AWARD NUMBER: CDMRP-16-0-BC151687

TITLE: The Role of Extracellular Vesicles in Metastasis

PRINCIPAL INVESTIGATOR: GABRIELA LOOTS

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
Livermore, CA 94550

REPORT DATE: OCTOBER 2018

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE OCTOBER 2018	**2. REPORT TYPE** Annual	**3. DATES COVERED** 1 Oct 2017 - 30 Sep 2018

4. TITLE AND SUBTITLE

The Role of Extracellular Vesicles in Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
CDMRP-16-0-BC151687

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Gabriela G. Loots

E-Mail: loots1@llnl.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lawrence Livermore National Laboratory
7000 East Avenue
Livermore, CA 94550-9698

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The proposed work addresses the dire need for new platforms for ESV isolation and precise, quantitative characterization of each cancerous ESV subpopulation's role in cargo transfer. Specifically, we aim to (1) optimize an existing microfluidic separation platform to efficiently isolate ESV subpopulations (from the cells and from each other) originating from breast cancer cell lines with a range of metastatic character, (2) engineer breast cancer cell lines with fluorescent and radiolabeled ESV sub-populations for individual tracking, and (3) use accelerator mass spectrometry (AMS), which allows extremely sensitive rare-molecule detection, to quantify low levels of tumor-derived RNA transferred via ESVs to osteoblasts. These bone cells represent the most common tissue target for breast cancer metastasis, and we will mimic ESV-mediated cancer invasion and metastasis by growing the cancerous and bone cells together in a trans-well cell co-culture system. The use of these molecular and physical tools in combination specifically to address cancer invasiveness and mechanisms of metastasis is unprecedented. This study will yield the first quantitative data on which ESV subpopulations (exosomes, MVs, or oncosomes) manipulate the tumor microenvironment, the ESV cargo transferred, and how this differs across the range of cell metastatic potential.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	18	**19b. TELEPHONE NUMBER** *(include area code)*

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- **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The release of extracellular vesicles (ESVs) from high grade, aggressive forms of human cancer cells into their surroundings has become increasingly recognized as a feature of tumor biology, but what promotes ESV release, what cargo different ESV subpopulations carry, and what roles ESV contents have in tumor progression remains largely unknown. One hindrance to the lack of progress has been the scarcity of methods available to purify large quantities of ESV subpopulations intact, without cellular contaminants or without damaging the cargo. A second hindrance has been the lack of quantitative methods for measuring very small amounts of DNA and RNA transferred from tumor cells to the surrounding cells. As cancer progresses, the surrounding microenvironment co-evolves with the tumor through continuous paracrine cross-communication, thus creating a dynamic signaling circuitry that promotes cancer initiation, growth, drug resistance, metastasis and ultimately organ failure and death. The stromal components that include endothelial cells, pericytes, fibroblasts, various classes of leukocytes, and extracellular matrix are likely to receive ‘executive signals’ from the tumor in the form of proteins, mRNAs, ncRNAs, miRNAs and DNA to promote phenotyping changes in the stromal components that benefit the tumor. If we can detect the signals propagated from the tumor cells to the stroma, we can begin to formulate new testable hypotheses on how cancer cells manipulate their microenvironment to develop an aggressive phenotype. To address these shortcomings, the project has three specific aims:

Aim 1. Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].
Aim 2. Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.
Aim 3. Use accelerator mass spectrometry (AMS) technologies to quantify low levels of tumor-derived RNA transferred via ESVs to osteoblasts, and characterize their functions in promoting invasion.

- **KEYWORDS:**

Breast cancer, extracellular vesicles, exosome, MDNA-MB-231, MCF7, MCF10A, metastasis

- **ACCOMPLISHMENTS:**
  - **What were the major goals of the project?**

	Year 1	Year 2	Year 3
Specific Aim 1. Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].			
Task 1A: Verify microfluidic separation performance of ESVs from host cells and debris using existing acousto-fluidic devices with each of the 3 breast cancer cell lines			
	1a: Generate mixed cell-vesicle samples by growing cells in serum-starved media; process samples through separation device at a range of flow and pressure-field parameters and assess separation efficiency and purity by cell counting, SEM and fluorescence		

	microscopy and qPCR.		
Task 1B: Determine optimal separation strategy for oncosome population in each of the 3 breast cancer cell lines; verify bead-complexed separation of populations			
	1b: In tandem with Task 1a, for each cell line, determine whether oncosomes are best grouped with “large” (cells) or “small” group (microvesicles and exosomes), i.e. conditions at which largest fraction of oncosomes are recovered.	Label subpopulation-specific ESV surface markers (e.g. CD63) with antibody-derivatized polystyrene microspheres and verify purity and separation efficiency of resulting subpopulation separation by qPCR and SEM.	
Milestone #1: Tabulate separation parameters (flow rate, actuation voltage and frequency) for purifying ESVs vs. host cells/debris for each of 3 cell lines; publication on acoustic device performance			
Task 1C: Design and fabricate acoustic separator chips specifically optimized for isolation of ESV subpopulations			
		1c: From results of Tasks 1a and 1b, generate new photo-masks, and fabricate new microfluidic devices, optimally configured to isolate ESV subpopulations, ideally in a single pass through the device.	
Milestone #2: Fabricate 30-50 new microfluidic devices based on optimized design parameters.			
Task 1D: Verify isolation and purification of multiple ESV subpopulations by optimized acoustofluidic device			
		1d: Using mixed cell-vesicle samples grown from serum-starved media, pass samples through optimized devices and assess separation efficiency, purity and recovery.	Continue using acoustic devices to generate pure ESV samples for supporting the efforts in Tasks 2 and 3.
Milestone #3: A functional automated separation platform capable of rapid recovery of individual ESV subpopulations from bulk cell culture samples.			
Milestone #4: Publication reporting results of platform development and novel findings on ESV shedding rates and quantities in breast cancer cell lines of different metastatic character.			
Specific Aim 2: ***Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.***			
Task 2A: Engineer [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)] cell lines to express fluorescent markers that discriminate exosomes from			

microvesicles.			
2a: Create DNA constructs that express CD63 or CD9 [exosome markers] fusion proteins with mKate, a far red fluorescent protein and CD40 or CD63 [microvesicle marker] fusion proteins with eGFP, an enhanced green fluorescent protein	Where available fusion protein clones will be purchased, transfected into MDA-MB-231; MCF7; MCF10A cells and selected for stable transfected cell lines. If not available for purchase, the full length cDNA clones will be obtained from IMAGE and subcloned to insert mKate or eGFP in frame to create fusion constructs	Stable transfected cell lines with one exosome and one microvesicle specific markers will be characterized using imaging, western blots, and immunoprecipitation to confirm the location of the fluorescent protein.	
Milestone #5: Create new MDA-MB-231, MCF7, MCF10A subclonal cell lines that express fluorescent markers that allow us to discriminate between exosomes (red) and microvesicles (green). Each breast cancer cell line will be positive for 2 transgenes [mKate+; eGFP+]			
Task 2B: Engineer [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)] cell lines to express a transgene for uracil phosphoribosyltransferase [UPRT].			
2b: Transfect DNA construct that expresses UPRT into MDA-MB-231; MCF7; MCF10A cells and select for stable cell lines.		MDA-MB-231; MCF7; MCF10A cells lines expressing mKate or eGFP fusion proteins will be transfected with UPRT vectors, and select for triple transgenic lines [mKate+; eGFP+; UPRT+]	
Milestone #6: Create new MDA-MB-231, MCF7, MCF10A subclonal cell lines that express fluorescent markers that allow us to discriminate between exosomes (red) and microvesicles (green) and also express UPRT. Each breast cancer cell line will be positive for 3 transgenes [mKate+; eGFP+; UPRT+]			
<u>Specific Aim 3:</u> ***Use Accelerator Mass Spectrometry (AMS) technologies to quantify low levels of tumor derived RNA transferred via ESVs to osteoblasts, and characterize their functions in promoting invasion***			
Task 3A: Determine if cancer cells derived microvesicles or exosomes carry RNA			
		3a. Different ESV subpopulations derived from triple transgenic cancer cell lines and cultured with ^{14}C-thiouracil will be isolated using microfluidic device and the ^{14}C-level will be quantified using AMS. Since only RNA will be labeled with ^{14}C, only	Quantify the amount of RNA packaged in ESV in the 3 triple transgenic cancer cell lines cultured with ^{14}C-thiouracil. Optimize culture conditions to enrich for ESV populations that carry RNA, to obtain sufficient RNA for

		ESV populations positive for ^{14}C will be used to isolate RNA and sequence the RNA	sequencing
Milestone #7: Determine which ESV subpopulation has mRNA cargo			
Task 3B: Determine if cancer cells derived microvesicles or exosomes are taken up by osteoblasts			
3b:			3b: Triple transgenic lines created in 2b will be co-cultured with osteoblasts, at different time points [6 hours, 24 hours, 48 hours, 96 hours] the RNA will be isolated from osteoblasts and ^{14}C will be quantified to determine if RNA was transferred from cancer cells to the bone cells. RNA will be further isolated and sequenced
Milestone #8: Identify whether tumor cells package mRNA randomly into ESVs, or whether there is a rationale and metastatic tumors package 'unique' mRNA species that are more likely to influence their environment			
Milestone #9: Publication reporting results of ESV differences in transferring RNA to osteoblasts, among breast cancer cell lines with different metastatic character.			

- **What was accomplished under these goals?**

Specific Aim 1*. Optimize an existing microfluidic platform developed at LLNL to efficiently separate different ESV subpopulations from different breast cancer cell lines with varying metastatic character [MDA-MB-231 (highly invasive); MCF7 (moderately invasive); MCF10A (non-tumorigenic)].*

In task 1b we proposed to isolate large oncosome populations (> 5 µm) from smaller microvesicle and exosome populations using immunological beads combined with acoustic separation. Defining oncosome population has been more challenging, therefore we have focused during this funding period on smaller microvesicle and exosome populations. Our proposed experimental plan using acoustic separation to isolate large oncosomes and immunologically labeled vesicle subsets in a single device was expected to streamline vesicle purification (Tasks 1b and 1c). Since we could not "find" oncosomes, we experimented with using acoustophoretic separation to isolate different ESV populations using immunological beads. In our last report we demonstrated the potential to use acoustophoretic separation to separate ESVs bound to beads and free ESVs. However, we determined that this method is not superior to existing immunological ESV purification using magnetic beads. Existing methods using functionalized magnetic beads proved to be superior in throughput as well as limiting the dilution of exosome samples. Furthermore, the dearth of isolation and purification techniques for ***functional*** exosomes has become apparent to us in the past year and we see a pressing need for methods optimized for basic science applications. Widely used exosome purification techniques using immunological markers are ill-suited for fundamental exosome research since they inherently select for specific subsets of exosomes, limiting our ability to draw general conclusions and interrogate the full spectrum of exosomes produced by various cells. Additionally, removing attached antibodies is challenging, thus there is need for label free vesicle purification for functional vesicle recovery. Furthermore, high forces generated using ultracentrifugation, the gold standard for exosome concentration, can affect exosome morphology and is expected to negatively impact exosome function[1-2]. Therefore, during this funding period we shifted our focus towards identifying label-free methods of concentrating and purifying exosomes as described below.

We have begun assessing different concentration and recovery methods for isolating functional exosomes. We have looked at two widely used methods of vesicle concentration and purification: Ultracentrifugation, the gold standard for exosome isolation[3], and ExoQuick, an ethylene glycol precipitation-based method. We are further investigating two size-based methods which have only recently been applied to vesicle purification: (1). InnovaPrep's Concentrating Pipette: This is an emerging filtration-based technique, which isolates particles on size selective filters and then recovers them using a high-pressure aerosol elution foam designed to gently and completely remove particles from the membrane; and (2). Concentrating and purifying exosome samples through dialysis and evaporation.

Comparison of different concentrating methods: Ultracentrifugation, Precipitation- ExoQuick, Filtration and Elution- Concentrating Pipette

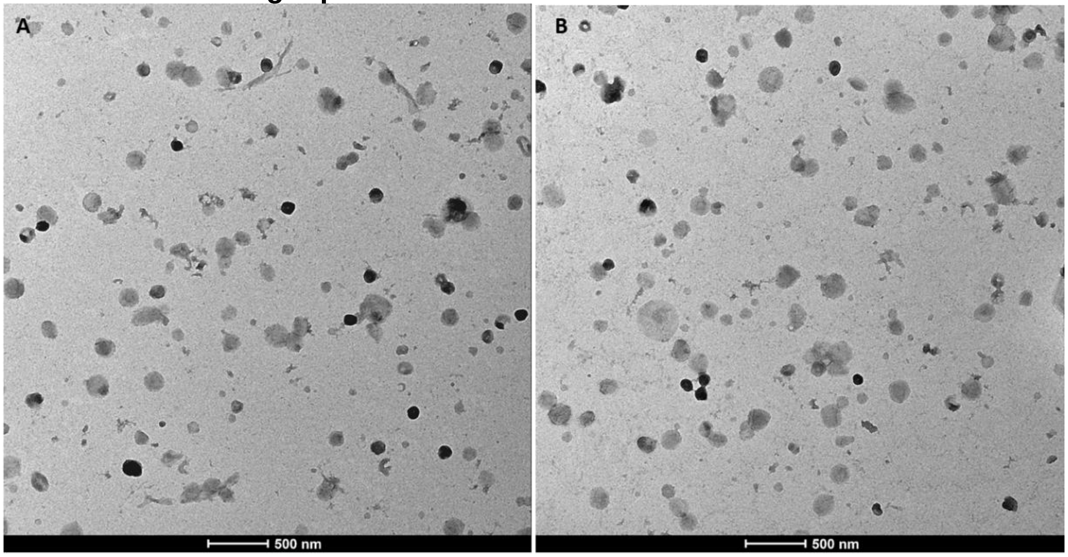


Figure 1: TEM images of ESVs isolated using one processing step A) concentrating pipette and B) ultracentrifugation.

To investigate different methods of label-free ESV concentration, we utilized transmission electron microscopy (TEM) analysis to assess each of these different techniques: Ultracentrifugation, Precipitation- ExoQuick, Filtration and Elution- Concentrating Pipette (Figure 1). Initial tests optimizing the extraction parameters using the Concentrating Pipette were performed using conditioned media from B16F10 cells, as B16F10 abundantly produce exosomes. Initial results measuring exosomes isolated by ultracentrifugation using dynamic light scattering showed more signal from exosomes isolated from B16F10s compared to numerous other cell lines including: MDA, E0771, PC3, or 4T1 cells. Thus, we used these for initial tests to optimize work flow and assay concentration techniques. Media was conditioned by serum starving cells for 24 hours (h) to induce exosome production and collected. Samples were spun on the benchtop to remove cell debris, then 0.2 um filtered, and processed via ultracentrifugation (200,000G for 2 hours), or using the concentrating pipette. To assess how multiple processing steps affect exosome isolation, additional samples were subject to two rounds of ultracentrifugation, and ultracentrifugation followed by using the concentrating pipette (Figure 2). After the first processing step, the concentrated exosomes were resuspended in the original volume and processed with the second processing step. All samples were eluted in 150 µL of buffer and stored at -80 C until TEM analysis. To analyze the particle size and concentration, we selected the best image for each condition and manually fit

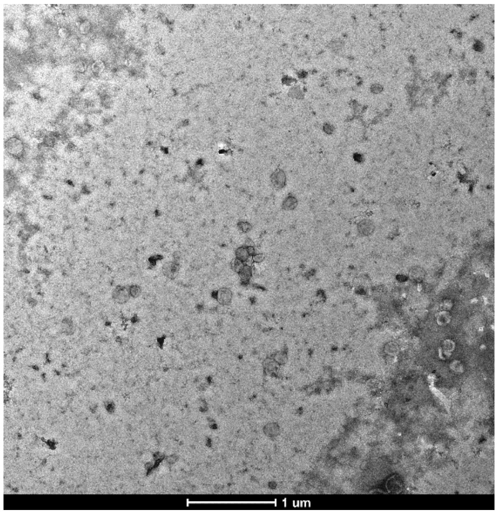


Figure 2: TEM images of ESVs isolated with two rounds of ultracentrifugation. No ESV were detected in samples processed by ultracentrifugation followed by the concentrating pipette.

ellipsoids to vesicles (Figure 3).

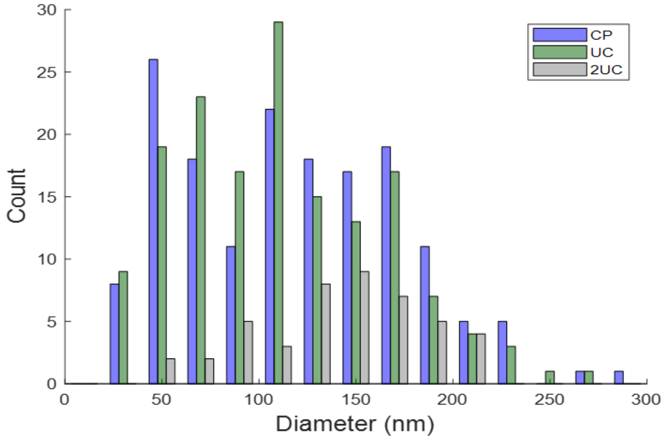


Figure 3: Sizes of ESVs collected via concentrating pipette (blue), ultracentrifugation (green) and two rounds of ultracentrifugation (gray). Note that a larger field of view was analyzed for the sample processed by two rounds of centrifugation. The number of ESVs per imaged area: concentrating pipette: 843 particles per mm^2; ultracentrifugation: 817 particles per mm^2; two rounds of ultracentrifugation: 143 particles per mm^2.

From these results we concluded:

- The quantity and size of vesicles is comparable between concentrating pipette and single round of ultracentrifugation.
- Multiple processing steps (two rounds of ultracentrifugation or ultracentrifugation followed by concentrating pipette) significantly reduces quantity and quality of vesicles. TEM images from both cases show increased debris and reduced counts of vesicles. No ESVs were identified in samples subjected to ultracentrifugation followed by the concentrating pipette, suggesting that the different vesicle populations are selected for using the different isolation techniques and processing with both techniques resulted in a loss of all ESVs.

Overall, these results suggested that the Concentrating Pipette may be an alternative method to isolate exosomes with performance comparable to ultracentrifugation. Thus, we moved forward with characterizing exosomes from breast cancer cells. We generated exosomes from 4T1 mouse breast cancer cell lines as described above. The 4T1 ESVs were isolated with more debris and at lower concentrations than those from B16F10 cells. This is in line with our previous results indicating that B16F10s produce greater amounts of ESVs. Figure 4 shows representative images of ESVs isolated using each technique. ESVs isolated via the concentrating pipette or the ExoQuick kit appeared somewhat more uniform in size than exosomes isolated using ultracentrifugation, which shows a large range of ESV sizes Figure 4a). However, due to the small sample size of imaged ESVs it was not possible to quantitate size differences between the different conditions.

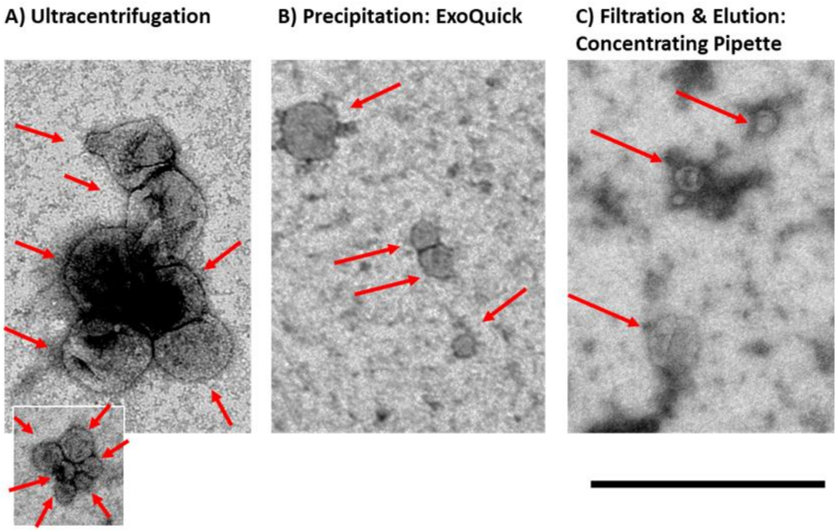


Figure 4: ESVs isolated from 4T1 breast cancer cells using different techniques. Red arrows indicate ESVs. Scale bar is 500 nm. A) Different fields of view of samples processed via ultracentrifugation showed dramatically different sized ESVs. Two different images are presented for the ultracentrifugation case to capture this.

Development of Evaporative Concentration and Dialysis

In task 1c we proposed to fabricate acoustic separator chips for the isolation of ESV subpopulations. Given the change of direction of the project, we instead worked towards developing methods and devices for ESV concentration and purification from conditioned cell media to complement our other work described. Key features of an ideal exosome isolation method for functional exosome recovery are:

1. At least 10-fold ESV concentration
2. Process multiple milliliters of sample in a reasonable time frame (<1 day).
3. Label-free
4. Removal of protein contaminants
5. Less force/more gentle than ultracentrifugation

Recently, hydrostatic filiation dialysis has been used for large volume concentration of concentrate exosomes from urine[4]. In this technique, commercial cellulose ester dialysis tubing is filled with the vesicle sample and suspended vertically in air. The hydrostatic pressure pushes the solvent and species smaller than the molecular weight cut off through the membrane while retaining larger species within the tubing. We sought to apply this technique to volumes suitable for small scale cell culture experiments to complement our other cell culture work. Mirroring their process, we experimented with using a model bead system with 200 and 1000nm fluorescent beads suspended in cell culture media to represent different vesicle populations, which could be quantitatively measured to determine recovery and concentration. To enhance the concentration rate, we experimented with applying pressure to the sample to enhance the pressure inside and outside of the tubing. We further found that performing these experiments in air causes the cellulose membrane to dry out which affects the pore size and membrane strength. Therefore, at higher pressures, it is necessary to submerge the device in buffer. We filled 300kDa cellulose ester dialysis tubing with 8-10 ml of sample and tested different applied pressures, both submerged in buffer or in air.

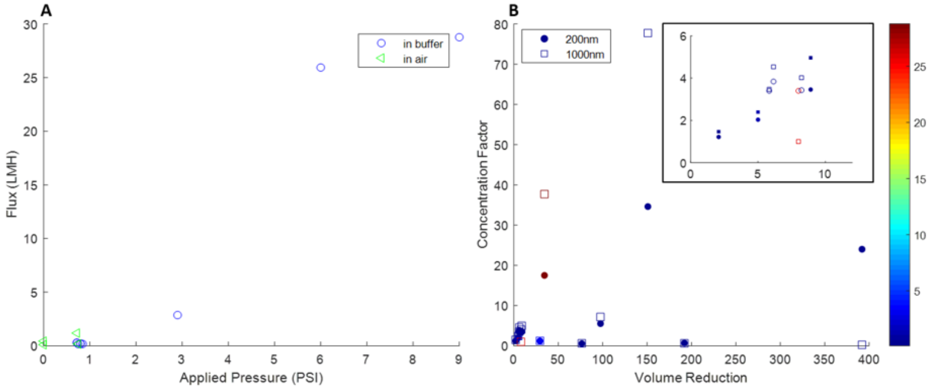


Figure 5: Preliminary tests with enhanced hydrostatic filtration. A) Flux through the membrane as a function of applied pressure demonstrates increased throughput at higher applied pressures. B) Concentration factor vs. Volume reduction for 200 and 1000 nm beads. Color represents flux in LMH, filled markers are performed in air, open markers are performed in buffer. Inset is enlarged plot at low volume reductions.

Samples collected in the membrane were compared to input samples by measuring fluorescence. Our initial tests demonstrate that enhanced throughput is possible by increasing the applied pressure to the sample (Figure 5A). These tests indicated that overall, larger particles were more efficiently retained and concentrated within the dialysis tubing as expected. At low volume reductions, we see an increase of concentration with increasing volume reduction (Figure 5B, inset). The concentration factor is slightly less than the volume reduction due to particle losses. We expect most loss particles are retained on the membrane rather than passing through the membrane, as no particles were detected in solution passing through the membrane, which is consistent with reported vesicle behavior using this technique[4]. At higher volume reductions results are extremely inconsistent. While it was possible to achieve extreme concentration and volume reduction (best results: 35x concentration of 200 nm beads, 78x concentration of 1000 nm beads and 150x volume reduction), numerous experiments resulted in little to no concentration (Figure 5B). When the volume is dramatically reduced, particles were observed sticking to the membrane and stuck in the region where the dialysis tubing was sealed and were not recovered reliably. Our results indicate that 10x vesicle concentration is possible, however, concentrating volumes to less than 1 ml is not feasible using this technique in its current form. While this method worked well for large, dilute urine samples, to adapt it for smaller cell culture volumes we need to increase the fluidic control and improve sample recovery.

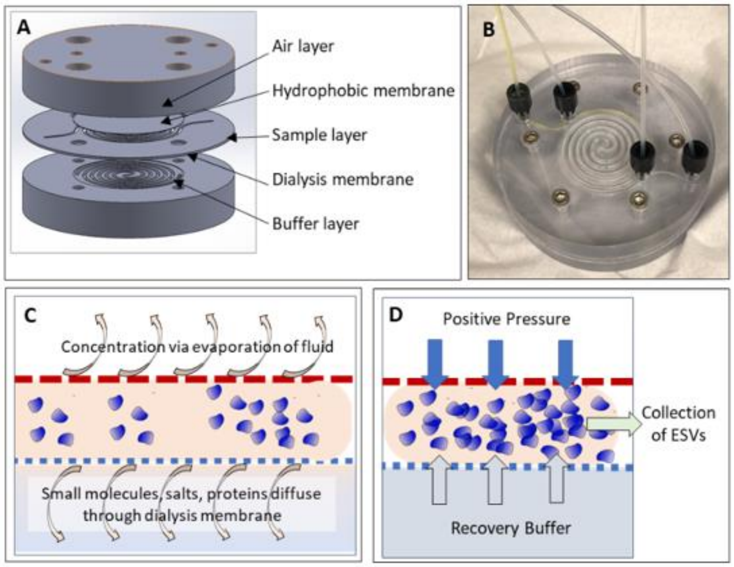


Figure 6: Second Generation Evaporative Dialysis Device. A) Exploded view of device stack with custom manufactured parts. B) Image of device with fluidic inputs and outputs connected for the sample and air [12]sample processing. D) Schematic of ESV recovery.

Thus, we developed a dialysis device with defined channels that allows us to precisely manipulate fluid on all sides of the membrane (Figure 6). The design of this device draws on microfluidic platforms for ESV isolation and filtration[5], [6] designed for diagnostic applications, but seeks to process larger volumes. To achieve gentle ESV isolation, we seek to employ evaporation for concentrating [7]–[9], and dialysis[10] to remove contaminants and maintain favorable conditions (i.e. keeping the osmotic pressure stable and limiting the co-concentration of salts and other species). We have further integrated this device with various automated fluidic handling valves, pressure pumps, vacuum sources, and syringe pumps which allows us to create well defined sample concentration and recovery procedures [11] to increase the repeatability and efficiency of ESV isolation.

Specific Aim 2: Engineer breast cancer cell lines with fluorescent and radiolabeled ESV subpopulations.

Towards our goal of engineering cell lines we have expanded the incorporation of our exosome labeling construct (pLLNL-exo-GFP) into remaining human cell lines. We successfully engineered a MDA-MB-231 cell line with a GFP fusion protein construct (Figure 7) as well as created new sublines of MCF-7-exo-GFP and MCF-10A-exo-GFP cancer cells. Through our initial success engineering a CD63-GFP fusion protein capable of fluorescently labeling ESVs, we devised a flow-cytometry based method to see if we could quantitatively track exosomes and their uptake in recipient cells. We were able to quantify exosome uptake in recipient cells at early timepoints (day 3 and day 14) and sequenced the RNA of those exosome-receiving cells using flow cytometry. This is an improvement over our originally proposed method as we are able to not only quantitatively track fluorescent exosome uptake, we were also able to study properties of recipient cells as this is a non-radiological based assay.

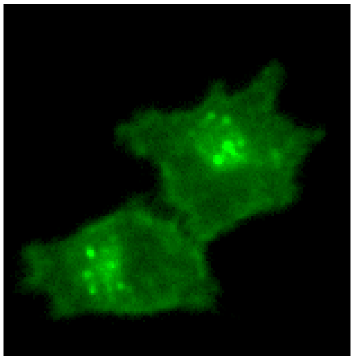


Figure 7. Representative images of transgenically labeled breast cancer cell lines, MDA-MB-231-exo-GFP.

Specific Aim 3: Use Accelerator Mass Spectrometry (AMS) technologies to quantify low levels of tumor derived RNA transferred via ESVs to osteoblasts, and characterize their functions in promoting invasion.

Towards our goal of characterizing exosome function in promoting invasion, we have:

- Tracked transfer of exosomes to recipient osteoblast cells using previously engineered MDA-MB-231-exo-GFP cell line
- Sequenced recipient osteoblast cells known to uptake MDA-MB-231-exo-GFP exosomes
- Isolated and characterized exosomes derived from all three breast cancer cell lines proposed: MCF-10A, MCF-7 and MDA-MB-231
- Sequenced exosome cargo and analyzed differences that relate to variation in cell line invasiveness
- Identified potential biomarkers packaged within highly invasive breast cancer exosomes that could be used to track disease progression

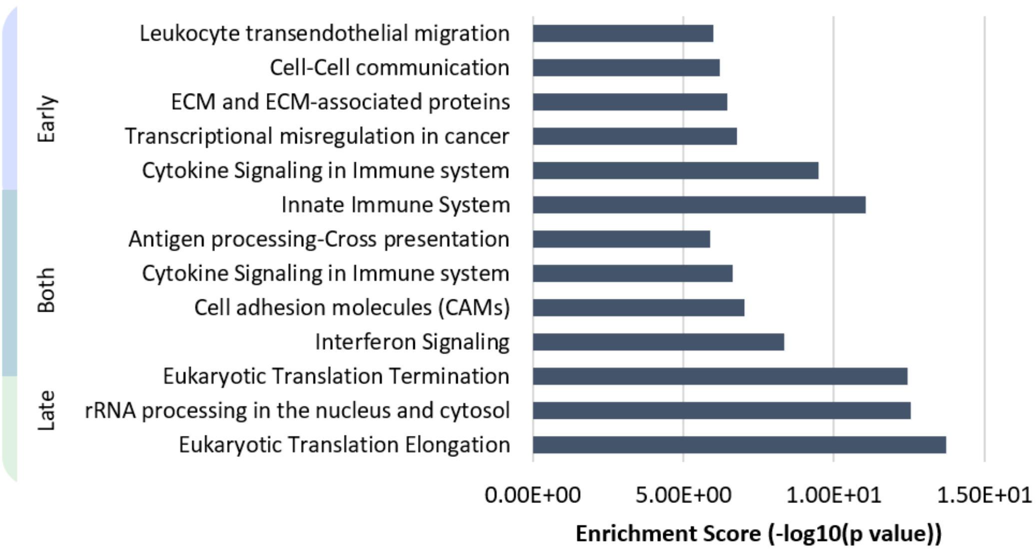


Figure 8. Upregulated gene ontologies in cancer exosome effected osteoblasts.

As referenced in Specific Aim #2, we have employed our genetically engineered MDA-MB-231-exo-GFP cell line to quantitatively track exosome uptake and sequence recipient cells.

In this study, we wanted to understand the transcriptional changes that take place following exposure to highly metastatic exosomes (MDA-MB-231-exo-GFP). First, we set up a co-culture transwell assay in which MDA-MB-231-exo-GFP cells were cultured on top of MC3T3 osteoblasts (recipient cells). At 3 days and 14 days of culture, osteoblasts were sorted into GFP+ and GFP- populations and both osteoblast populations were sequenced.

We found that upregulated genes in ESV-exposed populations following 3 days of co-culture correspond to extracellular matrix pathways and immune signaling, while genes involved in protein translation were significantly enriched follow 14 days of co-culture. Additionally, consistent with literature findings, we found seven key genes known to be associated with a cancer phenotype that were significantly differentially expressed at 3 days and 14 days of culture in cells that's exposed to MDA-MB-231-exo-GFP derived ESVs (Table 1).

Table 1. Enrichment of genes associated with cancer progression in GFP+ recipient cells at day 3 and day 14 of culture with endogenously fluorescent ESVs.

Gene	Function	Reference
Fgf5	Angiogenesis, metastasis	PMID: 28750499
Lmo7	Cell proliferation, migration, drug resistance	PMID: 28026121
Plau	Bone metastasis	PMID: 26317203
Notch1	Notch signaling	PMID: 29399967
Ncor2	Notch signaling	PMID: 29399967
Furin	Notch signaling	PMID: 29399967
Notch4	Notch signaling	PMID: 29399967

In the last fiscal year, we have been able to sequence changes induced by exosome uptake in recipient cells as well as characterize the exosomes themselves and sequence the ESV cargo specific to MCF-10A, MCF-7, MDA-MB-231 cells in an effort to better understand what transcriptional variation accounts for their differences in metastatic potential.

To study the effect of breast cancer exosomes on invasion, we first characterized the physical properties of these extracellular vesicles. We characterized particle size of exosomes secreted by each of the three breast cancer cell lines using Nanoparticle Tracking Analysis. This technique utilizes positional information to mathematically calculate particle size and quantity (Figure 9). This analysis revealed that exosomes derived from MCF-10A and MDA-MB-231 cells were similar in both size distribution as well as yield. MCF-10A and MDA-MB-231 cells produced particles approximately 350 nm in diameter while MCF-7 cells produced exosomes which were found to be smaller on average (~190 nm). Similarly, MCF-10A and MDA-MB-231 cells were also found to be similar when comparing amount of extracellular vesicle production. Both cell lines produced nearly three times less exosomes concentration when compared to MCF-7.

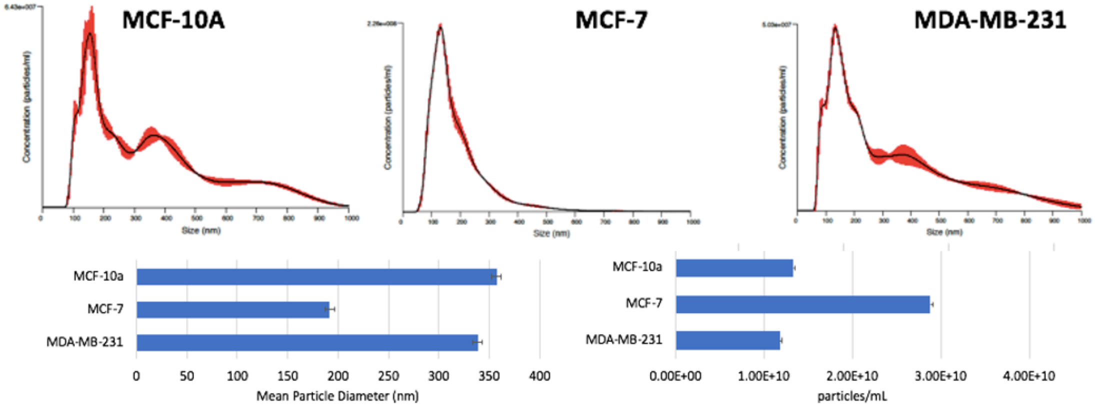


Figure 9. Nanoparticle Tracking Analysis of MCF-10A, MCF-7 and MDA-MB-231 cells.

Next, we wanted to understand what specific types of genetic cargo are packaged within extracellular vesicles. This was accomplished by performing RNA sequencing on exosomal RNA derived from all three breast cancer cells lines. Exosomes were isolated from ~4.0x10^7 cells per replicate using a polymer-based precipitation method (ExoQuick-TC). System Bioscience performed sequencing library preparation using their Exo-NGS service then sequenced on an Illumina NextSeq550 using single end, 75-bp reads. Three replicates were sequenced per cell line. Based on sequencing analysis, we found that the differences in cell behavior and potential for invasiveness lie not so much in the distribution of type of cargo that is packaged, but the identity of the cargo itself. Figure 10 shows that approximately one third of the genes encoded in extracellular vesicles regardless of cell line of origin correspond to non-coding regions of the genome, whereas the remaining two-thirds relate to protein-coding genes. Further, the non-coding regions represent genes from a variety of small RNAs (miRNA, snoRNA, snRNA, miscRNA, rRNA) and others (pseudogenes, antisense, etc). Similarly, the distribution of small RNAs within exosomes derived from all three breast cancer cell lines was comparable. Taken together, this data suggests that phenotypic differences in how each of the breast cancer cell lines behave *in vitro* and *in vivo* can be attributed to the differences in gene expression of specific genes.

Next, we wanted to distinguish between exosomes derived from metastatic cells (MCF-7) versus those from highly metastatic cells (MDA-MB-231) in both the number of non-coding genes differentially expressed as well as their identity (Figure 11). We found that there are 304 genes statistically significantly upregulated and 150 genes statistically significantly downregulated when comparing breast cancer exosomes (MCF-7 and MDA-MB-231) to normal, non-tumorigenic exosomes (MCF-10A). Interestingly, we found that that there are more differentially expressed genes between the MCF-7 compared to the MCF-10A exosomes than the MDA-MB-231 compared to the MCF-10A exosomes. This is likely due to the fact that both MDA-MB-231 cells and MCF-10A cells are derived from a similar gene cluster of triple negative breast cancer lineage.

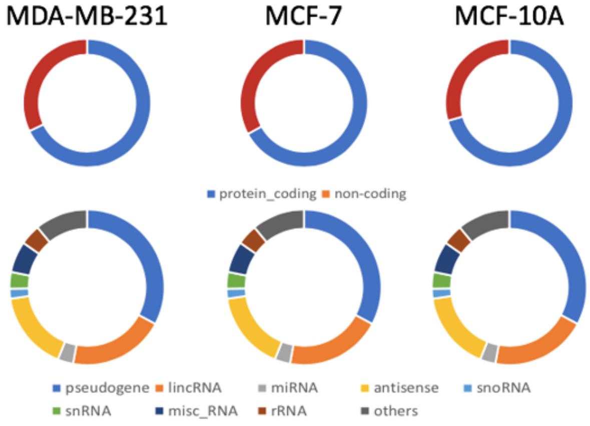


Figure 10. RNA sequencing of exosomes derived from MDA-MB-231, MCF-7 and MCF-10A cells.

Further investigating unique non-coding genes responsible for the metastatic versus the highly metastatic phenotype observed in the MCF-7 versus MDA-MB-231 cells respectively, we found the following microRNAs uniquely upregulated in MCF-7 exosomes (Table 2) and uniquely upregulated in MDA-MB-231 cells (Table 3). These microRNAs specific to metastatic and highly metastatic breast cancer can be further explored to evaluate specific function by which they confer a highly metastatic phenotype. Literature references shown in both Table ## and Table $$ provide evidence of other research efforts aimed at identifying the role of each of these microRNAs. These references aim to validate our findings, showing that other studies have also concluded that these microRNAs are related to a pro-metastatic phenotype.

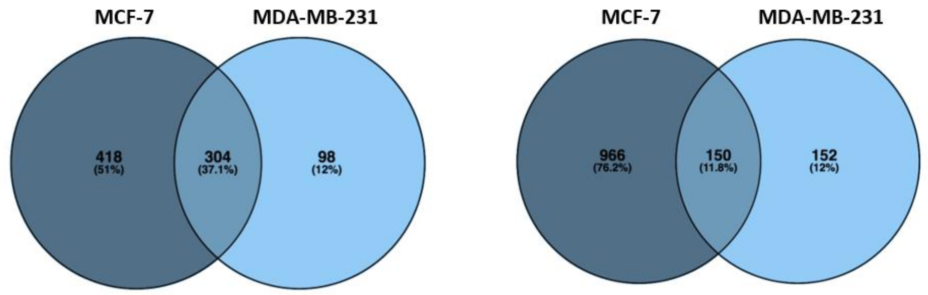


Figure 11. Overlapping non-coding genes upregulated (left) and downregulated (right) between exosomes derived from metastatic (MCF-7) and highly metastatic (MDA-MB-231) breast cancer cells.

Table 2. Upregulated microRNAs unique to metastatic MCF-7 exosomes.

miRNA	Potential Role	Reference
miR-200a	Regulates EMT in lung cancer	Future Oncol. 2018 Sep 13, ahead of print
miR-3609	Regulates cell adhesion, MAPK signaling	Cell Physiol Biochem. 44(5):1923-38.
miR-375	Tumor suppressor, cancer initiation	Cancer Letters, 438:126-132.
miR-489	Regulates migration and invasion	Oncotarget, 8:36410-22.
miR-542	Tumor suppressor, regulates EMT transition via targeting survivin	Biomed Pharmacother, 99:817-24.
miR-7641-1	Potential biomarker for multiple cancers	Scientific Reports, 7:8365.
miR-9-3	Potential tumor suppressor, enhances apoptosis	Molecular Cancer, 12:173.

Lastly, we wanted to compare patterns of gene expression across exosomes derived from all three cells types to identify if there are any patterns of gene expression that follow a clinical breast cancer disease progression. Progress towards early diagnosis and successful cancer screening depends on useful biomarkers and molecular identifiers that can be detected minimally invasively. Towards this objective, we have identified 21 potential biomarkers where gene expression is statistically significantly upregulated from a non-tumorigenic model (MCF-10A to a metastatic model (MCF-7) and further upregulated in a highly invasive model (MDA-MB-231), indicative of late stage disease/metastasis (Figure 12).

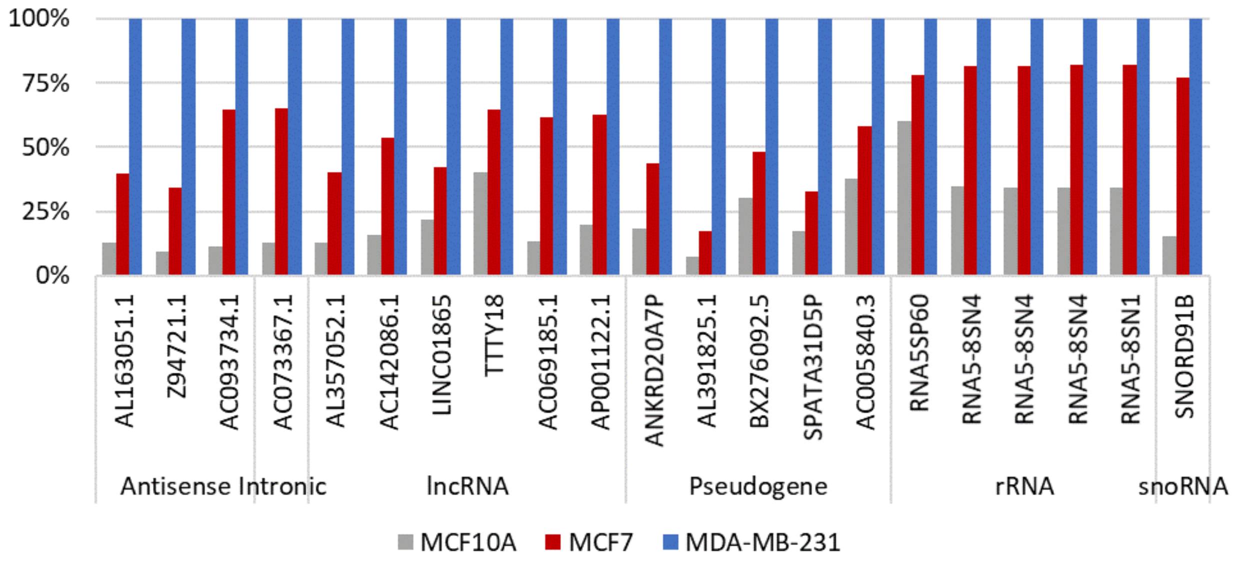
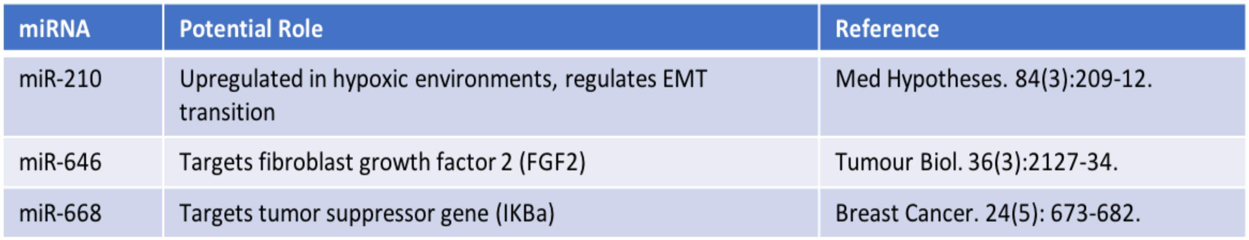


Figure 12. Potential candidate biomarkers based on patterns of gene expression in exosomes derived from cells of increasing invasiveness.

Table 3. Upregulated microRNAs specific to highly metastatic MDA-MB-231 cells.



miRNA	Potential Role	Reference
miR-210	Upregulated in hypoxic environments, regulates EMT transition	Med Hypotheses. 84(3):209-12.
miR-646	Targets fibroblast growth factor 2 (FGF2)	Tumour Biol. 36(3):2127-34.
miR-668	Targets tumor suppressor gene (IKBa)	Breast Cancer. 24(5): 673-682.

- **What opportunities for training and professional development has the project provided?**

Kelly Martin is a graduate student from Georgetown University who is conducting her Ph.D work at LLNL under Dr. Loots' mentorship. She has allocated approximately 30% of her research efforts towards this project. She has been involved with the isolation, characterization and fluorescent labeling of exosomes and microvesicles derived from various breast cancer cell lines. She has also presented research and received insightful feedback from the research community in a variety of forums including the Annual Cancer Research Symposium held at UC Davis Comprehensive Cancer Center and the American Association for Cancer Research (AACR) annual meeting.

- **How were the results disseminated to communities of interest?**

    - Nicholas R. Hum, Kelly A. Martin, Aimy Sebastian, Gabriela G. Loots. *TRANSCRIPTOME ANALYSIS OF OSTEOBLASTS FUSED WITH CANCER-DERIVED EXOSOMES;* Poster Presentation at AACR Annual Meeting, Chicago, IL, 4/14-18, 2018
    - Nicholas R. Hum, Kelly A. Martin, Aimy Sebastian, and Gabriela G. Loots. *Comparison of breast cancer exosomes from cell lines of varying metastatic potential;* Poster Presentation UC Davis Cancer Center Symposium, Davis, CA 9/27-28, 2018

- **What do you plan to do during the next reporting period to accomplish the goals?**
    - During the last year, the main focus will be on completing Aim 3, generating data outlined in Aim3A and Aim3B, publish results and present at AACR annual meeting and UCD Cancer Center Symposium.

- **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - *Nothing to Report*
    - **What was the impact on other disciplines?**
        - *Nothing to Report*
    - **What was the impact on technology transfer?**
        - *Nothing to Report*
    - **What was the impact on society beyond science and technology?**
        - *Nothing to Report*

- **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
    - **Changes in approach and reasons for change**

We proposed to isolate large oncosome populations (> 5 µm) from smaller microvesicle and exosome populations using immunological beads combined with acoustic separation. Our proposed experimental plan using acoustic separation to isolate large oncosomes and immunologically-labeled vesicle subsets in a single device was expected to streamline vesicle purification (Tasks 1b and 1c). Separation of bio-particles using acoustophoresis is less efficient for smaller sized particles. Focusing and separation degrades steeply as the particle size nears 1 µm due to competing acoustic streaming effects which increase mixing [13]. Given the fundamental limitations of acoustophoresis to isolate different ESV populations <1 µm, it is necessary to use immunological beads to increase the size and contrast factor of bound ESVs as discussed in our last report. However, we determined that using acoustophoretic separation with immunological beads is not superior to existing immunological ESV purification using magnetic beads. Existing methods using functionalized magnetic beads are superior in throughput as well as limiting the dilution of exosome samples. In our original proposal we expected acoustophoresis to be an ideal label free method for isolating populations of large oncosomes, but since we have not been able to “find” oncosomes, we have determined that pursuing acoustophoresis for isolation of small ESV populations is futile and unlikely to improve upon existing methods. Therefore, we chose to shift our focus towards identifying label-free methods of concentrating and purifying exosomes. In our last report, we detailed the separation parameters for purifying ESVs vs host/cell debris. However, since we have determined our method to isolate ESVs is not superior to established immunologic magnetic bead assays, we are instead pursuing evaluation of different ESV isolation and purification techniques. We are investigating three methods of vesicle concentration and purification: 1) Ultracentrifugation, the gold standard for exosome isolation[3], 2) ExoQuick, an ethylene glycol precipitation-based method, and 3) InnovaPrep's Concentrating Pipette, an emerging filtration-based technique, which isolates particles on size selective filters and then recovers them using a high-pressure aerosol elution foam designed to gently and completely remove particles from the membrane. We anticipate comparing concentration, recovery and morphology of ESVs isolated using each of these techniques.

Due to our change in direction, instead of generating a new generation of acoustophoretic separation devices, we will be focusing on the development of our evaporative dialysis device for exosome isolation and purification. Our revised tasks are:

Task 1B: Evaluate different ESV concentration techniques to recover functional ESVs.

Milestone #1: Comparison of ESV morphology, size and concentration using ultracentrifugation, precipitation and filtration.

Task 1C: Design and fabricate Evaporation-Dialysis devices for ESV concentration and purification.

Milestone #2: Demonstrate 10-fold particle concentration of >5 ml samples.

Task 1D: Verify concentration and purification of ESVs using Evaporation-Dialysis device.

Milestone #3: A functional ESV concentration platform capable of gentle, label-free ESV isolation and concentration from bulk cell culture samples, eliminating the need for ultracentrifugation

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Since we are seeking to develop a new method of ESV purification and concentration, we anticipate that the development will take longer than building off our existing acoustophoretic separation device. We are developing a platform which will be compatible with commercial filter membranes, to enable different types of membranes with different selectivity (size, charge, etc.) to isolate different ESV populations. However, since this is a new development effort, in the scope of this project we will be focusing on whole population ESV purification and concentration, rather than trying to separate different sub populations. We expect this developmental effort to generate a useful tool to reliably isolate a spectrum of ESVs without selecting for specific populations which are often lost or damaged by other isolation techniques.

- **Changes that had a significant impact on expenditures**
  - *No*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - *No*
- **Significant changes in use or care of human subjects**
  - *Not applicable*
- **Significant changes in use or care of vertebrate animals.**
  - *Not applicable*
- **Significant changes in use of biohazards and/or select agents**
  - *Not applicable*
-

- **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- *Nothing to Report*

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
  - **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	*Gabriela G Loots*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0001-9546-5561
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Loots was in charge of overseeing the project and collaboration with engineering group, met with team regularly [weekly] to discuss experimental design, data analysis, troubleshooting and future directions*
Funding Support:	*n/a*

Name:	*Maxim Shusteff*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Shusteff was in charge of overseeing the engineering component of this project, met with team regularly [weekly] to discuss experimental design, data analysis, troubleshooting and future directions*
Funding Support:	*n/a*

Name:	*Erika Fong*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Fong conducted all engineering, microfluidic experiments, met with the biologists regularly, optimized experimental design, collected data, analyzed data, troubleshooting*
Funding Support:	*n/a*

Name:	*Nicholas Hum*

Project Role:	*Biomedical scientist*
Nearest person month worked:	*1.5*
Contribution to Project:	*Mr. Hum in a biologist, he conducted cloning, culturing the cells, transfecting the cells, carrying our FACs analysis, isolating ESV via centrifugation*
Funding Support:	*n/a*

Name:	*Kelly Martin*
Project Role:	*Graduate Student*
Nearest person month worked:	3
Contribution to Project:	*Ms. Martin is a graduate student and has performed ESV isolation via ultracentrifugation, ESV characterization and cell culture.*
Funding Support:	*Livermore Graduate Scholar Fellowship*

Name:	*Deepa Murugesh Sosebee*
Project Role:	*Lab Technician*
Nearest person month worked:	*1.5*
Contribution to Project:	*Assisted with cell culture, FACS sorting, RNAseq analysis*
Funding Support:	*n/a*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - *Nothing to Report"*
- **What other organizations were involved as partners?**
    - *Nothing to Report*