining the size and quantity of nanoparticles in suspension, as has been extensively reviewed by Matthew Wright and Dr. Robert Carr [112]. The vast majority of publications use the light scattering properties of the nanoparticles to perform NTA (scNTA). However the same NTA measurements can be made using nanoparticles which fluoresce under the wavelength of the illumination source (fNTA). scNTA measurements provide the scattering intensity of each particle normalized to the brightest particle observed. Because the particles are free to move in suspension their position in the focal plane has a large influence on the apparent intensity, precluding absolute measurement. Thus nanoparticles of different composition cannot be distinguished by their scattering intensity except for contrived cases where the refractive index is quite different such as metal colloids mixed polymer beads. However the same NTA measurements can be performed by inserting a long pass or band pass filter in the optical path of the instrument at a wavelength greater than illumination sources and tracking the particles using their fluorescent properties. Particles which are fluorescent can be identified within heterogeneous populations and analysis of their size distribution. With growing interest in biological entities fNTA becomes an option for identification of distinct subsets of EVs in the same vein as flow cytometry is applied for sorting cells. The fNTA technique suffers from a lack of protocols for
thorough labeling of infrequently displayed biomarkers, and also from the lack of quantification present in more common biological techniques. A western blot positive for CD63 is simply that, positive that some CD63 protein was present in the sample, providing no indication of the frequency or density of the marker on EVs without more detailed quantification. There are only a few demonstrations of exploitation of the fluorescence-NTA (fNTA) method to identify specific surface biomarkers on EVs. Dragovic et al showed very clearly the presence of the placental marker NDOG-2 on placental EVs using quantum dot conjugated secondary antibodies, and careful separation of the labeled particles from the free quantum dots before analysis by size exclusion chromatography [18, 19]. In a similar strategy Gercel-Taylor et al showed the presence of both EpCAM and CD63 on EVs [21]. Nikitin et al investigated the labeling of spherical particles featuring tetraepitope complexes from the tobacco mosaic virus with both gold, and fluorescent antibodies, drawing important conclusions regarding the aggregation state, labeling efficiency and biological activity of their particles [113]. These examples raise interesting possibilities for f-NTA applications, however other literature regarding f-NTA experimentation is currently quite limited and its capabilities are not well characterized with regard to sensitivity. The lack of protocols for labeling nanoparticles, a challenge which presents substantially different challenges than labeling cells often complicates efforts. The potential of f-NTA to identify disease associated biomarkers on the surface and in the cargo of EVs demands a more careful description of its characteristic performance and expected limitations in order understand the proper interpretation of the measurements. The most pressing fundamental question regarding f-NTA is simply, what is the limit of sensitivity in terms of fluorescent molecules which
must be attached to a particle for accurate tracking, and by extension when a distribution of nanoparticles is unevenly loaded with a fluorophore will bias in the size distribution and number count results develop when comparing fNTA to scNTA measurements for the same particles. A reliable determination of the sensitivity for a single fluorophore would prove invaluable for the design of experiments and interpretation of results by other researchers. To estimate the number of probes which must be bound to single particle for fNTA measurement we employ the well characterized self-assembling cationic lipoplex nanoparticle (CLN). By controlling the amount of Cy3 labeled DNA available in the reaction mixture from 100%-1% CLNs can be formed with variable levels of Cy3 incorporated in their structure and the careful measurement of these particles formed at different loadings yields insightful results regarding the lower limit and influence of uneven loading. The work detailed in this chapter will be submitted to Nanomedicine, Scott Baldwin performed the experiments, Dr. Michael Paulaitis developed the model, Clayton Deighan analyzed the model, discussion of practical applications.

6.2 CLNs and fluorophore incorporation

CLNs are well understood multilamellar structures which self-organize under proper mixing conditions when cationic lipids are mixed with DNA. The negative charge of DNA against the positive charged lipid bilayer leads to multilamellar structure where each layer is comprised of either DNA or lipids. CLNs are known to form under a variety of conditions with the ratio of charge between the nucleic acid and lipids present being of critical importance, outside a narrow range of charge ratios the lipoplex will simply not form at all. The type of nucleic acid used to form the CLNs is not a critical factor, with
reports demonstrating the characteristic multilamellar structure with plasmids, dsDNA, and ssDNA of varying lengths [114-116]. In this chapter, we will use short 22 nucleotide ssDNA (ODN) and the same ssDNA conjugated to a single Cy3 (Cy3-ODN) at varying molar ratios to form CLNs. The mechanism of CLN formation is currently theorized as proceeding by a sequential layering mechanism, wherein an initial lipid nanoparticle forms with no DNA incorporated, with highly positive surface charge due to the cationic polymers, a layer of DNA (negative charge) coats the surface of the lipid particle recruiting another nearby lipid nanoparticle which lyse and wraps itself around the already DNA coated inner lipid particle. This layering proceeds for some time depending on the charge ratio in the formulation. Figure 29 gives a visual representation of this process. The CLNs make a useful particle for studying the properties of fluorescent loading because the number of Cy3-ODN molecules in the system is easily controlled during the formation and a model of their incorporation can be developed based on the measured particle size, interlayer spacing, and total ODN incorporation.
6.3 Results

CLNs measured by scNTA and fNTA give unimodal distributions. We used a cutoff of D less than or equal to 300nm for the upper size limit, which accounts for more than 99% of the nanoparticles by number. The scattering distribution is shifted slightly to larger sizes. We posit the reason for this difference is intensity variation with particle size. In Rayleigh scattering the intensity varies as a sixth power function of diameter, whereas the fluorescent intensity will vary with the number of Cy3-ODNs incorporated, thus some third power function of diameter related to the particle volume. This scattering relationship increases the likelihood of larger particles occluding smaller ones from measurement in the light scattering mode. This effect can be mitigated by lowering the total nanoparticle

![Overlay of 100% Cy3-ODN loaded CLNs](image1)

Figure 30: Total Particle Size Distributions as determined by fNTA (blue), and scNTA (orange, note the shift in scNTA measures to slightly larger sizes.

![Model Fit Using Hypergeometric Distribution n*Cy3=100](image2)

Figure 31: Model fit result using a characteristic fNTA size distribution in 10nm bins and a value of n*Cy3=100.
concentration in a measurement but not eliminated entirely, and as shown above in Figure 31 the difference between fluorescence and scattering concentration measurements also depends on the fluorescent dye loading. The scNTA distribution depicted in Figure 30 is not impacted by the loading level. Under scatter glare patterns from larger particles lead to noise by creating areas in the image of similar intensity to a scattering particle, which then rapidly disappear and reappear giving frequent short incomplete particle tracks which can trigger high noise warnings and in turn occlude small particles from accurate tracking. Cryo-TEM images of the CLNs showed a characteristic multilamellar structure, confirming CLN formation.

In order to determine the threshold sensitivity we must show that the ODNs are randomly distributed without regard for the presence of Cy3. To determine such we employ two types of CLN preparations. Loadings describe those CLNs which are formed with varying molar ratios of ODN and Cy3-ODN mixed before the CLN formation is prepared. These will be compared to dilutions, mixtures of two preparations of CLNs formed with only ODN or Cy3-ODN respectively. The dilutions should show linear response in the concentration ratio ([fNTA]/[scNTA]) curve with increasing percentage of Cy3-ODN, this is confirmed in Figure 32. Figure 31 shows the results from the loadings, a sigmoidal curve bounded by 0 on the lower end and 1 on the upper end with loading preparations at

Figure 32: Dilutions for three different loading levels. 10% (triangles), 40% (squares), and 100% (circles). The linear behavior of the concentration result differentiates the phenomena of dilution from that of formation under different loading of Cy3-ODN [120].
greater than 60 mol percent Cy3-ODN resulting in [fNTA]/[scNTA] ratios near 1, indicating that at 60% loading virtually every particle contains a sufficient number of Cy3 molecules to be accurately tracked. Below 60% loading the [fNTA]/[scNTA] decreases because not every particle formed carries sufficient Cy3 to be tracked by fNTA, the effect of decreasing Cy3-ODN loading percentage is pronounced in the loadings from 10-60% dropping the [fNTA]/[scNTA] from 1 at 60% to .21 at 10%. For Cy3-ODN loadings less than 10% [fNTA]/[scNTA] values continue to decrease and, corresponding to 0% loading and thus a [fNTA] result of 0. The sigmoidal response to Cy3-ODN loading percentage indicates that the ODNs are randomly distributed, if they were distributed based on the presence of Cy3 the response would be linear instead. The concentration ratio near 1 for high loadings confirms that all particles observed under scatter contain ODN. More critically the sigmoidal curve can be modeled to determine the number of Cy3-ODN which need to be incorporated into a CLN to provide sufficient signal for tracking, n*<sub>Cy3</sub>. The fit of the model results in a n*<sub>Cy3</sub> value of 100, meaning that a minimum of 100 Cy3 molecules need to be enclosed in a particle for sufficient intensity to be tracked by fNTA, the necessity that the particle be visible for enough frames to complete the tracking is built in. The model incorporates the size distribution of CLNs (in 10nm bins, for reasons discussed in detail below), a hypergeometric distribution regarding the formation of the particles without replacement of Cy3-ODN in solution determines the probability that any individual particle of diameter D will harbor n*<sub>Cy3</sub> Cy3-ODN or greater within each loading preparation. The total particles containing n*<sub>Cy3</sub> for each loading divided by the [scNTA] is fit to the [fNTA]/[scNTA] data. The total available ODN molecules, Cy3-ODN (N*ODN) and ODN (N<sub>ODN</sub>), available for
formation at each D are used to determine the probability a Cy3-ODN will be incorporated at any site in a CLN of diameter D. Using the 10nm binned data we can model each bin as adding a layer of surface area for the ODN to occupy, we then set a packing factor (nm²/ODN) such that the mass balance around the total ODN in the system is closed. When observing the fit of the model it is important to differentiate between the experimentally observed values and the estimates of the model, the model will never allow for a particle which is set to contain less than n_{ODN} at size D to contain more than expected, this assumption may bias to the model to larger particle sizes in the distribution particularly when looking at ratios of size between fNTA and scNTA. In order to run the model we employ a characteristic number fraction distribution for the loaded CLNs based on the average of the observed distributions at 100% loading for both fNTA and scNTA shown in Figure 30. This helps the model fit the trend of the data rather than fluctuating with exactly the observed size distribution at each fluorescent loading level. We can test the value of n^*Cy3 determined by the fit to [fNTA]/[scNTA] by looking at the ratio of probabilistic average size in each loading also, as shown in Figure 33. We note that over all of the loadings down to 10% the ratios of ratio of average size between scatter and fluorescent is less than 1, that is, the mean of the size distribution in the scatter
The measurement was greater, up until the 10% loading. The consistent ratio of the averages between modes up until 10% loading justifies the use of a characteristic distribution for determination of n*Cy3 because the particles should form to the same size distribution during each loading preparation as the charge ratio of DNA to cationic lipid is constant. The upturn in ratio is driven by both the NTA measurement method and the effect of photobleaching of smaller CLNs loading with size. To validate its size a particle must be tracked by the software for a certain number of consecutive frames based on the calculated size, with large particles requiring more frames to validate. As the loading percentage decreases the total number of particles with much more than n*Cy3 incorporated decreases, and the particles which become susceptible to photobleaching first are the smaller CLNs as they are more likely to have nCy3 very near n*Cy3 as they can accommodate far less Cy3-ODN. This accommodation factor can be understood by considering the increase in ODN accessible volume with each layer added to a CLN, from one to five layers each additional layer increases the ODN content by 20%, and at 15 layers approximately the maximum observed size, the outer layer accounts for 10% of the ODN content. The effect of photobleaching in the low level loadings is of greater influence on the mean size than mode because the larger particles, which will spend more time in the measurement before bleaching will show a corresponding increase in concentration in the determined size distribution, while the small particles are under represented dragging the mean size away from the mean size of the characteristic distribution. The disagreement in n*Cy3 between the two fits, being 100 by the [fNTA]/[scNTA] fit and approximately factor of three lower at 35 in the mean size fit may be driven by fitting the data to the least reliable points in the mean sizes data (lowest
loadings), or the inherent bias to larger sizes. It is safest to label n*Cy3 as roughly equal to 100 molecules and characterize this result an upper approximation which may serve as a guide to how many fluorophores one needs to load in a single particle to accurately track by fNTA. In the following sections we will consider in greater depth the model itself, and the practical application of these results.

6.4 Modeling Fluorophore Inclusion in a CLN

In order to effectively model the concentration ratio \([fNTA]/[scNTA]\) for each loading we must develop a model regarding the likelihood that at least \(n^*\) Cy3-ODNs will be incorporated in a single CLN during the formation. The results of NTA provide a tremendously useful data set for this solution, as the size distribution is number weighted by relative concentration directly from the measurement. This number weighted distribution is not accessible by orthogonal sizing techniques, for comparison the transformation of the intensity weighted distribution in dynamic light scattering to a number count weight is fraught with error. The power in the number weighted distribution is it allows for accurate modeling of geometric implications of Cy3-ODN loading for particles of different sizes, where the volume fraction of all particles size \(D\) and thus the relative amount of Cy3-ODN incorporated is easily calculated. The discussion and calculations which follow are based upon the mathematical description for fNTA developed by Dr. Michael Paulaitis in Appendix B.

Let there be a true size distribution for the CLNs independent of measurement method, \(\pi(D)\), where \(D\) is the diameter of the CLN and \(\pi\) (\(D\)) the associated probability of finding a particle at size \(D\). Because we know from Figure 31 and Figure 33 that a size dependent fNTA bias occurs with decreasing Cy3-ODN loading we can use the average size
distribution of the 100% loaded fNTA measurement to as a characteristic size distribution \( \pi (D) \), shown in Figure 30. From the characteristic size distribution, total nanoparticle concentration \( (N_{p,\text{tot}}) \), and known total molecules of ODN \( (N_{\text{ODN}}) \) and Cy3-ODN \( (N^*_{\text{ODN}}) \) the fraction of all ODN available for formation of particles of each size is estimated. Understanding the total ODN \( (n_{\text{ODN}}) \) in a single particle represents the number of “trials” for incorporation of a Cy3-ODN, and we want to predict the total number of CLNs incorporating \( n^*_{\text{Cy3}} \) or greater as the value of \( N^*_{\text{ODN}} \) decreases.

6.4.1 A mass balance on the total ODN

The value of \( n_{\text{ODN}} \) is proportional to the volume of the particle, which is straightforward from the multilamellar geometry. Using high resolution Cryo-TEM data we can probe the total volume accessible to the ODN in solution, and thus the loading level based on a packing factor as shown in Appendix B. In light of the physical situation (multilamellar CLNs) the value of \( n_{\text{ODN}} \) can alternatively be approximated based on the number of layers in the nanoparticle, the specific surface area associated with each layer, and a simple effective packing area of each ODN \( (\text{nm}^2/\text{ODN}) \), as has been done in the following calculations. The effective packing area of ODN is determined such that a mass balance is closed on each particle synthesis around the total ODN in the system. In the 1nm binned data the same approach is taken but we use a volume packing factor, surprisingly the volume packing factor is virtually the same as the This makes the value of ODN incorporation efficiency (and thus the true value of \( N_{\text{ODN}} \)) a critical parameter, as the true value of \( n^*_{\text{Cy3}} \) is linearly related. As an upper limit approximation the value of \( n^*_{\text{Cy3}} \) reported here is for the case of all ODNs being in lipoplex after formation, if it is realized that say 50% of the ODNs are left out of lipoplex the value of \( n^*_{\text{Cy3}} \) will respond linearly.
[118]. The results between either method of calculation for the value of \( n_{\text{ODN}} \) per particle are agreeable showing less than 10% difference for particles greater than 50nm.

### 6.4.2 Binomial or Hypergeometric Distribution?

When modeling the uptake of Cy3-ODN into a single particle it is at first simple to think of a binomial distribution wherein the probability of a “success” or selection of a Cy3-ODN can be calculated as the mol fraction of Cy3-ODN in the formulation. This assumption may not hold in the region below 10% loading, where the frequency of Cy3-ODN is greatly decreased and a hypergeometric model, which accounts for trials “without replacement” may be more appropriate. Appendix B calls for the cumulative hypergeometric distribution that a particle containing \( n_{\text{ODN}} \), will contain more than \( n^*_{\text{Cy3}} \) when formed in a mixture containing \( N_{\text{ODN}} \) and \( N^*_{\text{ODN}} \) based on the total particle volume fraction associated with the particle size \( D \).

Interestingly the assignment of \( N_{\text{ODN}} \) and \( N^*_{\text{ODN}} \) based on the scale factor times \( n_{\text{ODN}} \).
N\textsubscript{ODN} is not influential on the cumulative hypergeometric distribution function.

Assigning values of N\textsubscript{ODN} and N\textsubscript{\textbullet{ODN}} based on a simple scaling factor on n\textsubscript{ODN} reveals this clearly in Figure 35. This means that probability of Cy3-ODN incorporation should be well approximated by a binomial distribution with the probability of success equal to the mol fraction of Cy3-ODN used, this is confirmed in Figure 34 which shows that regardless of model choice the results for a test value of n\textsubscript{Cy3} are virtually identical, with only subtle differences orders of magnitude below significance. In light of these results we can use either distribution with confidence as the fit is not ultimately consequential, but will move forward with the hypergeometric distribution as the uptake of Cy3-ODN is best represented by the distribution “without replacement”.

6.4.3 The bin size of characteristic distribution does not affect the model fit

Data collected by NTA can be exported in user specified bins which represent the resolution of the size distribution and concentration measurement, the bin size can be set as low as one nanometer. When performing evaluations based on the binned data the resolution of the distribution could be an influencing. Consider π (D) between D=30nm and D=40nm at 1nm resolution, when calculating the area or volume weighting of each bin the small change in diameter effects a significant change in the surface area or volume. This growth increases the value of n\textsubscript{ODN} and thus the likelihood a particle will surpass the loading of n\textsubscript{Cy3}. When instead all particles from D=30nm to D=40nm are considered identical the total particles in the bin are given the same probability of containing n\textsubscript{Cy3} and thus as loading decreases this data input may result a sharp drop in
concentration as a whole bin particles being eliminated at once when the probability of loading \( n^*_{Cy3} \) becomes remote for a particular value of \( D \). By the same token the 1nm resolution of \( \pi(D) \) results in more precise calculation of the cumulative distribution which might depress the value of \( n^*_{Cy3} \) at best fit. However we find the value of \( n^*_{Cy3} \) is equivalent for both data inputs and the comparable results between different bin sizes are illustrated graphically in Figure 36. We also find that the resolution of \( \pi(D) \) does not impact the results comparing between hypergeometric and binomial distribution either, thus only the hypergeometric distribution will be used from here on. With the lack of meaningful impact on the result deciding what binning is most appropriate becomes a philosophical discussion. One can argue that by performing NTA the particle diameter reported is the equivalent spherical hydrodynamic diameter, this sizing includes the hydration shell around the nanoparticle, and has some level of broadening induced by variations in track length of individual particles (although only “valid tracks” are used to form the size distribution). The CLNs are by nature layered structures, the accessible volume changes as layers are added which is well captured with the 10nm binned size distribution. Thus \( \pi(D) \) may be more appropriately modeled by using 10nm bins. A counter argument could be made that the multilamellar structure is

\[ Figure 36: The influence of bin size, notice that each model fit, gray or orange is essentially the same for the given value of \( n^*_{Cy3} = 100 \) \]
not identical on every particle, and numerous TEM images [114] show a wide variation in the layer by layer structure where some layers may form incompletely. Thus it is plausible that the 1nm bins more accurately reflect the true nature of π (D), where particles of virtually every size are formed by virtue of incomplete layering. In either case the fit of the model to the data is equivalent.

6.4.4 What drives the difference in value of $n^*_{Cy3}$ when comparing diameters vs concentrations?

We note in the results that the model result for $n^*_{Cy3}$ is lower by a factor of three when fit to the diameter ratio between fNTA and scNTA measurements as compared to the concentration ratio. The choice of bin width is again not influential in this fitting as shown in Figure 37. It is not entirely clear what causes the difference in fitting between the two methods, where the expected ratio of mean diameters is consistently higher in the model than the data at the value of $n^*_{Cy3}$ which provides a good fit to the concentration ratio data. While the value of $n^*_{Cy3}$ is within the same order of magnitude this result makes it difficult to state exactly what the value is. The same mass balance is closed in both cases, but clearly one of the models is missing something about the encapsulation in the particles. The fNTA
measurements at loadings less than 10% are arguably the weakest data points statistically, as would be expected with the decreased number of tracks and the obvious bias to larger particle sizes. This hypergeometric model fits the data at high loadings well with little regard for the value of \( n^*_{\text{Cy3}} \) so it is possible we see an artifact of trying to force a fit on the weakest section of the data, whereas the concentration approach is much more sensitive to changes in the high loadings. Lastly it might be possible that all smaller lipoplexes form first and thus have a better chance of containing the requisite \( n^*_{\text{Cy3}} \) molecules of Cy3-ODN, which are depleted by the time more layers have been added, this would lead to an unexpectedly low ratio of mean diameters which is clear in the resulting \( n^*_{\text{Cy3}}=35 \) by mean ratio fit, compared to \( n^*_{\text{Cy3}}=100 \) by the concentration ratio fit. The total volume fraction associated with each layer grows significantly with size and in combination with the

6.5 Practical applications and considerations for other fluorophores

The practical application for determination of this value is a clear understanding of how many fluorescent molecules need to be concentrated in a particle to provide sufficient signal for accurate tracking by NTA, the value of \( n^*_{\text{Cy3}} \) reported here is specific to the Cy3 fluorophore, this CLN particle system, and the particular NTA instrument configuration, but with this value of \( n^*_{\text{Cy3}} \) a reasonable estimation can be made for \( n^*_{\text{Cy3}} \) regarding other labeling methods and probes for use in NTA. We can give further consideration to the meaning of \( n^*_{\text{Cy3}} \) by examining the optical arrangement of the NanoSight Instrument used to measure. The equipment features a laser excitation source at 532nm and the fluorescent signal is collected after passing through a 565 nm long pass filter. Cy3 is an orange fluorescent dye with a maximum excitation at 554nm, the
excitation is 52% of maximum under the 532nm laser source used in these experiments. The emission signal is collected through the 565nm long pass filter will result in an additional 30% signal reduction, leaving 35% of the original signal available for tracking. For comparison AlexaFluor 488 (AF488), when excited with a 488 nm laser and imaged through a 500nm long pass filter would have 72% of its total signal available, more than double the Cy3. This also does not account for the inherently greater quantum yield of AF488, thus in a similar application one could expect that the value of $n^*_{Cy3}$ for AF488 would be less than that determined in this optical arrangement for Cy3. A different dye which was less optimal with a chosen laser and emission filter would in turn give a higher values of $n^*_{Cy3}$. All calculations in this section were made using Life Technologies Spectra Viewer.

It should be recognized that the determination of $n^*_{Cy3}$ presented herein is specific to the Cy3 fluorophore, the CLN particle system, and the specific instrument configuration utilized in the experiments. The optical arrangement of the NanoSight instrument included a 532nm excitation source, giving 52% of maximum excitation for Cy3, and the data was collected through a 565nm long pass filter, leaving a total signal at 35% of the maximum. To consider $n^*$ for other fluorophores or particle systems the relationship between the optical arrangement of the measurement instrument, optimum excitation and emission wavelengths, and characteristic brightness of the fluorophore will alter the value of $n^*$ though we suspect it is within the same order of magnitude for most common fluorophores.
6.6 Comments on antibody mediated labeling of EVs

In the course of my work at the Analytical Cytometry Shared Resource at Ohio State I had the opportunity to attempt to specifically label surface markers on EVs with various groups, none of which were distinctly unsuccessful. Despite this I would like to take a moment to discuss some of the considerations that should be made with regard to optimization of these labeling techniques. Labeling nanosized EVs is more difficult than labeling cells, the dense micron sized cells can be flooded with antibody and easily pelleted by a quick centrifugation to separate them from unbound antibody and/or excipient fluorophore. The size signature of the cell either under the microscope or on the forward and side scatter channels of a flow cytometer give indirect confirmation that the body under inspection is in fact an intact cell. The EVs are difficult to remove from unbound antibody, requiring chromatography or preparative ultracentrifugation [19]. It is difficult if not impossible to judge the frequency of a specific biomarker within a heterogeneous population of EVs, making the calculation of an appropriate antibody concentration for complete binding difficult. Some antibodies have equilibrium positions where more than 90% is free is solution when the marker of interest is saturated. The minimization of free fluorophore (and thus antibody in the case of labeled primaries) in the solution is critical to success, thus it is useful to consider the number of EVs, the relative display of the marker, and in turn how many antibodies should be incubated with an EV sample. Aliquot EVs so that an appropriate number for analysis (on the order of $10^9$ total) are contained in a small volume. Using separate samples add several different concentrations of antibody, to account for potentially wide variations in antibody display. If the frequency of a specific marker is less than 1 EV in 1000 it becomes quite unlikely
that labeled particles will be reliably measured by fNTA because of the timescale required to complete 200 valid tracks. It is possible to use secondary antibodies to “amplify” the signal of a single bound primary, or other biointeractive molecules such as biotinylated antibody and streptavidin conjugated fluorophore. Based on the sensitivity to Cy3 calculated in this work, one could estimate the number of fluorophores needed for a different marker and in turn the demands on signal amplification if the frequency/display on a single EV is quite low.

6.7 Conclusion

In summary this chapter discusses the difficulty involved with making accurate fNTA measurements, particularly when the true size distribution and fluorophore content of nanoparticles are unknown. Regarding the sensitivity of fNTA I am comfortable stating the lower limit of sensitivity for a 532nm laser/565 LP filter equipped instrument is on the order of 100 molecules of Cy3 per nanoparticle. While Cy3 is not particularly well suited to this optical arrangement, the number of fluorophores that are seemingly required for accurate tracking needs to be considered for other applications including the labeling of EVs. The method of titration utilized in this work could be easily mimicked in a different model system, and values of n* for other fluorophores and optical arrangements documented.
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Chapter 2


Appendix A: NTA SOP for Analytical Cytometry Shared Resource

NanoSight SOP at ACSR

By: Clayton Deighan

This document will cover all aspects of NanoSight as it pertains to sample preparation, data acquisition, analysis, and operation for the NS300 instrument and NTA 3.0 software.

What is the NanoSight?

The NanoSight is a light scattering and image processing instrument which measures number concentration, and size distribution for particles in suspension in the size range 10-2000nm. It is suitable for use with virtually any material type and buffer condition, given that the buffer is verified to be free of native particles. The technique is based on capturing videos of Brownian motion of nanoparticles in solution, and based on this motion and the number of particles observed in each frame the software tracks the particle motion to determine the size of each particle according to the Stokes-Einstein equation.

It is worth noting that the form of the Stokes-Einstein equation uses the mean squared displacement in two dimensions, while the particles are observed moving in a three dimensional space. This however is of no consequence as the two and three dimensional forms of the Stokes-Einstein equation are perfectly equivalent in their result.

The instrument uses a laser diode as the illumination source, the laser passes through a prism which defines it shape as it enters the sample chamber. The depth of the laser is similar to the focal depth of the 20x objective used to observe the particles, this setup is the key to the technique as particles which are out of focus do not scatter any light, allowing those which are illuminated to be tracked frame by frame in a video and their size resolved based on the observed Brownian motion. The sample chamber is fitted with a peltier element which can control the temperature of the sample chamber between five degrees below ambient and 50 C, the temperature for each frame of video captured is recorded whether temperature control is being used or not, this combined with viscosity of the suspending medium leaves on the diffusion coefficient unknown, and from the diffusion coefficient the equivalent hydrodynamic diameter is calculated.

The images that appear on screen are not resolved images of the particles, that would be impossible with the 20x objective. Rather they are visualized as point scatterers whose movement can be tracked to extract the size.

What types of samples can NanoSight measure?

NanoSight is largely insensitive to material type, polymeric nanoparticles, liposomes, protein aggregates, colloidal metals, quantum dots, self assembling particles, microvesicles and other subcellular bodies can all be sized effectively in an NTA
measurement [3]. NanoSight is extremely sensitive to concentration, and requires that samples be diluted to an appropriate concentration before they can be effectively measured. The lower size limit of the measurement is determined by the scattering efficiency of the particles of interest, with 10\text{nm} for colloidal metals and other species of high refractive index, and 40\text{nm} for biologics. The lower limit of the concentration measurement is $10^7$, driven by the inability to complete enough tracks when the concentration is lower. The upper limit of concentration is $10^9$, driven by the inability to properly resolve the movement of neighboring particles, and at times the propensity of the background subtraction feature to remove too many of the dim particles in the viewing window, leading to erroneous counting. Methods of assessing whether a measurement should be accepted will be discussed later.

**How should samples be prepared?**

Sample preparation is easy, and the choice of suspending medium is largely inconsequential to the final result and it is the duty of the user to ensure that the medium is appropriate for their particles of interest. All samples should be prepared to 1 ml final volume for measurement. The most important part of preparing samples is moving them from (often) high concentration to the $10^8$ particles/ml range necessary for measurement. This may require several orders of magnitude dilution and it is recommended to use recently calibrated pipettes, and to dilute no more than 100 times in any single step.

For a series of measurements the user must provide enough suspending medium to dilute all of the samples to 1 ml volume, prime, flush, and clean the instrument after use, this adds up to roughly 50 ml per 10 samples. It is important to verify that the suspending medium is virtually particle free by running it through the instrument with the camera active, at which time no more than 5 particles should be visualized with the camera turned to its highest setting. For fluorescent nanoparticles the particle free medium requirement is relaxed, but it still must be verified that the suspending medium does not autofluoresce.

Operational procedure (requires 10ml luer syringe for flushing/cleaning, and 1ml luer syringes for running samples.

**Priming the equipment and software start up**

1. Turn on the computer tower by pressing the power button in the upper corner of the front panel, no password is required and it will boot to the desktop.
2. On the left hand side of the NS300 turn on the black power switch, the power indicator lights will come on, and the filter wheel lights should roll through one time (you can hear the filter wheel spin also).
3. Turn on the NTA 3.0 software on the desktop, the screen below should open up.
4. In the lower left hand corner the Hardware Status panel (green box above) to tell you the state of the instrument, and confirm connectivity with all the components, FOCUS H/W, SYRINGE PUMP H/W, FILTER WHEEL H/W, and TEMPERATURE H/W should all show up in green text. The heater should be off when you start the instrument, and the temperature will be reported only when the module is in the instrument.

5. With the system powered up you must select a top plate for the experiments you plan to run, there are 4 sprung screws which hold either top. The decision for which one to use (plastic or metal) can be dictated by your application, for aqueous solutions of mM salt concentration, and pH 4-7 the plastic will work fine. For solvents, strong acids or bases, or solutions which may have high concentrations of particles it is recommended to use the metal top plate. To attach the top plate to the laser module use the four spring loaded screws, start each screw and then hand tighten (NEVER USE TOOLS) each screw in a diagonal pattern until they stop. The same screws work with either top plate.

6. With the top plate of choice attached to the laser module slide the laser into the machine and flip the red lever at the end of the stage guides.

7. There are two hoses coming from the right side of the instrument, the luer tipped one will be used to introduce new samples, and flush between runs. The other should be placed in the waste collection bucket, but do not allow it to sit in the expelled fluid in the waste collector, it should drip from above into the collection volume.

8. To prime the instrument turn the module so that the gold connection grid is pointing up, the idea is air will be less likely to be trapped in the chamber, draw up 7-9ml of your clean medium, attach the syringe to the luer fittings and slowly inject the solution, you will see the sample chamber fill from top to bottom, a slight tilt to point the out port upwards as the chamber fills can be helpful to eliminate any trapped bubbles. Verify that no leaks have occurred in any of the hoses, connections or between the top plate and optical flat.

9. When you see the solution emptying into the waste container insert the now primed laser block into the instrument and turn the red lever to lock it in place. The module light will illuminate on the front of the instrument to indicate it is in
place properly. When inserting the module be aware and do not damage the objective lens by ramming the module into it. At this point the module is primed and ready for sample introduction.

**Sample Analysis**

Before introducing the sample, verify the suspending medium is free from contaminating particles by turning on the camera with the module in place. Click the start camera (green box) button as indicated below, the default camera level is 7 (blue box), 7 is rarely useful, to check for the presence of particles in the medium turn the camera up to level 16. If there are a lot of particles present push on the 10ml syringe to flush more suspending medium through the system, if the particles cannot be eliminated then the medium is contaminated with particles and should not be used for NanoSight experiments. The acceptable level of contaminating particles can be debated based on the concentration of the samples to be measured, but no particles in the field of view is most desirable.

When the sample chamber is sufficiently clean draw up the 1ml of diluted sample for measurement. One of the critical steps for quickly measuring multiple samples is good technique when switching from the flush syringe to the sample syringe to prevent the introduction of air. The luer port should never be left open to the air, when you have the sample drawn into the syringe remove the 10ml flush syringe and hold the port vertical and carefully drip two drops of the sample into the port before inserting the syringe. Watch carefully for a bubble when loading the syringe, if one appears, simply pull it back into the sample and tap it to the plunger end, keep the camera on and level at 16 during sample injection and wait for the particles to arrive, they should be visible by the time there is .4-.3ml left in the syringe.

**IF THE PARTICLES DO NOT ARRIVE**

1. Adjust the focus, roll the knob on the side of the machine top to bottom, by choosing the advanced tab in the software, and then the focus tab you can see the exact position of the objective.
2. Remove the laser block and visually inspect for bubbles.
3. If it seems like only a few particles arrived the sample may be too dilute, decrease the dilution factor.

When the particles have arrived on screen stop the syringe injection, the particles should quickly stop advancing. There should be .3-.4ml remaining in the syringe when the particles are in view. Place the syringe in the syringe pump. The brass button on the side of the screw will allow the plunger holder to slide freely so that it can be positioned
appropriately for the size of the syringe. The plunger of the syringe sits in the slot that is backed by a plate with two thumbscrews, tighten the thumb screws to lock the syringe in place.

To run the syringe pump go to the hardware tab, then select syringe pump, the units are arbitrary. With the infuse rate set to 1000, start the syringe pump until the particles move quickly across the screen, this is necessary to properly engage the syringe with the pump. Press stop, change the inject value to 30 and then click start again, the particles should slowly advance (5-10 sec residence time) from left to right across the screen.

With the particles flowing use the black focus knob to set the rough focus, it is often useful to move between out of focus on both sides of the particles to determine the best position, when the particles look like nice round vibrating spheres use the fine focus to make any final adjustments. In polydisperse samples it is often impossible to have every particle perfectly in focus, blurring and diffraction rings on some of the particles will be present in most samples. Try to set the focus such that the smallest/dimmest particles visible have the clearest image.

To set the camera level increase it to maximum 16 and work down, minimize the number of particles which show green false colored centers as this is indicative of saturation, always use the highest camera level possible. The camera level can be related to material type, in metals and other high refractive index materials camera levels under 10 can easily visualize 100nm particles. As a check one can use 100nm polystyrene spheres diluted 1000:1 in water, they should be visible at camera level 5-7. Biologics and other samples with low refractive index often require camera settings 13+ in order to be visualized. The screen gain does not influence the measurement and does not need to be adjusted.

With the camera and focus set and the syringe pump running it is now time to create an SOP to take the measurement and produce results.

In the SOP tab shown below there are several options for users to set

Number of Captures- 5 is sufficient for almost all samples, in experiments with low concentration more than 5 may be used. The upper limit is 10. For samples which are largely monodisperse 3 captures can be sufficient to establish agreement between the run to run distributions.

Length of Capture- Chosen by the number of particles visible on the screen if 30+ particles are visible 30 seconds is sufficient, <30 particles use 60 seconds.

Date and time name- leave checked, appends the date and time of capture to the filename.

Process after capture- leave checked, will prompt you to process files immediately after capture.

Process settings each file- leave unchecked, process all videos under the same settings.

Export after process- leave checked, this will prompt you for data export after the measurement is complete.
Temperature can be activated and set by choosing the boxes. It is not necessary for any measurement, but can be a controlled experimental parameter.

Base File Name- Click the … button to open the file save routing. There are two locations appropriate for saving files Documents ->NanoVideos->Your Folder or D:// ->Your Folder. If it is your first time running samples, make a folder with your name it either of the two directories. Within my folder I arrange by experiment name and date. The location selected for the base file is where all of the associated data will be exported to. Name each measurement and as a good practice include the dilution factor used in the file name, this will prevent confusion later on. Save the filename and continue.

Open the Advanced menu (pictured below) and check continuous syringe pump flow, then choose the same syringe pump flow rate of 30. If your solution requires the use of different viscosity, uncheck Use Water and input the the desired value in cP. Click OK to close the dialogue box and save the settings.

At this time the script is completely prepared, the camera and focus are set, and the syringe pump is running. Click create and run script to start the measurement, the dialogue box below will pop up, enter the relevant information and any remarks, all the information entered will be included on the PDF report of the results. When the information is complete press OK to start the script, the current step will be highlighted green in the Script control panel and the current repetition (for multi video runs) is displayed just below the script panel. After the videos have finished capture the software will automatically advance to processing, a dialogue box will prompt you to select the Detection Threshold, the detection threshold can be thought of as the signal/noise discrimination, when set too low many spots which are not particles will be falsely attempted to be tracked, and when it is set too high dim particles may be missed in the tracking. As the detection threshold setting is change the red and blue crosses on the screen will move, marking different particles, a good video will have almost all of the
particles marked with red crosses. To see how a setting will work throughout a video grab the slider just below the video viewing window and drag it forward. For many videos detection threshold settings of 3-5 are appropriate, if the software indicates high noise during processing, increase the detection threshold. Keep a careful eye on the processing, if you notice that many diffraction rings or blurred areas around particles are creating false tracks and centres increase the detection threshold a bit more. Once again it is not necessary to change the screen gain as it has no influence on the results. When a satisfactory detection threshold is set, allow the videos to process, at the conclusion the export dialogue pictured below box will pop up, always select Include PDF-letter, and Include Experiment Summary (excel csv file). If videos for use in presentations are desired, choose Microsoft WMV 10 sec, each video file will be converted to a 10 sec wmv at much smaller size. The results files and videos will be stored in the same folder at the base filename directory.

With the data exported the measurement and processing of one sample is complete. Turn the camera back on and flush the sample chamber with 3-5ml of clean diluent before loading the next sample. For each new sample it is only necessary to change the base filename and then click Run on the script panel to take the measurement.

**Clean Up**

When sample runs are complete for your time period, flush the sample cell clean with diluent, and then follow that with 5-7ml of DI water. Making sure the waste tube is removed from the collection remove the sample cell and use reverse flow on the syringe to remove the water from the system, this action requires slow and steady back pressure. When the fluid is drained remove and discard the syringe. Remove the four screws from the top plate and store them in the appropriate container. Wipe the optical flat and top plate dry with a kim wipe and replace them in the instrument for the night. Close the software, turn the power switch on the side of the instrument, and shut down the computer. To remove your data please provide a jump drive, data left on the computer longer than three months will be removed.
Appendix B: Mathematical Description of ODN loading into CLNs by Dr. Michael Paulaitis.

Modeling the dependence of fNTA on the number of bound fluorophores

Let's suppose a “true” nanoparticle size distribution exists independent of the measurement of that distribution by nanoparticle tracking analysis using fluorescence (NTA) or light scattering (soNTA) to track and count the nanoparticles. We will represent this size distribution by $\pi(D)$, the “true” probability of finding a nanoparticle with a diameter, $D$. We can also define the “true” total number of nanoparticles in a sample, $N_{\text{prot}}$, such that the “true” number of nanoparticles with a diameter, $D$, is

$$N(D) = \pi(D)N_{\text{prot}} \quad .$$

(1)

Now define two conditional probabilities that characterize the measurement of this “true” size distribution by fNTA and soNTA. For fluorescence tracking, this conditional probability is $p_f\left(n_{\text{eq3}}|D\right) > n_{\text{eq3}}^{*}|D\right)$ with $n_{\text{eq3}}|D\right)$ the number of Cy3-labeled ODN molecules in a nanoparticle with diameter, $D$, and $n_{\text{eq3}}^{*}$ the threshold value of Cy3-labeled ODN molecules required for fluorescence tracking. The threshold value is independent of the nanoparticle diameter. For tracking by light scattering, this conditional probability is $p_{\text{sc}}\left(v_{\text{sc}}|D\right) |D\right)$ with $v_{\text{sc}}|D\right)$ the nanoparticle scattering volume, which does not depend on $n_{\text{eq3}}|D\right)$ or $n_{\text{eq3}}^{*}$.

With these definitions in hand, we can define the nanoparticle concentrations measured by fluorescence and light scattering tracking. For fluorescence tracking,

$$[\text{fNTA}] = \frac{N_{\text{prot}}}{V_s} \int_0^\infty \pi(D) p_f\left(n_{\text{eq3}}|D\right) > n_{\text{eq3}}^{*}|D\right) dD \quad ,$$

(2)

and for nanoparticle tracking by light scattering,

$$[\text{soNTA}] = \frac{N_{\text{prot}}}{V_s} \int_0^\infty \pi(D) p_{\text{sc}}\left(v_{\text{sc}}|D\right) |D\right) dD \quad .$$

(3)

Note that the dependence on $D$ has been dropped for $n_{\text{eq3}}$ and $v_{\text{sc}}$ in these equations and below to simplify the notation. Since we are interested in the ratio of the two concentration measurements as a function of $n_{\text{eq3}}$, we can treat Eq. 3 as a constant, independent of the number of Cy3-labeled ODN molecules in a nanoparticle,

$$\frac{[\text{fNTA}]}{[\text{soNTA}]} = C \int_0^\infty \pi(D) p_f\left(n_{\text{eq3}}|D\right) > n_{\text{eq3}}^{*}|D\right) dD \quad .$$

(4)
with $C$ defined from the experimental observation that $N_{p,sc}/V_{c}$ measured by nNTA at 100% Cy3-ODN loading and by scNTA is essentially identical for $D \leq 300$.

$$C^{-1} = \int_0^{\infty} \pi(D) f_{\text{meas}}(\kappa;\rho)\,d\kappa D \approx 1.$$ (5)

We now express the integrand of Eq. 4 in terms of $n_{\text{obs}}(D)$, the total number of Cy3-labeled and unlabeled ODN molecules in a nanoparticle with a diameter, $D$:

$$\pi(D) f_{\text{meas}}(\kappa;\rho)\,d\kappa D \approx \sum_{k=n_{\text{obs}}+1}^{n_{\text{obs}}} p(D|n_{\text{obs}} = k),$$ (6)

where $p(D|n_{\text{obs}} = k)$ is the nNTA nanoparticle size distribution measured at 100% Cy3-ODN loading (Fig. 1 in the main text) and the summation is the cumulative probability of finding more than $n_{\text{obs}}$ Cy3-ODN molecules given that a nanoparticle with diameter $D$ contains $n_{\text{obs}}$ Cy3-labeled and unlabeled ODN molecules. We evaluate this cumulative probability by applying the hypergeometric probability distribution [1] to each term,

$$p(D|n_{\text{obs}} = k) = \frac{h(k;\phi_p(D) N_{\text{ODN}}, \phi_p(D) N_{\text{ODN}}^* - n_{\text{obs}}, n_{\text{obs}})}{\phi_p(D) N_{\text{ODN}}} \frac{\phi_p(D) N_{\text{ODN}}^* - n_{\text{obs}}}{n_{\text{obs}} - k}.$$ (7)

where $N_{\text{ODN}}$ and $N_{\text{ODN}}^*$ are the total numbers of ODN molecules and Cy3-ODN molecules, respectively, added in the lipoplex nanoparticle formulation, and $\phi_p(D)$ is the volume fraction of nanoparticles with diameter $D$, and

$$\left(\frac{n}{\rho}\right) = \frac{n}{\rho} \left[\frac{n}{\rho} - \rho\right].$$ (8)

Thus,

$$\frac{[\text{nNTA}]}{[\text{scNTA}]} = \int_0^{\infty} p(D|n_{\text{obs}} = k) \left[1 - H(n_{\text{obs}})ight] \,dD,$$ (9)

where $H(n_{\text{obs}})$ is the cumulative hypergeometric distribution function:

$$H(n_{\text{obs}}) = \sum_{k=0}^{n_{\text{obs}}} h(k;\phi_p(D) N_{\text{ODN}}, \phi_p(D) N_{\text{ODN}}^* - n_{\text{obs}}, n_{\text{obs}}).$$ (10)

The ratio of mean nanoparticle diameters measured by nNTA and scNTA is likewise given by

$$\frac{\langle D \rangle_{\text{meas}}}{\langle D \rangle_{\text{sc}}} = \frac{\int_0^{\infty} D^2 p(D|n_{\text{obs}} = k) \left[1 - H(n_{\text{obs}})ight] \,dD \,dD}{\int_0^{\infty} \pi(D) f_{\text{meas}}(\kappa;\rho)\,d\kappa D \,dD} \times \frac{\int_0^{\infty} \pi(D) f_{\text{meas}}(\kappa;\rho)\,d\kappa D \,dD}{\int_0^{\infty} \pi(D) f_{\text{meas}}(\kappa;\rho)\,d\kappa D \,dD}.$$ (11)
Lastly, to obtain an expression for \( n_{\text{ODN}}(D) \), we estimate the volume accessible to the ODNs in the multilamellar structure of the lipoplex nanoparticles, which we characterize by three parameters derived from cryo-TEM images of the lipoplex nanoparticles [2, 3]: (1) a core radius, \( R_{\text{core}} \approx 12 \text{ nm} \), containing no ODN-accessible volume, (2) the spacing between bilayers, \( \Delta R \approx 5.7 \text{ nm} \), and (3) the thickness of the space between adjacent lipid bilayers, \( \sigma = 3.0 \text{ nm} \). The total volume accessible to ODNs in the interstitial space between the bilayers of a nanoparticle with diameter, \( D = 2R \), is therefore,

\[
V_{\text{layers}} = \frac{\pi \varphi \sigma}{3} \left[ R^3 + (R - \Delta R)^3 + (R - 2\Delta R)^3 + \ldots + (R - \varphi \Delta R)^3 \right]
\]

\[
= \frac{\pi \varphi \sigma}{3} R^3 \sum_{k=0}^{\infty} \left( 1 - k \sigma \right)^3
\]

(12)

where \( \sigma = \Delta R/R, I = (R - R_{\text{core}}) / \Delta R \), and \( \varphi \) is a packing efficiency. The summation on the right side of Eq. 12 can be written as

\[
\sum_{k=0}^{\infty} \left( 1 - k \sigma \right)^3 = 2I^2x^3 + lx^2 + 6x + 6
\]

and therefore,

\[
V_{\text{layers}} = \frac{\pi \varphi \sigma}{3} \frac{\Delta R}{R} \left[ 2 \left( 1 - \frac{R_{\text{core}}}{R} \right)^3 + \left( 1 - \frac{R_{\text{core}}}{R} \right) \frac{\Delta R}{R} - 6 \left( 1 - \frac{R_{\text{core}}}{R} \right) + 6 \right]
\]

(13)

Note that as \( R \to R_{\text{core}} \), then \( V_{\text{layers}} \to \frac{\pi \varphi \sigma}{3} R_{\text{core}}^3 \), and in the limit of \( R \to \infty \), then \( V_{\text{layers}} \to \frac{\pi \varphi \sigma}{3} R^3 \). The corresponding total number of ODN molecules in a nanoparticle with diameter, \( D = 2R \), is obtained by multiplying this accessible volume by \( \rho \), the average ODN number density:

\[
n_{\text{ODN}}(D) = \rho \varphi \frac{2}{3} \frac{\Delta R}{R} \left[ 2 \left( 1 - \frac{R_{\text{core}}}{R} \right)^3 + \left( 1 - \frac{R_{\text{core}}}{R} \right) \frac{\Delta R}{R} - 6 \left( 1 - \frac{R_{\text{core}}}{R} \right) + 6 \right]
\]

(14)

with \( \rho \) specified by satisfying a material balance for the ODN molecules:

\[
\epsilon N_{\text{ODN}} = N_{\text{ODN}} \int_{0}^{\infty} \rho f_{\text{D}}(D) \delta_{\text{D}} - n_{\text{ODN}}(D) dD
\]

\[
\int_{0}^{\infty} \rho f_{\text{D}}(D) dD = N_{\text{ODN}}
\]

where \( \epsilon \) is the measured efficiency of loading free ODN in solution into the nanoparticles.

References

