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Extracellular Shed Vesicle Isolation - InnovaPrep® Concentrating Pipette, Ultracentrifugation, and Systems Biosciences ExoQuick®

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Extracellular Shed Vesicle Isolation - InnovaPrep® Concentrating Pipette, Ultracentrifugation, and Systems Biosciences ExoQuick®

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Isolation and purification techniques for ***functional*** exosomes are limited, 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 the 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].

We have begun assessing different concentration and recovery methods for 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 a size-based method which has only recently been applied to vesicle purification: 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.

To investigate different method of label free ESV concentration we compared TEM images collecting using the different techniques: Ultracentrifugation, Precipitation- ExoQuick, Filtration and Elution- Concentrating Pipette.

Initial tests optimizing the extraction parameters using the Concentrating Pipette were performed using conditioned media from B16F10 cells, as B16F10 appear to be high producers of 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 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 at 200,000G for 2 hours,
- InnovaPrep's Concentrating Pipette using ultrafiltration tips, or
- System Biosciences's ExoQuick precipitation isolation kit following kit instructions.

To assess how multiple processing steps affects exosome isolation, additional samples were subject to two rounds of ultracentrifugation, and ultracentrifugation followed by using the concentrating pipette. 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 -

80C until TEM analysis. To analyze the particle size and concentration, we selected the best image for each condition and manually fit ellipsoids to vesicles.

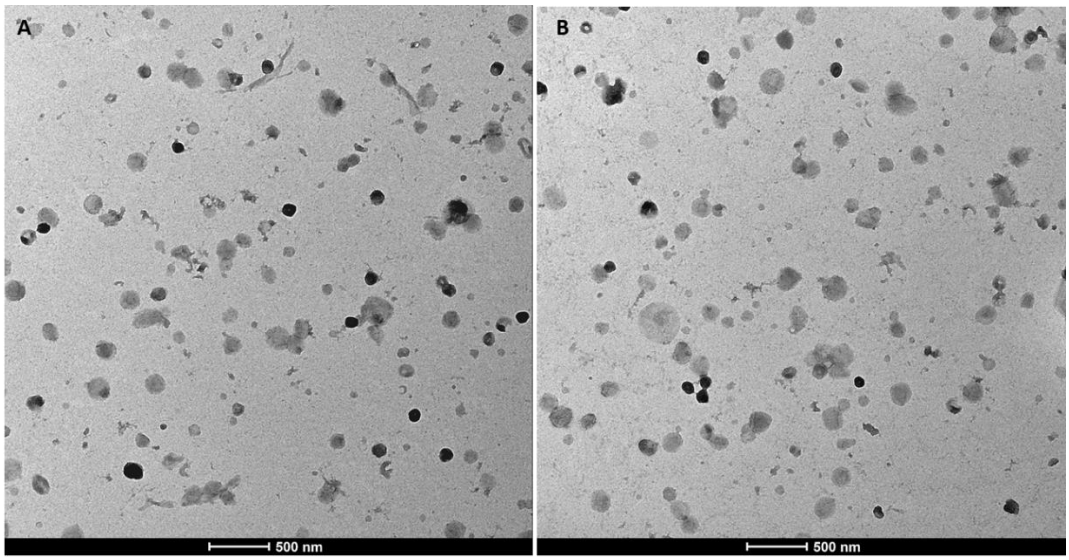

Figure 1: TEM images of ESVs isolated using one processing step A) concencentrating pipette and B) ultracentrifugation.

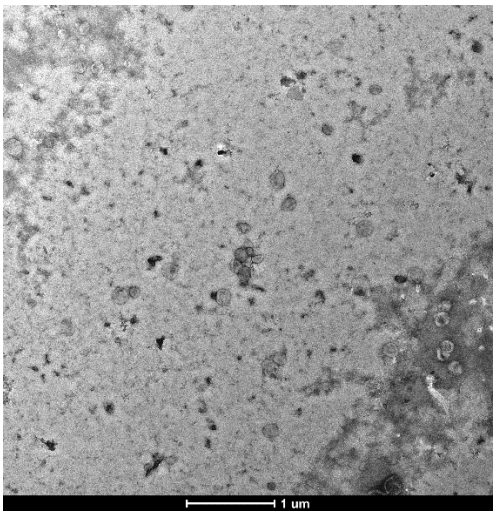

Figure 2: TEM images of ESVs isolated with two rounds of ultracentrifugation. No ESV were detected in samples processed by ultracentrifugation followed by the concentrating pipette.

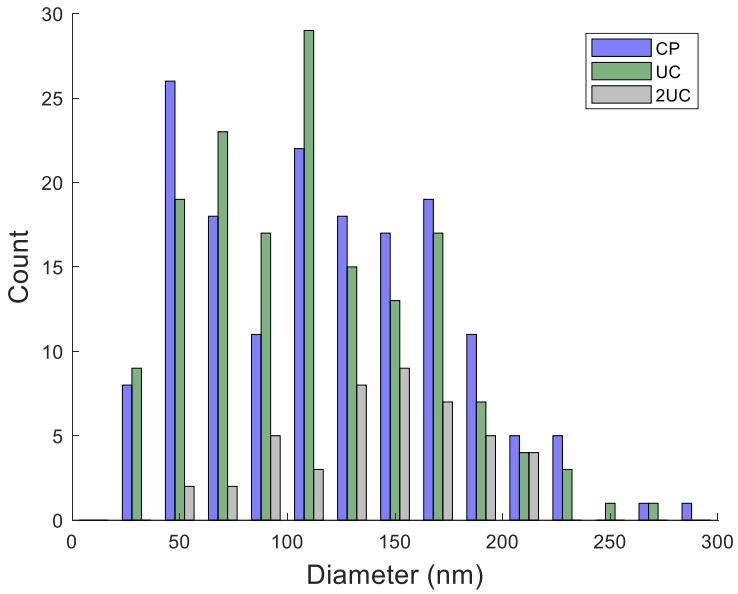

Figure 3: Sizes of ESVs collected via concentrating pipette (blue), ultracentrifugation (green) and two rounds of ultracentrifugation (gray). Note that a larger field of view was analyzed for the sample processed by two rounds of centrifugation. The number of ESVs per imaged area: concentrating pipette: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

From these results we conclude:

- 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 maybe an alternative method to isolate exosomes with performance comparable to ultracentrifugation. Thus, we moved forward characterizing exosomes from breast cancer cells. We generated from 4T1 mouse breast cancer cell lines as described above. The 4T1 ESVs were isolated with more debris and at lower concentrations than the B16F10 ESVs. 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, as see in Figure 4a. However, due to the small sample size of imaged ESVs it was not possible to quantitate size differences between the different conditions.

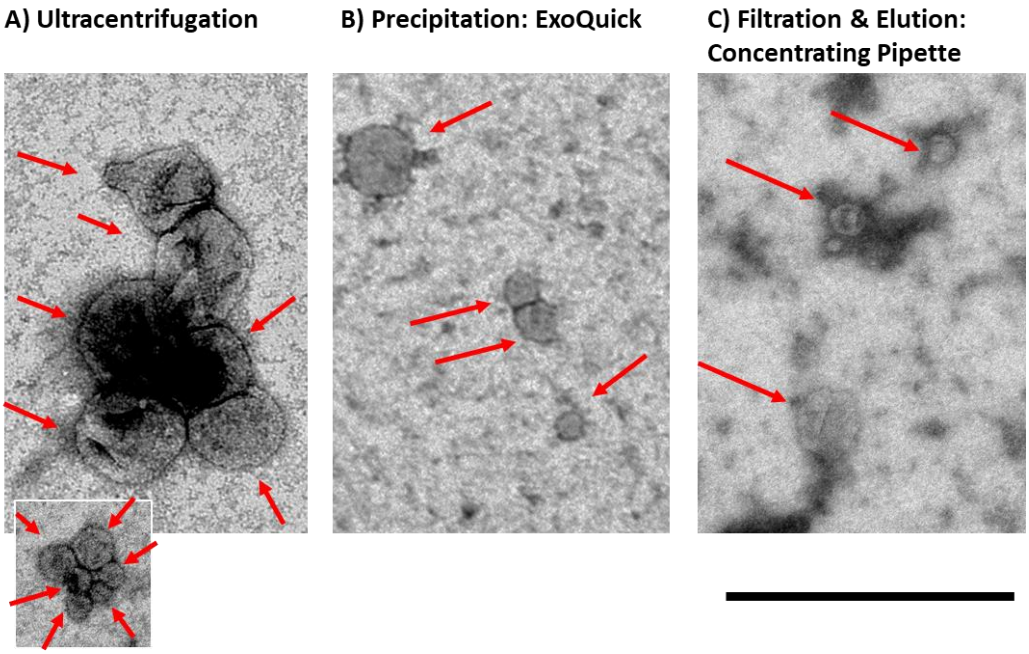

Figure 4: ESVs isolated from 4T1 breast cancer cells using different techniques. Red arrows indicate ESVs. Scale bar is 500 nm. A) Different fields of view of samples processed via ultracentrifugation showed dramatically different sized ESVs. Two different images are presented for the ultracentrifugation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

Concentrating Pipette Operation

Overall the concentrating pipette operation was user friendly, and easy to use. However, sample processing varied somewhat between samples, and often stopped before the sample was completely processed, requiring the user to manually restart. Diluting the sample, and/or adjusting the flow settings only marginally improved operation in this aspect. Overall, for vesicle samples the average throughput was 1 ml/minute.

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