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UNIVERSITY OF CALIFORNIA,
IRVINE

A Novel Approach to Extracellular Vesicle Isolation Using Immunoaffinity and Acoustic Microstreaming Microfluidic Device

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Iember Hemben

Thesis Committee:
Professor Abraham Lee, Chair
Assistant Professor Michelle Digman
Assistant Professor Jered Haun

2019

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DEDICATION

To

The most remarkable Tiv woman I know
Descended from elephants
Dr. Kwaghdoo Atsor Bossuah

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ABSTRACT OF THE THESIS

A Novel Approach To Extracellular Vesicle Isolation Using Immunoaffinity and Acoustic Microstreaming Microfluidic Device

By

Iember Hemben

Master of Science in Biomedical Engineering

University of California, Irvine 2019

Professor Abraham P. Lee, Chair

Early cancer detection results in higher survival rates for patients. Circulating tumor cells (CTCs) traveling through the blood can be used for early cancer diagnosis. Unfortunately, these CTCs are rare (as low as 1 CTC per billion red blood cells), limiting their use in early diagnostic applications. A substantial amount of research suggests a correlation between the presence of cancer and the circulation of EVs, but detailed knowledge on the role of EVs is still lacking due to inefficient methods in EV isolation. The current "gold standard" for EV isolation is ultracentrifugation. Ultracentrifugation produces low yield, contain damages cells, has poor reproducibility and low purity.

Microfluidic technologies are an attractive alternative for EV isolation due to their low-cost, low sample and rapid throughput. We propose a quick, low-sample microfluidic solution to EV isolation with efficiencies that rival the gold standard of ultracentrifugation. Using acoustic microstreaming and immunoaffinity capture methods, we report isolation of EVs for downstream analysis at high efficiencies and faster turnaround times, fewer steps and less equipment than UC.

CHAPTER 1: Introduction

1.1. Problem Statement

With an estimated count of 9.6 million deaths in 2018, cancer is one of the leading causes of death globally. Responsible for one in six deaths worldwide, it is likely that everyone has encountered someone affected by this disease[1]. Furthermore, a vast majority of cancer-related deaths (70%) occur in low and middle-income countries where resources and treatment are scarce. According to a study completed by the Public Health England – National Cancer Registration and Analysis Service, the chances of survival after cancer diagnosis are much higher for patients diagnosed at Stage 1. Out of 30,400 female patients diagnosed with melanoma, those diagnosed at Stage 1 had a 100% net-survival rate after one year. Patients diagnosed at Stage 4 yielded a 50% survival rate after one year. These statistics suggest that early detection of cancer could literally be a matter of life and death for many patients[2].

The importance of circulating tumor cells (CTCs) in cancer diagnosis has recently gained traction. CTCs can serve as a precursor to metastasis, provide genotypic and/or phenotypic information of a cancer and even offer 'real-time' tumor bioposies through simple blood tests[3,4]. While CTCs have the potential to aid in cancer screening protocols and guide prognosis, their rarity is an obstacle to their diagnostic application. With values as low as 1 CTC per billion blood cells, the reality is CTC capture is synonymous to the 'needle in a haystack' paradigm[5]. Thus, to address cancer diagnosis as early and accessibly as possible, a noninvasive, novel approach to identifying cancerous cells is needed.

1.2. Proposed Solution

The excessive release of extracellular vesicles (EVs) from cancer cells implies that EVs may play a significant role in tumor progression[6]. The rapid growth of cancer can be attributed to the cells ability to release factors which promote cancer growth and metastasis in the extracellular microenvironment. Extracellular vesicles have been shown to modulate tissue microenvironments by encouraging matrix remodeling and angiogenesis[7]. Unlike CTCs, quadrillions of EVs are constantly being circulated throughout the body, and cancer patients have exhibited higher concentrations of EVs. Specific markers associated with cancer permits the enrichment of cancer related EVs, opening doors for the use of cancer EVs in liquid biopsies. EVs have significant potential as a noninvasive biomarker for early detection, diagnosis and prognosis of cancer patients.

A substantial amount of research suggests a correlation between the presence of cancer and the circulation of EVs, but detailed knowledge on the role of EVs is still lacking. This gap in knowledge is largely attributed to inefficient methods in EV isolation. Accounting for 56% of implemented EV isolation techniques, the most common method of EV isolation is ultracentrifugation. Ultracentrifugation (UC) consists of a series of increasing centrifugation cycles that can require forces as high as 1,000,000xg[8]. Though ultracentrifugation is a low-cost

technique for EV isolation, it still has a myriad of shortcomings including low RNA yield, impurities, damage to EVs and low reproducibility[9].

Microfluidic technologies are an attractive alternative for EV isolation due to their low-cost, low sample and rapid throughput. Despite these advantages, microfluidic devices account for a measly 3.5% of published papers on EV isolation while ultracentrifugation accounts for 66.6% [9]. We propose a quick, low-sample microfluidic solution to EV isolation with efficiencies that rival the current gold standard of ultracentrifugation.

1.3. Scope of Report

This investigation details the motivation, design, methods, results, and discussion of a novel approach to EV isolation using an acoustic-streaming microfluidic device. An overview of the importance of EVs in cancer research and the current standards for EV isolation are provided in this report along with a comparison of the proposed microfluidic approach and ultracentrifugation. The verification of successful EV capture is corroborated by immunofluorescence, TEM imaging, flow cytometry and RNA quantification. Methodologies for the two approaches evaluated in this report are detailed. Subsequent findings are included in the report and the significance is discussed. A summary of the results, limitations, and future work conclude this report.

1.4. Summary of Conclusion

The results of this study demonstrate the combination of acoustic microstreaming and immunoaffinity capture as a promising alternative to ultracentrifugation for the isolation of EVs. The proposed method boasts faster turnaround, higher sample yields, less working sample and less specialized equipment required.

CHAPTER 2: Background

2.1. Extracellular Vesicles

It is well-known that cells release EVs of endosomal and plasma membrane origin. The size, function and composition of these EVs are diverse in nature. “Extracellular vesicle” is a blanket term that includes microvesicles and exosomes[10]. While details behind the differences between microvesicles and exosomes are still developing, the general approach to separating these two types of EVs is based on origin, lipid composition and size. Microvesicles have a diameter of 100-1000 nanometers and originate via outward budding and fission of the plasma membrane. Exosomes are much smaller in size, ranging from 40-150 nanometers in diameter. Produced internally, exosomes are created when membrane-bound compartments (endosomes) within a eukaryotic cell bud inward and fill the luminal space of the endosome with small vesicles. This endosome filled with vesicles is referred to as a multivesicular body (MVB) and the vesicles it holds are as known as exosomes. When the MVB fuses with the plasma membrane, it releases

exosomes into the extracellular space (Figure 2.1[7]). Exosomes are of higher interest than microvesicles, however, they can contain overlapping information. In this report, we will use the terms “exosome” and “extracellular vesicle” interchangeably.

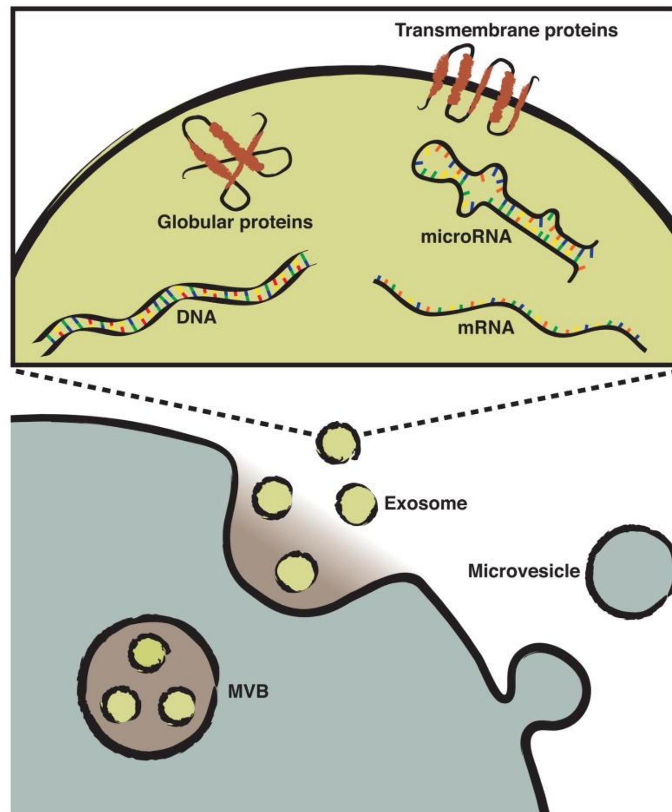


Figure 2.1 – Extracellular Vesicle Formation[7]

Recent discovery of the role of exosomes in intracellular communication has brought EVs to the forefront of cancer research. Originally nicknamed cellular “garbage bags”, the main function of exosomes was thought to be waste removal. However, similar to how one can learn a lot about the way a person lives based on their trash, much can be derived from a cell’s microenvironment based on the contents of its exosomes. Composed of a lipid bilayer that mirrors the cell they originated from, exosomes can contain DNA, RNA and proteins. Exosomes essentially mirror the “biological fingerprint” of their parent cell[8]. Several components of plasma membranes can be found in the lipid composition of exosomes including cholesterol, phosphatidylserine and saturated fatty acids. Exosomes are enriched with proteins associated with membrane transport/fusion, heat shock proteins, tetraspanins, epithelial cell adhesion molecules, MHC Class II proteins among others. All species of RNA have been identified in exosomes along with fragments of both single and double-stranded DNA[6].

To further understand the role of extracellular vesicles, specifically exosomes, in cell-to-cell communication and unlock their potential as disease biomarkers, efficient and reproducible EV isolation technologies that do not require large volumes or high turnaround time is needed.

2.2. Isolation Methods

Most biology related research labs are equipped with centrifuges. The use of a centrifuge is simple to implement, and the actual centrifugation process does not require much intervention once it has begun. The accessibility and ease of use for centrifugation is likely the reason ultracentrifugation is still the gold standard for EV isolation despite its shortcomings. The procedure is simple, but the UC process is lengthy, requires specialized equipment and the isolation efficiency is dependent on several factors such as the rotor type, sample viscosity and centrifugation acceleration. Sample purity is another concern with UC, compensated by repeated ultracentrifugation and microfiltration to further purify the sample. However, increasing the purity of the sample results in additional loss of sample quantity. Furthermore, exosomes tend to stick together, a feature that decreases efficiency of exosome isolation due to aggregate formation and pellet compaction at high ultracentrifugation speeds. In addition to these concerns, ultracentrifugation protocols differ widely, causing low reproducibility[9].

It is speculated that high speeds have a negative effect on exosomes. A comparison of UC to commercial kit exosome isolation reveals that exosomes isolated via UC produce significantly larger particles. This phenomenon may be attributed to proteins and other contaminants fusing with exosomes[11]. One study found that an increase in serum volume did not equate to an increase in exosome samples isolated by UC[12]. Additionally, in the same study, a western blot analysis revealed the UC samples were significantly contaminated with albumin. High protein contamination is a prevalent issue in UC samples [12–14].

Ultrafiltration is another alternative to UC, but it comes with a different set of challenges. Non-specific protein binding to filtration membranes, vesicle deformation resulting from materials forced though the membrane, and membrane clogging are drawbacks to the ultrafiltration process [15–17].

Commercial kits such as ExoQuick are attractive due to their high yields and simplified protocol. However, the commercial kits are expensive, produce low protein yields[18] and can require overnight incubation. Non-exosomal particle isolation such as contaminants and polymeric materials are observed in samples isolated using commercial kits, making this method a poor choice for downstream analyses that requires pure exosome samples[8,13,17].

Immunoaffinity-based capture methods for EV isolation have exhibited significant potential in their purity and high selectivity. Their high specificity feature has the ability for characterization and specific isolation of target exosome populations[19]. This method has been proven effective by several researchers, but it is still lacking a simple microfluidic solution capable of integrating an immunoaffinity approach with minimal preparation and harvesting protocol steps[19–22]. Exosome elution is another drawback of immunoaffinity-based capture method, as most elution methods result in some loss of sample.

2.3. Microfluidic Technologies

As mentioned in the introduction of the report, most victims of cancer-related fatalities reside in low-income areas. This statistic has driven an increase in microfluidic technologies, which offer low procedural costs and point-of-care diagnostic solutions. Microfluidic solutions have permeated the diagnostic field, including this area of EV research.

Several microfluidic devices have demonstrated the ability to isolate sub-micron particles. Many of the current proposed designs exhibit benefits of a microfluidic approach such as high purity, faster reaction times, and low cost, but they also increase in complexity. Additional equipment and logistics such as external pumps, magnets, layered microchips, variable flowrate and multiple inlets for various reagents give rise to daunting protocols[23–27]. Microfluidic EV isolation platforms such as a microfluidic membrane filtration system[28] and nanopillars[29] circumvent the issues of vesicular damage and expensive equipment present in conventional EV isolation methods. However, the design of these approaches inherently introduces challenges associated with device clogging.

In this report, we detail a microfluidic device that requires minimal additional equipment, quick turnaround times, simple fabrication methods and an EV capture efficiency surpassing ultracentrifugation.

Table 2.1 – Comparison of common EV isolation methods

EV Isolation Method	Pros	Cons	Ref.
Ultracentrifugation	Simple implementation, widely used method, no additional chemicals	Time consuming, multiple centrifugation steps, multiple centrifuges required, specialized equipment, low yield, damage to cells, poor reproducibility	8,9,11–14,30
Ultrafiltration	Simple procedure	Sample loss to membrane, damage to cells, clogging	9,15–17
Commercial kits (i.e. ExoQuick)	Simple procedure, short experiment run time, EV preservation, no specialized equipment	Cost of kit, contaminated sample, low protein yields	8,9,11,13,17
Microfluidic	Shorter experiment run time, high purity, low sample, EV preservation	Specialized equipment, complex protocols	9,23–29

CHAPTER 3: Research and Design Methods

3.1. Lateral Cavity Acoustic Transducer

Acoustic microstreaming is a phenomenon that occurs when bubbles trapped by a liquid phase are excited by acoustic energy. The acoustic energy causes the air/liquid interface to oscillate, resulting in a first-order periodic flow (U_s) located at the interface and a second-order bulk flow (U_b) within in the oscillatory boundary layer[31]. The lateral cavity acoustic transducer (LCAT) is an acoustic microstreaming microfluidic device equipped with on chip pumping, enrichment and size-based trapping capabilities. The size-based capture capabilities of the LCAT are attributed to the distance between the open and closed microstreaming vortices, referred to as the d_{gap}. Particles with a diameter larger than $2*d_{gap}$ are trapped within the closed the streamlines while particles smaller than the threshold will pass through the vortex and continue with the bulk flow[32,33]. A paper completed in 2018 by Garg et. al, demonstrates the use of LCAT to enrich, sort, and labels cells within whole blood. Figure 3.1 taken from this paper illustrates the basic principles that allow LCAT to perform multi-step processes on target particles within a microchannel[31,33–36].

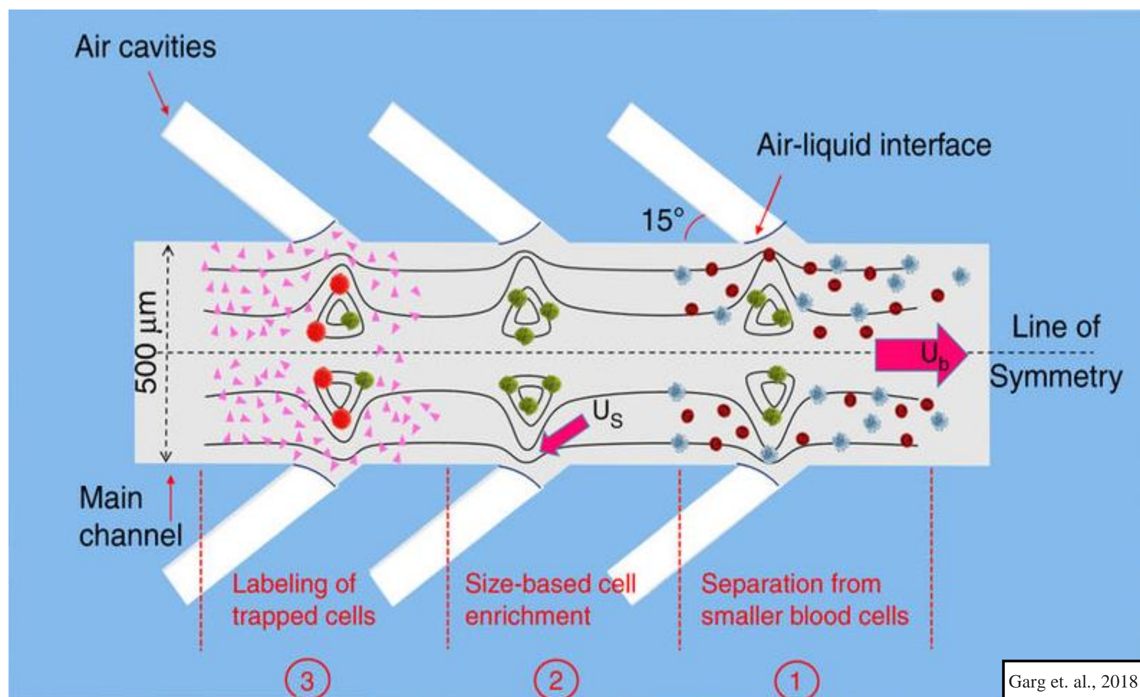


Figure 3.1 – LCAT Diagram: sorting, enrichment and labeling of trapped cells from Garg et. al., paper[33]

The LCAT device consists of hydrophobic PDMS channels bonded to a glass slide (Figure 3.2). Primed channels create air/liquid interfaces at the angled lateral cavities. The lateral cavity is the main feature that drives the functionality of the device. A piezoelectric transducer

topped with a ceramic pad is run at 50.2kHz frequency to transfer acoustic energy through the glass slide. Ultrasound gel placed in between the glass slide and the piezoelectric transducer increases the coupling efficiency. Introducing a voltage to the piezoelectric transducer transfers acoustic energy to the air/liquid interfaces, which oscillate and pump fluid through the PDMS microchannels.

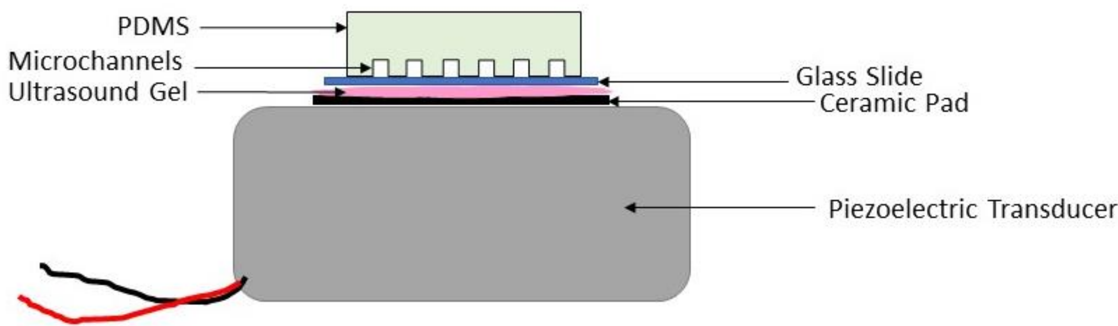


Figure 3.2 – LCAT Experimental Set-up

3.2. Bead-based Sandwich Assay

The functionality of LCAT is based on the liquid-gas interfaces formed by air trapped in dead-end side channels. When this interface oscillates, it creates two streaming flows: a streaming viscous flow velocity (U_s) that is the highest at the outer edge of the microstreaming vortices and a bulk flow (U_b). There is a critical open streamline that borders closed streamlines at end of the air-liquid interface vortex. In between this open and closed streamline is a gap referred to as the d_{gap}. Particles larger than 2*d_{gap} are trapped within the vortex. The equation for the d_{gap} is as follows: $d_{gap} = \frac{U_b}{U_s}\frac{W}{2}$, where U_b is the bulk streaming velocity, U_s is the streaming viscous flow velocity, and W is the width of the channel. LCAT has demonstrated the ability to directly capture red blood cells in whole blood (5-7 µm wide) and perform on-chip sorting and immunolabeling [33]. Theoretically, the size-based capture approach used to sort and enrich red blood cells can be applied to capture smaller particles, but the minuscule size of exosomes makes them a challenging target for direct capture. As a result, we opted for a bead-based sandwich assay approach, merging LCAT's acoustic microstreaming with immunoaffinity capture methods.

Streptavidin-coated beads were conjugated with biotin-anti-human CD63. CD63 is a known biomarker of exosomes[37–39], thus this tetraspanin protein was chosen for the analysis. The streptavidin bead and anti-CD63 conjugate was mixed into fresh plasma to directly capture EVs expressing the CD63 biomarker. The plasma is then pumped through LCAT to capture the beads, which can be either lysed for genotypic analysis or eluded for phenotypic analysis. Figure 3.3 contains an overview of this process.

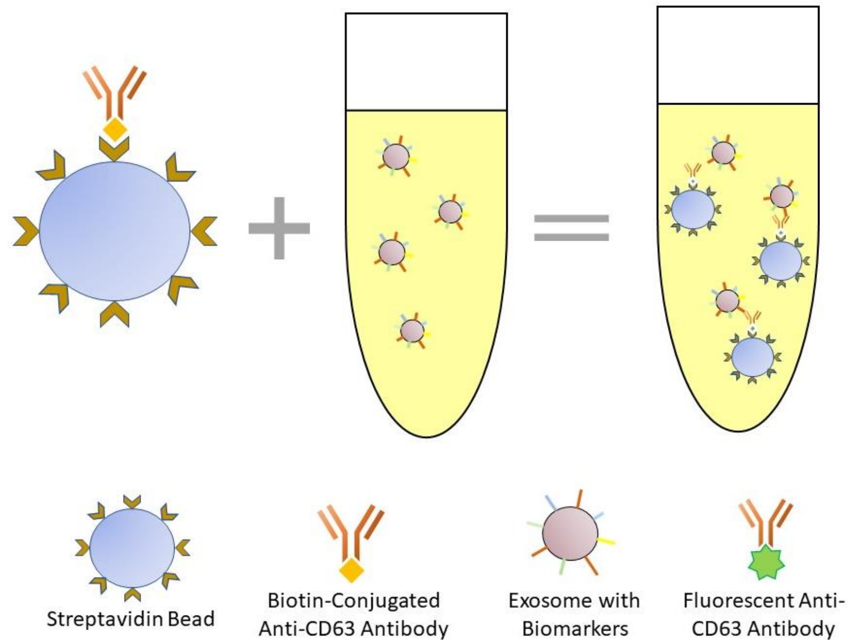


Figure 3.3 – Bead-Based Sandwich Assay for Capture of EVs

3.3. Device Fabrication Methods

LCAT was fabricated using standard soft lithography methods. A silicon wafer was cleared of debris using a nitrogen gun and negative photoresist SU-8 2050 was spin coated per the manufacturer's protocol for a channel height of 100 µm. After spin coating, the silicon wafer was soft-baked, exposed, post baked and developed. Developed wafers were inspected under a microscope, then hard baked at 200°C and treated with silane overnight. Polydimethylsiloxane (PDMS) base and curing agent mixed at an 11.5:1 ratio was poured onto the silicon wafer mold and the mixture was degassed in a desiccator. Following degassing, the PDMS was cured overnight at 65°C. After overnight curing, the hardened PDMS channels were cut and carefully peeled from the mold. A 4 mm biopsy punch was used to create the inlet and outlet holes. The device was cleaned and bonded with a thin cover slip using standard plasma procedure. The plasma bonded device was placed on a hotplate set at 65°C overnight to allow oxygen plasma treated PDMS to become hydrophobic.

3.4. EV Isolation Experiments

Spherotech streptavidin-coated beads were obtained and washed by gently centrifuging for 10 minutes and replacing the supernatant with an equivalent amount of PBS buffer. A stock solution of streptavidin-coated polystyrene microspheres conjugated with biotin-anti-human CD63 was created and stored in the refrigerator for use over the duration of the study. The same stock solution was used for experiments.

Blood plasma from the UCI Institute for Clinical and Translational Science (ICTS) was used for all experiments under UCI's Institutional Review Board approval. Blood samples from healthy patients were centrifuged at 3500 RPM for 10 minutes, per standard plasma separation procedure. The streptavidin bead and biotin-anti-CD63 conjugate was introduced to 1 mL of fresh plasma and incubated for 10 minutes. From this 1 mL of plasma, as little as 90 ul of plasma was necessary for the experiment. 30 µl of plasma was introduced to the inlet and a syringe was used to manually prime the first half of the microchannels. LCAT devices with larger channel widths can self-prime (automatically create interfaces when fluid is introduced to inlet), but the resistance in the 250 µm channel width device requires a partial prime to introduce the interfaces. Once the device has been partially primed, it is able to fully prime itself using the piezoelectric transducer. Partially priming the device assists in minimizing any human variability associated with the manual priming process, resulting in more stable interfaces. During the experiment, the inlet and outlet are monitored. The inlet is replenished periodically in 30 µl increments and the waste in the outlet is removed periodically to prevent an overflow. The waste is harvested throughout the experiment to quantify how much sample is lost to the outlet during RNA quantification. After flowing 90-120 µl, 30 µl of PBS wash buffer is pumped through the device. The beads trapped in the device are pushed to the outlet and harvested for analysis along with the collected waste (Figure 3.4).

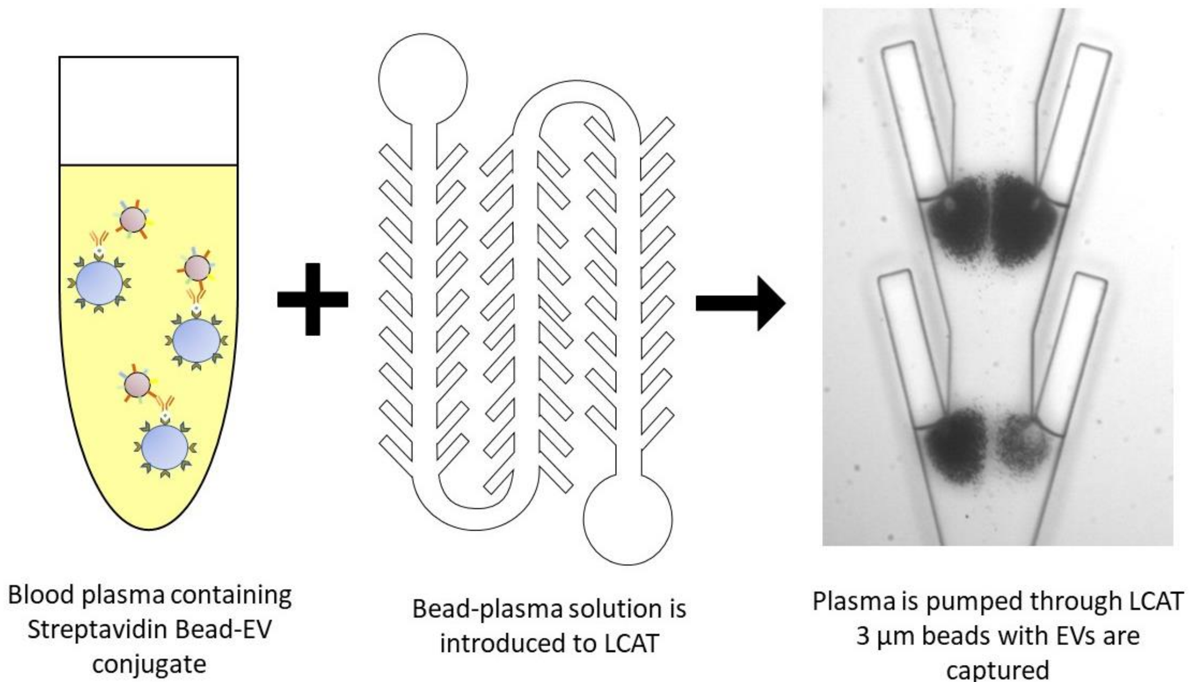


Figure 3.4 – EV Isolation Using LCAT

Ultracentrifugation was also performed on blood plasma obtained from UCI ICTS. A protocol was adapted from Miltenyi Biotech for characterization of exosomes. Figure 3.5 compares the LCAT and UC protocols for EV isolation.

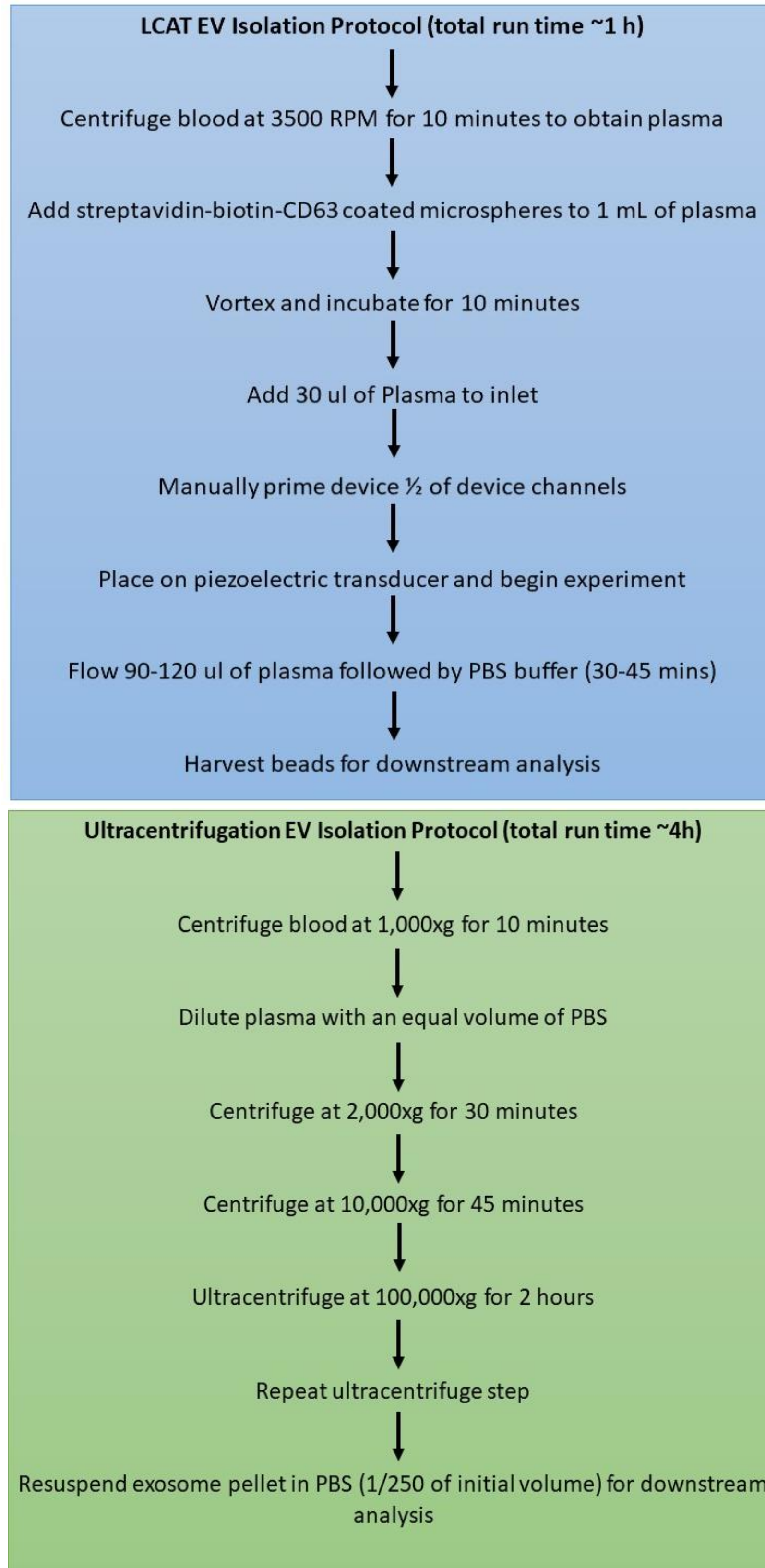


Figure 3.5 – LCAT vs UC EV Isolation Protocol

3.5. Device Optimization

Several iterations of LCAT were tested for extracellular vesicle isolation. Lee lab has published several papers demonstrating that the size-cutoff for LCAT trapping capabilities can be adjusted by decreasing the width of the main channel [32,33,40]. However, reducing the main channel width also increases the resistance within the channel and can cause the vortices to interfere with one another. The high resistance also effects the device's flowrate and increases the force required to prime the device, two factors that can affect the stability and longevity of the device during an experiment. A 250 µm width device was chosen due to its ability to remain stable and consistently flow 90-120 µl of plasma under 1 hour while still capturing smaller beads.

Considering the bead capture efficiency is dependent on the device, bead optimization was completed in conjunction with the LCAT channel width optimization. 10 µm beads were originally tested due to 100% trapping efficiency in 250 µm channel devices. Surprisingly, RNA extraction analyses of the LCAT EV isolation using 10 µm beads did not produce any results. The group speculated that the large surface area of the 10 µm beads causes the surface of each individual bead to be less saturated with exosomes. Though the details of this phenomenon are not certain, experimental observation revealed that the same RNA extraction analysis that failed to produce results with 10 µm beads was able to produce results using 2 µm and 3 µm beads. Consequently, these sizes were chosen and compared for bead size optimization.

The hypothesis for optimal bead size considers LCAT's lower capture efficiency of smaller diameter particles. Due to a lower trapping efficiency in a 250 µm device, it was hypothesized that 3 µm beads would yield more favorable results in the LCAT device than 2 µm beads. Experimental observations and RNA isolation results revealed that at the same concentration (and even at higher concentrations) 2 µm beads demonstrate lower capture efficiency on average (Figure 3.6). From this information, 3 µm beads were chosen for the next optimization step: bead concentration.

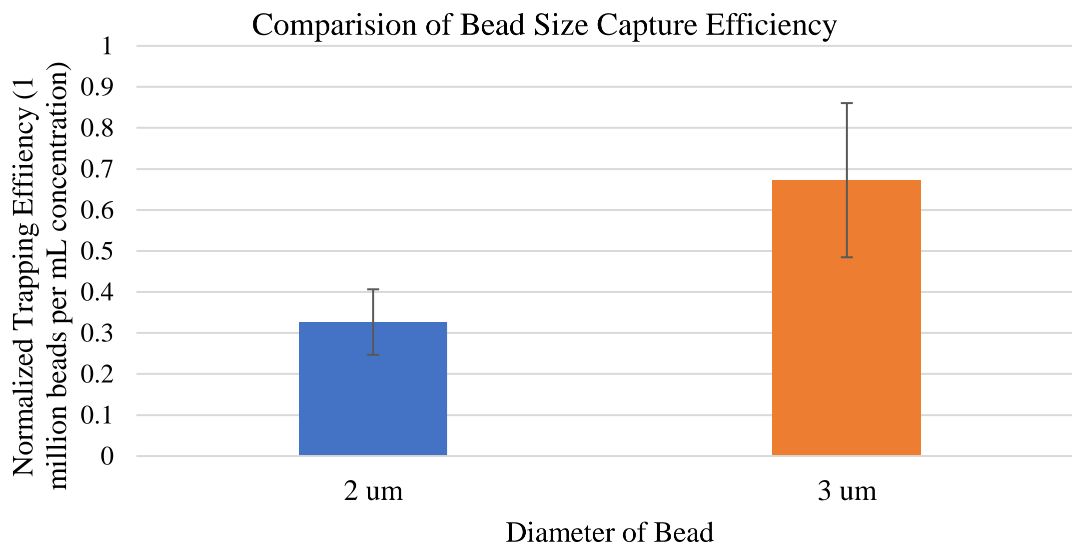


Figure 3.6 – Bead Size Optimization Results

For concentration optimization, 3 µm beads were tested at 1 million, 5 million, and 10 million beads per mL. When LCAT vortices become overly saturated, they become unstable and release beads, causing more beads to be lost to the waste outlet. Introducing an excessive concentration of beads to a plasma sample with a finite amount of exosomes means each individual bead will be less saturated with exosomes. These two factors are significant because they influence the following hypothesis: increasing the bead concentration will decrease the trapping efficiency by encouraging sample loss through unstable, overly saturated vortices and undersaturated beads. Figure 3.7 illustrates the accuracy of this prediction.

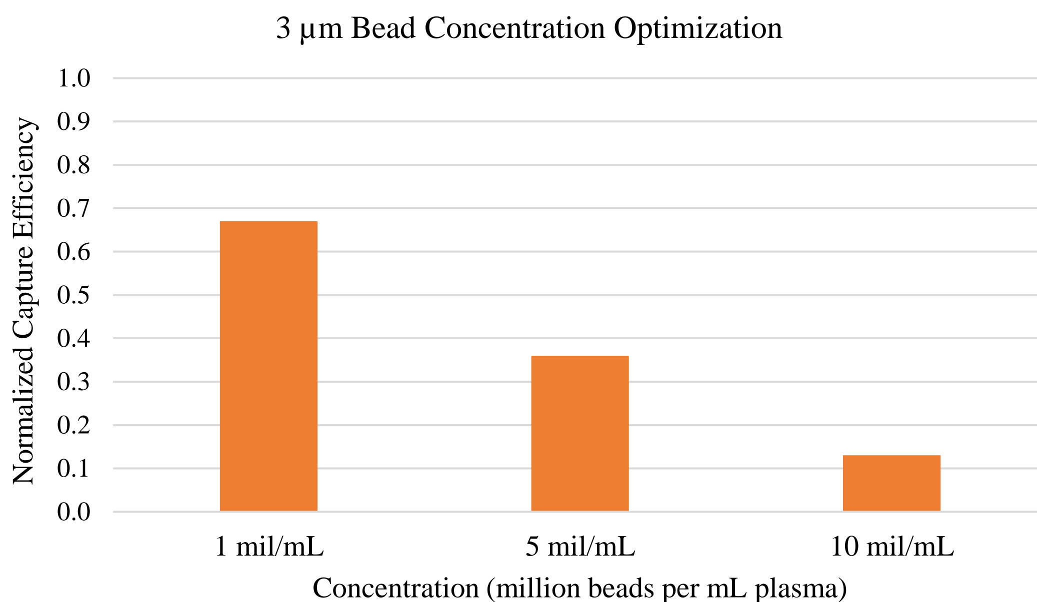


Figure 3.7 – Bead Concentration Optimization Results

CHAPTER 4: Results and Discussion

In order to verify the presence of extracellular vesicles, RNA isolation, flow cytometry and TEM imaging were performed on LCAT and UC isolated EV samples for comparison.

4.1. Visual detection via immunofluorescence

Several methods were used to verify the presence of exosomes. The first and most simplistic method was by visual detection. Immunofluorescence is an immunoassay technique in which a detector antigen or antibody is labeled with fluorophores[41]. Immunofluorescence allows for the staining of specimen that contain the targeted molecule. Fluorescein isothiocyanate (FITC) is a commercially available fluorescent probe widely used for biological molecule conjugates. FITCs popularity is attributed to its bright fluorescence, easy preparation, and low nonspecific binding[41]. FITC conjugated with anti-human CD63 was chosen for this application

because EVs express CD63 on their surface[13,37,42]. After the volume of plasma was finished pumping through the device, FITC was added to the inlet. A small sample of the harvested beads were placed under a fluorescent microscope for imaging. As seen in Figure 4.1 beads imaged under the fluorescent lamp are stained fluorescent green suggesting the successful capture of exosomes on the surface of the bead.

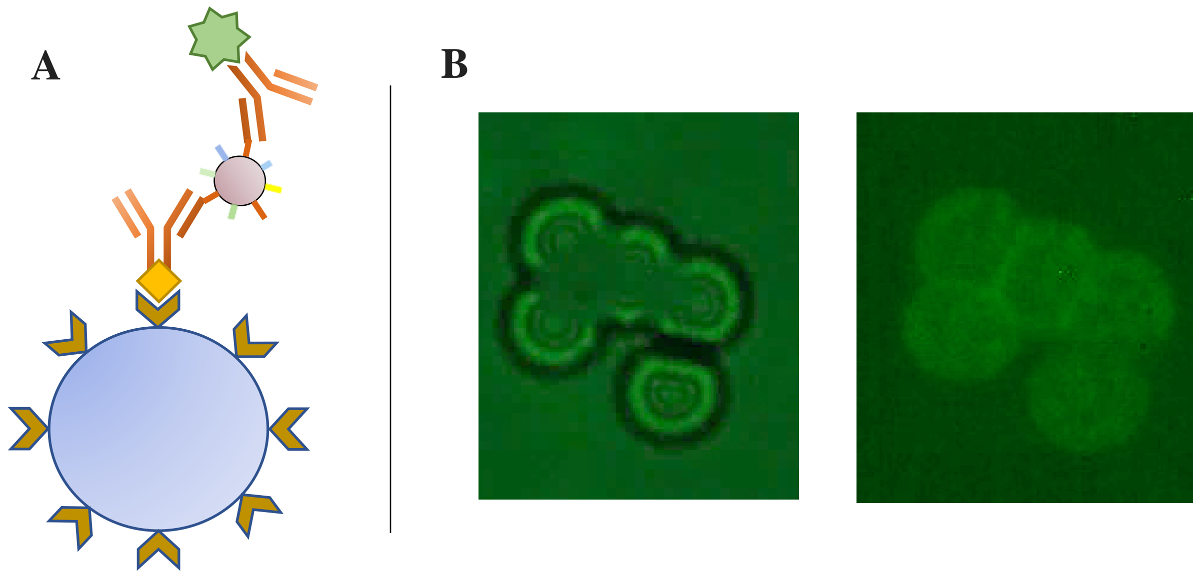


Figure 4.1 – Fluorescent Imaging: **A**: Schematic of bead conjugate with exosome and anti-CD63 FITC. **B**: Bright field and dark field images of LCAT captured EV-bead conjugates with anti-CD63 FITC fluorescent labeling. *Right*: Bright field image of 3 µm beads at 40X. *Left*: Fluorescent image of 3 µm beads at 40X

4.2. RNA Quantification

Following visual detection, the next EV verification method implanted was verifying the presence of RNA in a captured bead sample. Quantification of RNA is a common method for identifying the presence of extracellular vesicles [18,24,43–45]. RNA was extracted from EV samples using Trizol RNA isolation kit and evaluated using Quibit RNA High Sensitivity (HS) Assay kit. Trizol is a method for simultaneous isolation of RNA, DNA and proteins from a biological sample[46]. Separated into three phases, the top RNA aqueous phase was extracted for Quibit quantification and the DNA and proteins were discarded. Following RNA extraction, the highly selective Qubit RNA HS Assay Kit does not quantify DNA, protein or free nucleotides, providing confidence that the Qubit Fluorometer is only quantitating RNA in sample.

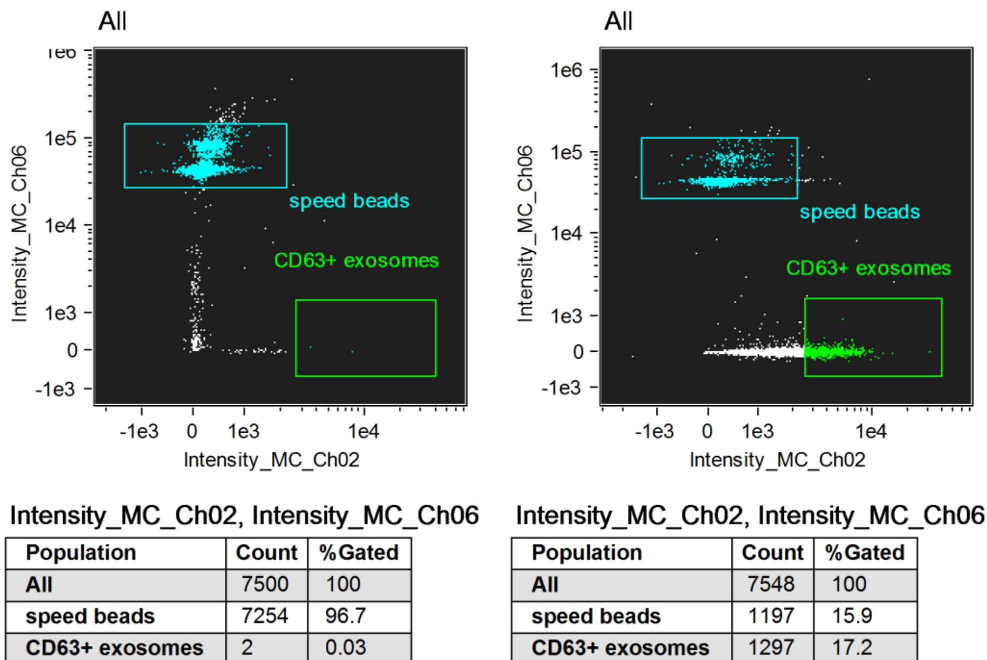
RNA quantification was performed on three samples following experimentation: captured beads, waste collected throughout the experiment and plasma sample that has not undergone EV isolation. Table 4.1 includes the raw values provided by the Qubit fluorometer analysis. RNA extraction was also performed on a UC sample for comparison.

Table 4.1 – RNA Quantification Results

	Trial A	Trial B	Trial C	Trial D	UC
Bead Solution RNA (ng/ul)	1.61	0.256	1.39	0.403	0.724
Waste RNA (ng/ul)	0.828	0.306	0.41	1.51	
Plasma (ng/ul)	2.81	0.351	1.53	0.84	1.67
Efficiency	57%	73%	91%	48%	43%

4.3. Flow Cytometry

Used to measure both physical and chemical characteristics of individual particles, flow cytometry is an incredibly valuable tool for single cell characterization[46]. Particles inside a flow cytometer are focused and passed through an "interrogation point" individually. A laser is focused at the interrogation point. When a particle passes the laser, light is emitted in all directions. These light signals are analyzed in the computer and a histogram is created from the resulting data. The laser excites fluorescent labeled particles to a higher state. These fluorophores emit light energy at higher wavelengths following excitation[46,47]. This mechanism is the basis behind the use of flow cytometry to analyze FITC-CD63+ EV samples. Figure 4.2 displays flow cytometry results from a blank sample and a sample of plasma with streptavidin-CD63 bead conjugate.



Intensity_MC_Ch02, Intensity_MC_Ch06

Population	Count	%Gated
All	7500	100
speed beads	7254	96.7
CD63+ exosomes	2	0.03

Intensity_MC_Ch02, Intensity_MC_Ch06

Population	Count	%Gated
All	7548	100
speed beads	1197	15.9
CD63+ exosomes	1297	17.2

Figure 4.2 – Flow Cytometry Results: Streptavidin-beads with Exosomes. *Right:* Blank Sample. *Left:* Sample with CD63+ conjugated beads and exosomes.

4.4. TEM Imaging

Transmission electron microscopy (TEM) is an imaging technique that transmits electron beams through an ultrathin specimen. When the diffraction pattern of electrons transmitted through the specimen are focused, a magnified image of the specimen materializes[48,49].
In order to perform TEM imaging on sub-micron particles attached to microspheres, the exosomes must be removed from the microspheres. An EV elution protocol was adapted to break the biotin streptavidin bonds and release EVs for further analysis[50].

Isolated exosome samples were fixed to a carbon mesh grid and stained used uranium acetate. Following staining, the sample ready for TEM imaging. TEM images of exosome samples isolated by UC and LCAT are found in Figure 4.3 and Figure 4.4. Refer to Figure 4.5 for details on the TEM imaging protocols.

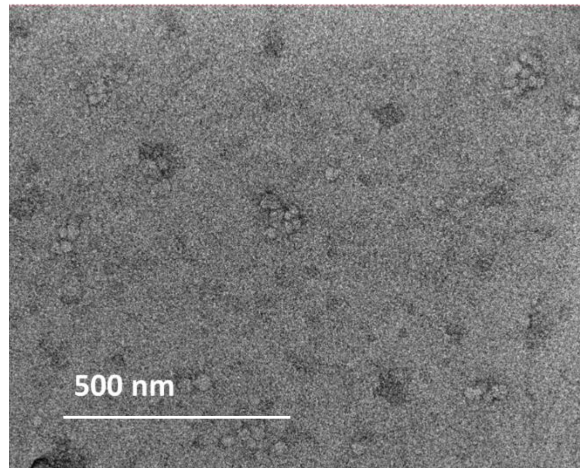


Figure 4.3 – TEM Imaging of UC Isolated EV Sample: three EVs are identified by yellow arrows.

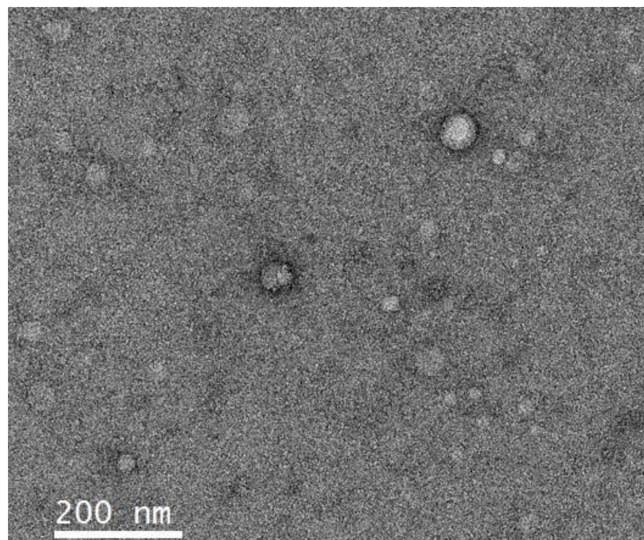
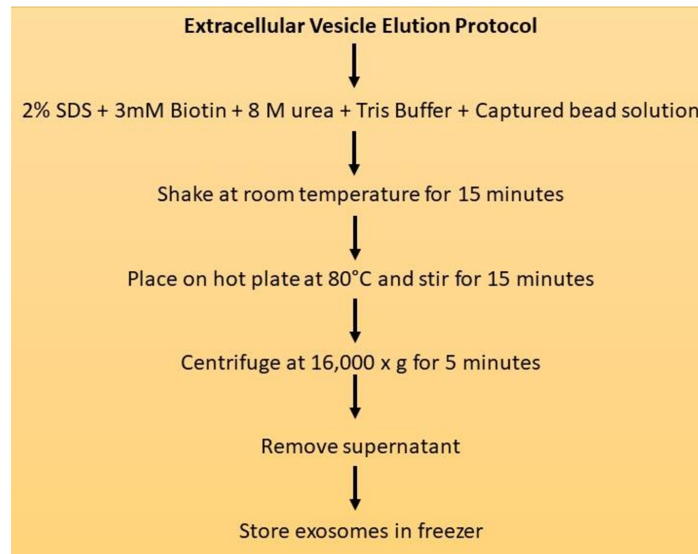


Figure 4.4 – TEM Imaging of LCAT Isolated EV Sample



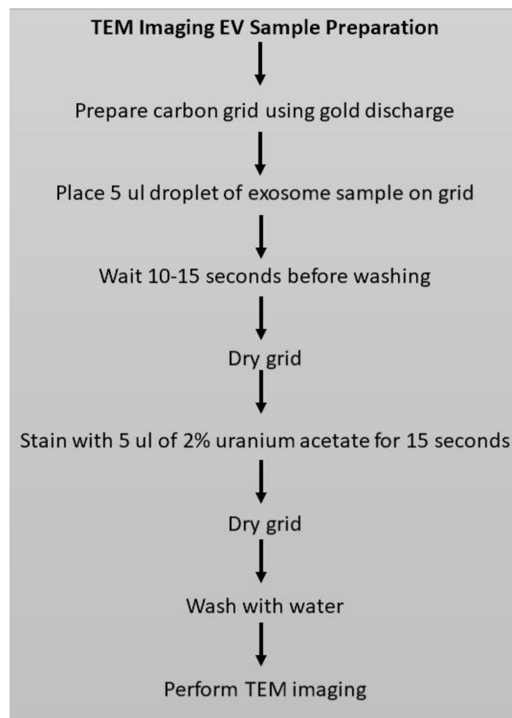


Figure 4.5 – Exosome Elution and TEM Imaging Protocols

CHAPTER 5: Conclusion

5.1. Discussion of Results

Despite emerging alternatives to EV isolation, ultracentrifugation remains the gold standard. Current proposed microfluidic solutions demonstrate higher efficiencies and faster reaction times at the sacrifice of simplicity and accessibility of the necessary equipment. The presence of EVs isolated by LCAT and UC methods were successfully verified using immunofluorescence, RNA quantification, flow cytometry, and TEM imaging. Both methods are able to produce EV samples for downstream analysis, however, advantages of the LCAT approach include a reduction in time, protocol steps, equipment, and sample loss. The average EV isolation efficiency for 3 µm beads in an LCAT device was 67%, compared to the average efficiency of 5-25% for UC[30]. Based on this analysis, LCAT is a viable microfluidic solution for EV capture, outperforming UC in terms of experiment time, isolation efficiency, and working volume (Table 5.1).

Table 5.1 – Comparison of LCAT and UC Results

Parameter	LCAT Bead Based Assay	Ultracentrifugation
Experiment Time	30-45 minutes	4 hours
Average efficiency	40-70%	5-25% (39)
Sample Volume	1 mL of plasma	5 mL of plasma
Specialized Equipment	- LCAT chip - Centrifuge capable of 3500 RPM - Piezoelectric transducer	- Different centrifuges capable of 3 levels serial centrifugation (1,000-2,000xg, 10,000xg, and 100,000xg) - High performance centrifuge tubes - Specialized rotors compatible with specialized ultracentrifuge tubes

5.2. Limitation of the Study

While the LCAT protocol utilized in this study produced favorable results, more experiments to further optimize the device could have reduced the standard deviation in the LCAT EV isolation efficiency. Time being the greatest limiting factor, this study did not investigate why larger beads were unable to recover large quantities of exosomes despite having higher bead trapping efficiencies within the LCAT device. This study was also unable to evaluate and compare the viability of EVs isolated using both methods.

5.3. Future Work

Isolation of extracellular vesicles is only the first step of LCAT sub-micron particle analysis. This study focuses on using CD63, a biomarker present in both healthy and tumor derived EVs, but beads conjugated with a tumor specific antibody could potentially isolate tumor-derived exosomes from a population of exosomes. The next direction is to use LCAT to capture tumor derived exosomes. A VEGF kit can be used to verify if the sample captured by the exosomes contains the growth factor present in cancer cells. Proof of this concept could lead to use of LCAT for on-chip isolation and immunolabeling of tumor-related exosomes from plasma. LCAT has demonstrated its ability to perform enrichment, sorting and immunolabeling of red blood cells from whole blood. The integration of the previous LCAT applications with LCAT exosome isolation has the potential to provide on-chip immunolabeling of cancer derived exosomes.

The future of diagnostics is microfluidic. From accessibility to quick reaction times, the advantages of true lab-on-a-chip methodologies are endless. There is still much work to be done to integrate the multifaceted capabilities of LCAT into a POC device for early cancer detection, but the results of this study demonstrate that the research is moving in a promising direction. The work described in this report moves us a step closer to understanding and capitalizing on the role of EVs in cancer behavior.

CHAPTER 6: References

1. World Health Organization. Cancer: Key FActs. https://www.who.int/news-room/fact-sheets/detail/cancer. Published 2018. Accessed October 4, 2018.
2. John S, Broggio J. Cancer survival in England: national estimates for patients followed up to 2017. Office for National Statistics - Great Britain. https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancersurvivalinengland/nationalestimatesforpatientsfollowedupto2017. Published 2019.
3. ASHWORTH, TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Aust Med J*. 1869;14:146. http://ci.nii.ac.jp/naid/10027663080/en/. Accessed May 30, 2019.
4. Krebs MG, Hou JM, Ward TH, Blackhall FH, Dive C. Circulating tumour cells: Their utility in cancer management and predicting outcomes. *Ther Adv Med Oncol*. 2010;2(6):351-365. doi:10.1177/1758834010378414
5. Eroglu Z, Fielder O, Somlo G. Analysis of circulating tumor cells in breast cancer. *JNCCN J Natl Compr Cancer Netw*. 2013;11(8):977-985. doi:10.6004/jnccn.2013.0118
6. Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest*. 2016;126(4):1208-1215. doi:10.1172/JCI81135
7. Kastelowitz N, Yin H. Exosomes and microvesicles: identification and targeting by particle size and lipid chemical probes. *Chembiochem*. 2014;15(7):923-928. doi:10.1002/cbic.201400043
8. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics*. 2017;7(3):789-804. doi:10.7150/thno.18133
9. Konoshenko MY, Lekchnov EA, Vlassov A V, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed Res Int*. 2018;2018:8545347. doi:10.1155/2018/8545347
10. Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200(4):373-383. doi:10.1083/jcb.201211138
11. Helwa I, Cai J, Drewry MD, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One*. 2017;12(1):e0170628-e0170628. doi:10.1371/journal.pone.0170628
12. Caradec J, Kharmate G, Hosseini-Beheshti E, Adomat H, Gleave M, Guns E. Reproducibility and efficiency of serum-derived exosome extraction methods. *Clin Biochem*. 2014;47(13-14):1286-1292. doi:10.1016/J.CLINBIOCHEM.2014.06.011
13. Van Deun J, Mestdagh P, Sormunen R, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles*. 2014;3(1):24858. doi:10.3402/jev.v3.24858
14. Musante L, Saraswat M, Ravidà A, Byrne B, Holthofer H. Recovery of urinary nanovesicles from ultracentrifugation supernatants. *Nephrol Dial Transplant*. 2013;28(6):1425-1433. doi:10.1093/ndt/gfs564
15. Merchant ML, Powell DW, Wilkey DW, et al. Microfiltration isolation of human urinary exosomes for characterization by MS. *PROTEOMICS - Clin Appl*. 2010;4(1):84-96. doi:10.1002/prca.200800093
16. Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. *Methods*. 2015;87:3-10. doi:10.1016/J.YMETH.2015.02.019

17. Lobb RJ, Becker M, Wen SW, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell vesicles*. 2015;4:27031. doi:10.3402/jev.v4.27031
18. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int*. 2012;82(9):1024-1032. doi:10.1038/KI.2012.256
19. Rana S, Yue S, Stadel D, Zöller M. Toward tailored exosomes: The exosomal tetraspanin web contributes to target cell selection. *Int J Biochem Cell Biol*. 2012;44:1574-1584. doi:10.1016/j.biocel.2012.06.018
20. Tauro BJ, Greening DW, Mathias RA, et al. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods*. 2012;56(2):293-304. doi:10.1016/J.YMETH.2012.01.002
21. Taylor DD, Zacharias W, Gercel-Taylor C. Exosome Isolation for Proteomic Analyses and RNA Profiling. In: Humana Press; 2011:235-246. doi:10.1007/978-1-61779-068-3_15
22. Kalra H, Adda CG, Liem M, et al. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. 2013:3354-3364. doi:10.1002/pmic.201300282
23. Santana SM, Antonyak MA, Cerione RA, Kirby BJ. Microfluidic isolation of cancer-cell-derived microvesicles from hetergeneous extracellular shed vesicle populations. *Biomed Microdevices*. 2014;16(6):869-877. doi:10.1007/s10544-014-9891-z
24. Dudani JS, Gossett DR, Tse HTK, Lamm RJ, Kulkarni RP, Carlo D Di. Rapid inertial solution exchange for enrichment and flow cytometric detection of microvesicles. *Biomicrofluidics*. 2015;9(1):014112. doi:10.1063/1.4907807
25. Chen C, Skog J, Hsu C-H, et al. Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab Chip*. 2010;10(4):505-511. doi:10.1039/B916199F
26. Lee K, Shao H, Weissleder R, Lee H. Acoustic Purification of Extracellular Microvesicles. *ACS Nano*. 2015;9(3):2321-2327. doi:10.1021/nn506538f
27. He M, Crow J, Roth M, Zeng Y, Godwin AK. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. *Lab Chip*. 2014;14(19):3773-3780. doi:10.1039/c4lc00662c
28. Davies RT, Kim J, Jang SC, Choi E-J, Gho YS, Park J. Microfluidic filtration system to isolate extracellular vesicles from blood. *Lab Chip*. 2012;12(24):5202-5210. doi:10.1039/c2lc41006k
29. Wang Z, Wu H, Fine D, et al. Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. *Lab Chip*. 2013;13(15):2879-2882. doi:10.1039/c3lc41343h
30. Dorsey R, Emmett G, Salem H. Ricin. *Handb Toxicol Chem Warf Agents*. January 2015:347-360. doi:10.1016/B978-0-12-800159-2.00027-0
31. Lee AP, Patel M V, Tovar AR, Okabe Y. Microfluidic AireLiquid Cavity Acoustic Transducers for On-Chip Integration of Sample Preparation and Sample Detection. 2010. doi:10.1016/j.jala.2010.05.002
32. Tovar AR, Lee AP. Lateral cavity acoustic transducer. *Lab Chip*. 2009;9(1):41-43. doi:10.1039/b812435c
33. Garg N, Westerhof TM, Liu V, Liu R, Nelson EL, Lee AP. Whole-blood sorting, enrichment and in situ immunolabeling of cellular subsets using acoustic microstreaming.

Microsystems Nanoeng. 2018;4:17085. doi:10.1038/micronano.2017.85
34. Patel M V, Nanayakkara IA, Simon MG, Lee AP. Cavity-induced microstreaming for simultaneous on-chip pumping and size-based separation of cells and particles. *Lab Chip*. 2014;14(19):3860-3872. doi:10.1039/c4lc00447g
35. Huang P-H, Nama N, Mao Z, et al. A reliable and programmable acoustofluidic pump powered by oscillating sharp-edge structures. *Lab Chip*. 2014;14(22):4319-4323. doi:10.1039/c4lc00806e
36. Wang C, Jalikop S V, Hilgenfeldt S. Efficient manipulation of microparticles in bubble streaming flows. *Biomicrofluidics*. 2012;6(1):12801-1280111. doi:10.1063/1.3654949
37. Trajkovic K, Hsu C, Chiantia S, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science*. 2008;319(5867):1244-1247. doi:10.1126/science.1153124
38. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol 2007 96*. 2007;9(6):654. doi:10.1038/ncb1596
39. Lamparski HG, Metha-Damani A, Yao J-Y, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods*. 2002;270(2):211-226. doi:10.1016/S0022-1759(02)00330-7
40. Nivedita N, Garg N, Lee AP, Papautsky I. A high throughput microfluidic platform for size-selective enrichment of cell populations in tissue and blood samples. *Analyst*. 2017;142(14):2558-2569. doi:10.1039/c7an00290d
41. Clark Brelje T, Wessendorf MW, Sorenson RL. Multicolor Laser Scanning Confocal Immunofluorescence Microscopy: Practical Application and Limitations. *Methods Cell Biol*. 2002;70:165-249e. doi:10.1016/S0091-679X(02)70006-X
42. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*. 2009;4(4):e5219. doi:10.1371/journal.pone.0005219
43. Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2(1). doi:10.3402/jev.v2i0.20360
44. Lässer C. Exosomal RNA as biomarkers and the therapeutic potential of exosome vectors. *Expert Opin Biol Ther*. 2012;12(sup1):S189-S197. doi:10.1517/14712598.2012.680018
45. Li M, Zeringer E, Barta T, Schageman J, Cheng A, Vlassov A V. Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philos Trans R Soc Lond B Biol Sci*. 2014;369(1652). doi:10.1098/rstb.2013.0502
46. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clin Chem*. 2000;46(8):1221 LP - 1229. http://clinchem.aaccjnls.org/content/46/8/1221.abstract.
47. Orfao A, Ruiz-Arguelles A, Lacombe F, Ault K, Basso G, Danova M. Flow cytometry: its applications in hematology. *Haematologica*. 1995;80(1):69-81. http://www.ncbi.nlm.nih.gov/pubmed/7758996. Accessed May 14, 2019.
48. Bozzola J, Russell L. The Transmission Electron Microscope. In: *Electron Microscopy: Principles and Techniques for Biologists*. Second Edi. Jones & Bartlett Publishers; 1999:149-162.
49. Egerton R. *Physical Principles of Electron Microscopy: An Introduction to TEM, SEM,*

and AEM. Springer US; 2011. https://books.google.com/books?id=t6TTgqp3O5MC.

50. Rybak J-N, Scheurer SB, Neri D, Elia G. Purification of biotinylated proteins on streptavidin resin: A protocol for quantitative elution. *Proteomics*. 2004;4(8):2296-2299. doi:10.1002/pmic.200300780

1. World health organization - https://www.who.int/news-room/fact-sheets/detail/cancer
2. Office for national statistics (GB) - https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancersurvivalinengland/nationalestimatesforpatientsfollowedupto2017
3. Ashworth T. (1869) A case of cancer in which cells similar to those in the tumors were seen in the blood after death. Aust Med J 14: 146–149
4. Krebs, M. G., Hou, J. M., Ward, T. H., Blackhall, F. H., & Dive, C. (2010). Circulating tumour cells: their utility in cancer management and predicting outcomes. *Therapeutic advances in medical oncology*, *2*(6), 351–365. doi:10.1177/1758834010378414
5. Eroglu, Z., Fielder, O., & Somlo, G. (2013). Analysis of circulating tumor cells in breast cancer. *Journal of the National Comprehensive Cancer Network : JNCCN*, *11*(8), 977–985.
6. http://jcb.rupress.org/content/jcb/200/4/373.full.pdf (extracellular vesicles: exosomes, microvesicles and friends)
7. Exosomes and Microvesicles: Identification and Targeting By Particle Size and Lipid Chemical Probes - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4098878/
8. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4811149/ - The biology and function of exosomes in cancer - Raghu Kalluri
9. Progress in Exosome Isolation Techniques - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5327650/
10. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends - Maria Yu. Konoshenko, 1 , 2 Evgeniy A. Lekchnov, Alexander V. Vlassov, 1 and Pavel P. Laktionov 1 , 2https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5831698/
11. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents - Inas Helwa,#1 Jingwen Cai,#1 Michelle D. Drewry https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5256994/
12. Reproducibility and efficiency of serum-derived exosome extraction methods – Josselin Caradec, Geetanjali Kharmate Elham Hosseini-Beheshti Hans Adomat https://www.sciencedirect.com/science/article/pii/S0009912014004056?via%3Dihub
13. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling - Jan Van Deun, Pieter Mestdagh, Raija Sormunen, Veronique Cocquyt, Karim Vermaelen, Jo Vandesompele, https://www.tandfonline.com/doi/full/10.3402/jev.v3.24858
14. Recovery of urinary nanovesicles from ultracentrifugation supernatants - Luca Musante Mayank Saraswat Alessandra Ravidà Barry Byrne https://academic.oup.com/ndt/article/28/6/1425/1836669

15. Microfiltration isolation of human urinary exosomes for characterization by MS - Michael L. Merchant David W. Powell Daniel W. Wilkey https://onlinelibrary.wiley.com/doi/full/10.1002/prca.200800093
16. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes - Douglas D.TaylorSahilShah https://www.sciencedirect.com/science/article/pii/S1046202315000924?via%3Dihub
17. Optimized exosome isolation protocol for cell culture supernatant and human plasma - Richard J. Lobb,1,2 Melanie Becker,1 Shu Wen Wen,1 Christina S. F. Wong https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4507751/
18. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers - M. Lucrecia Alvarez Mahdieh Khosroheidari RupeshKanchi Ravi1Johanna K.DiStefano https://www.sciencedirect.com/science/article/pii/S0085253815556755?via%3Dihub
19. Toward tailored exosomes: The exosomal tetraspanin web contributes to target cell selection - Sanyukta Ranaa, Shijing Yuea, Daniela Stadel a, Margot Zöller
20. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes - Bow J.Tauro David W.Greening Rommel A. Mathias Hong Ji Suresh Mathivanan Andrew M.Scott Richard J.Simpson https://www.sciencedirect.com/science/article/pii/S1046202312000187?via%3Dihub
21. Exosome isolation for proteomic analyses and RNA profiling. - Taylor DD1, Zacharias W, Gercel-Taylor C https://link.springer.com/protocol/10.1007%2F978-1-61779-068-3_15
22. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma - Hina Kalra Christopher G. Adda Michael Liem Ching-Seng Ang Adam Mechler Richard J. Simpson Mark D. Hulett Suresh Mathivanan https://onlinelibrary.wiley.com/doi/full/10.1002/pmic.201300282
23. Microfluidic isolation of cancer-cell-derived microvesicles from hetergeneous extracellular shed vesicle populations https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4624394/
    a. Steven M. Santana, Marc A. Antonyak, Richard A. Cerione, and Brian J. Kirby
24. Rapid inertial solution exchange for enrichment and flow cytometric detection of microvesicles- Jaideep S. Dudani,1,a) Daniel R. Gossett,1,2 Henry T. K. Tse,1,2,b) Robert J. Lamm,1 Rajan P. Kulkarni,3,4,c) and Dino Di Carlo https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4320146/
25. Microfluidic isolation and transcriptome analysis of serum microvesicles - Chihchen Chen,a Johan Skog,b Chia-Hsien Hsu,a Ryan T. Lessard,b Leonora Balaj,b Thomas Wurdinger,bc Bob S. Carter,d Xandra O. Breakefield,b Mehmet Tonera and Daniel Irimia https://pubs.rsc.org/en/content/articlelanding/2010/LC/B916199F#!divAbstract

26. Acoustic Purification of Extracellular Microvesicles - Kyungheon Lee†, Huilin Shao†, Ralph Weissleder†‡, and Hakho Lee* https://pubs.acs.org/doi/10.1021/nn506538f
27. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology† https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4161194/
    a. Mei He,a,§ Jennifer Crow,a Marc Roth,a Yong Zeng,corresponding authorb and Andrew K. Godwin
28. Microfluidic filtration system to isolate extracellular vesicles from blood. - Davies RT1, Kim J, Jang SC, Choi EJ, Gho YS, Park J. https://www.ncbi.nlm.nih.gov/pubmed/23111789/
29. Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. - Wang Z1, Wu HJ, Fine D, Schmulen J, Hu Y, Godin B, Zhang JX, Liu X https://www.ncbi.nlm.nih.gov/pubmed/23743667
30. Microfluidic Air-Liquid Cavity Acoustic Transducers for On-Chip Integration of Sample Preparation and Sample Detection - Abraham P. Lee, Ph.D., Maulik V. Patel, Armando R. Tovar https://journals.sagepub.com/doi/pdf/10.1016/j.jala.2010.05.002
31. Cavity-induced microstreaming for simultaneous on-chip pumping and size-based separation of cells and particles - Patel MV1, Nanayakkara IA, Simon MG, Lee AP https://pubs.rsc.org/en/content/articlelanding/2014/LC/C4LC00447G
32. A reliable and programmable acoustofluidic pump powered by oscillating sharp-edge structures. Huang PH1, Nama N, Mao Z, Li P, Rufo J, Chen Y, Xie Y, Wei CH, Wang L, Huang TJ. https://pubs.rsc.org/en/content/articlelanding/2014/LC/C4LC00806E#!divAbstract
33. Efficient manipulation of microparticles in bubble streaming flows - Cheng Wang,a) Shreyas V. Jalikop, and Sascha Hilgenfeldt https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3365321/
34. Lateral cavity acoustic transducer - Armando R. Tovar*a and Abraham P. Lee https://pubs.rsc.org/en/content/articlepdf/2009/lc/b812435c
35. Whole-blood sorting, enrichment and in situ immunolabeling of cellular subsets using acoustic microstreaming - Neha Garg, Trisha M. Westerhof, Vick Liu, Robin Liu, Edward L. Nelson & Abraham P. Lee https://www.nature.com/articles/micronano201785
36. Trajkovic K, Hsu C, Chiantia S et al (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319:1244–1247
37. Valadi H, Ekstrom K, Bossios A et al (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659
38. Production and characterization of clinical grade exosomes derived from dendritic cells Henry GLamparskiAnitaMetha-DamaniJenq-YuanYaoSanjayPatelDi-HweiHsuCurtisRueggJean-BernardLe Pecq https://www.sciencedirect.com/science/article/pii/S0022175902003307?via%3Dihub
39. Russell Dorsey, George Emmett, Harry Salem, Chapter 27 - Ricin, Handbook of Toxicology of Chemical Warfare Agents (Second Edition), Academic Press, 2015,

Pages 347-360,
http://www.sciencedirect.com/science/article/pii/B9780128001592000270)

40. Chapter 5 - Multicolor Laser Scanning Confocal Immunofluorescence Microscopy: Practical Application and Limitations, T.Clark BreljeMartin W.WessendorfRobert L.Sorenson https://www.sciencedirect.com/science/article/pii/S0091679X0270006X
41. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. Logozzi M1, De Milito A, Lugini L, Borghi M, https://www.ncbi.nlm.nih.gov/pubmed/19381331
42. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research - Kenneth W. Witwer, Edit I. Buzás, Lynne T. Bemis, Adriana Bora, Cecilia Lässer, Jan Lötvall https://www.tandfonline.com/doi/full/10.3402/jev.v2i0.20360
43. Exosomal RNA as biomarkers and the therapeutic potential of exosome vectors - Lässer C https://www.ncbi.nlm.nih.gov/pubmed/22506888
44. Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers - Mu Li,† Emily Zeringer,† Timothy Barta, Jeoffrey Schageman, Angie Cheng, and Alexander V. Vlassovhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4142023/
45. Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage - Hummon A Lim S Difilippantonio M Ried T https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4721573/
46. Flow Cytometry: Principles and Clinical Applications in Hematology - Michael Brown, Carl Wittwer http://clinchem.aaccjnls.org/content/46/8/1221
47. Flow Cytometry: Its Applications In Hematology - A Orfao, A Ruiz-Arguelles, F Lacombe, K Ault, G Basso, M Danova Haematologica January 1995 80: 69-81; http://www.haematologica.org/content/80/1/69.full.pdf+html
48. Electron Microscopy: Principles and Techniques for Biologists, By John J. Bozzola, Lonnie Dee Russell https://books.google.com/books?id=zMkBAPACbEkC&lpg=PR21&ots=AcO-3tnEI4&dq=transmission%20electron%20microscopy%20principle&lr&pg=PP1#v=onepage&q&f=false
49. Physical principles of electron microscopy, RF Egerton - 2005 – Springer https://link.springer.com/content/pdf/10.1007/978-3-319-39877-8.pdf (also downloaded)
50. Purification of biotinylated proteins on streptavidin resin: A protocol for quantitative elution - Rybak J Scheurer S Neri D Elia G https://www.ncbi.nlm.nih.gov/pubmed/15274123
51. A high throughput microfluidic platform for size-selective enrichment of cell populations in tissue and blood samples - Nivedita Nivedita,a Neha Garg,bc Abraham P. Leebcd and Ian Papautsky https://pubs.rsc.org/en/Content/ArticleLanding/2017/AN/C7AN00290D#!divAbstract