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Review Article

Integrated systems for exosome investigation

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ABSTRACT

Extracellular vesicles, including exosomes, are currently being investigated to better understand their biogenesis and biological functions. There is also a rapidly growing interest in utilizing exosomes present in patient biofluids for molecular diagnostics in the clinic. Exosomes are natural shuttles of RNA and protein cargo, making them attractive as potential therapeutic delivery vehicles. Here, we describe the methods for using the latest tools and technologies to study exosomes to better understand their roles in cell-to-cell communication, for discovery of clinical biomarkers and to engineer exosomes for therapeutic applications.

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1. Introduction

1.1. What are exosomes and microvesicles?

Extracellular microvesicles (EMVs) are an intriguing class of secreted, nano-sized cellular organelles used by cells for intercellular communication [1]. There are a number of different types of EMVs which can be roughly classified based on their internal cellular origin prior to secretion. The largest of the EMVs are secreted directly from the plasma membrane and range in diameter sizes from 200 nm up to and over 1000 nm [2]. These are classified as ectosomes; the most interest generated by vesicles within this classification has been those secreted from cancer cells, which have been termed “oncosomes” [3]. Secreted microvesicles that originate from the endosome are deemed “exosomes” and are highly abundant in many biofluids including serum/plasma, urine, amniotic fluid, cerebral spinal fluid and saliva. Exosomes are typically smaller than ectosomes and oncosomes, with sizes ranging from 30 nm up to approximately 200 nm in diameter [4].

1.2. What is so exciting about exosomes and microvesicles?

EMVs are a rich, untapped treasure chest for biomarker discovery and development [5,6]. Since exosomes and microvesicles can reflect the cell's RNA and protein content from which they are secreted, there is a substantial opportunity for creating molecular

diagnostics and prognostics as well as real-time monitoring of therapies based on patient biofluid exosome analysis – the highly valued “Liquid Biopsy” [7]. Exosomes are also naturally occurring nanoshuttles that are capable of delivering their cargo to cells in close proximity, analogous to a snowball fight between two opposing cells. Exosomes can also deliver their contents to cellular destinations distant from their point of origin, and they can even pass through the blood–brain barrier [8,9]. This article will focus primarily on extracellular vesicles and describe methods for their isolation, detection, quantitation, and characterization of their biomarkers using integrated exosome research toolsets from System Biosciences (SBI). We end with a perspective on the future use of exosomes as therapeutic shuttles to deliver engineered RNA, protein, and pharmaceutical cargo while also being programmed with surface ligands for targeting specific cellular destinations.

2. EMV isolation techniques

There is a variety of different ways to isolate exosomes. The choice of protocol depends on the sample source (tissue culture media, malignant ascites fluid, urine, plasma, or another biological fluid), the amount of sample, and what the downstream application(s) for the exosomes is after isolation: RNA analyses, proteomics, biological assays, etc. Due to the small size of EMVs, isolating them from biofluids can be challenging. There are a number of validated methods for retrieving EMVs from various biofluids including ultracentrifugation [10], ultrafiltration [11], chromatography [12], polymer-based precipitation [13–16] and affinity capture on antibody-coupled magnetic beads [17]. Each

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method has certain benefits and drawbacks depending upon the exosome isolation scale, purity and ease desired for a particular application.

2.1. Ultracentrifugation

For a number of years, ultracentrifugation has been the method of choice for concentrating and isolating exosomes [18]. Both differential and density gradient ultracentrifugation (UC) are used, and either method can be paired with size filtration. Because the density of their cargo alters how exosomes behave under differential centrifugation, this method is not able to completely separate exosomes by particle size. In addition, extravesicular protein complexes and aggregates, lipoproteins, and other contaminants may also be pelleted by a high-speed spin. Density centrifugation can separate out these extravesicular contaminants, but may still be unable to separate exosomes from other extracellular vesicles of similar density. While widely used, ultracentrifugation has several drawbacks, especially for biomarker studies or diagnostics using clinical samples. Ultracentrifugation is time consuming, potentially involving multiple overnight spins, requires sample volumes that preclude use on low-volume clinical samples or rodent systems, is not suitable for processing a large number of samples, and requires access to an ultracentrifuge. For exosomes in cell culture media, the typical protocol calls for the cell culture supernatant to be centrifuged (300×g for 10 min; 2000×g for 20 min to eliminate dead cells; and 10,000×g for 30 min to remove debris) and then pelleted by ultracentrifugation at 100,000×g for 70 min at 4 °C. Recent studies indicate that exosomes isolated by UC may have more cellular debris contaminants than other isolation methods [19]. Additionally, using UC may cause vesicle rupture, resulting in vesicle loss during the high velocity ultracentrifugation process, a phenomenon referred to as the “splat factor” [19].

2.2. Ultrafiltration and size exclusion chromatography

Both Ultrafiltration (UF) and size exclusion chromatography techniques use a solid matrix by which exosomes can be fractionated and isolated. UF procedures can be used to produce highly-purified EMVs [20] when compared to other methods. This exosome isolation method utilizes nanomembrane concentrators equipped with semipermeable polyethersulfone membranes. An example column kit for this application is the Vivaspin 20, 100 kDa MWCO from GE Life sciences which has been used to isolate exosomes from urine [21]. Drawbacks of UF include difficulty in removal of remaining proteins that adhere to the nanomembrane and elution of exosomes from the membrane. Size exclusion chromatography using Sepharose 2B or CL-4B packed columns has been successfully used to fractionate exosome from biofluids [22]. Individual fractions are collected and then subjected to centrifugation at 100,000×g for 1 h; therefore, the UC “splat factor” would occur with this method of isolation as well. Both UF and chromatography may not offer the scalability desired for number of samples for clinical applications or routine laboratory investigations.

2.3. Polymeric precipitation

One alternative technology invented by SBI in late 2009 is exosome isolation through precipitation using polymers. The technology works by capturing and collecting exosomes of a certain size range (60–150 nm) in “polymer nets” that can be recovered by a simple, low speed centrifugation on the bench top at 1500×g. This technology is commercially available from System Biosciences (SBI) under the trade names ExoQuick® and ExoQuick-TC® kits. Once the exosome pellet is obtained, the supernatant containing excess polymer is removed and the exosomes can

then be resuspended in a suitable solution, such as PBS. This resuspension process dilutes the residual polymer in the exosome pellet enough to dissolve the polymer net and liberate intact exosomes. The entire process can take as little as 30 min with exosome yields that are higher than ultracentrifugation, chromatography and antibody-coupled magnetic beads methods [23]. When working with plasma samples, it is recommended to first pre-treat the plasma with recombinant thrombin to coagulate the fibrinogens. A short spin is then used to pellet the coagulation factors and the supernatant that is more “serum like” is suitable for standard exosome precipitation using ExoQuick. Any exosome type from any biofluid from any species can be isolated using this ExoQuick technology. An example image of exosome pellets from cell culture media is shown in Fig. 1.

2.4. How does ExoQuick work?

The polymer formulation used in the ExoQuick technology forms a network when introduced at a certain salt condition and incubated at 4–5 °C. Exosomes as individual nanoparticles would require high gravitational force through ultracentrifugation to pellet out of solution, but by gathering them in polymer bags or nets formed by polymer branches, these extracellular nanovesicles can be recovered quite easily at low gravitational centrifugal forces. We like to use the analogy of free marbles versus marbles collected in a mesh bag: the bagged marbles would have a higher density as a group than any individual marble as depicted in Fig. 2. This polymer net bag excludes very large microparticles in favor of

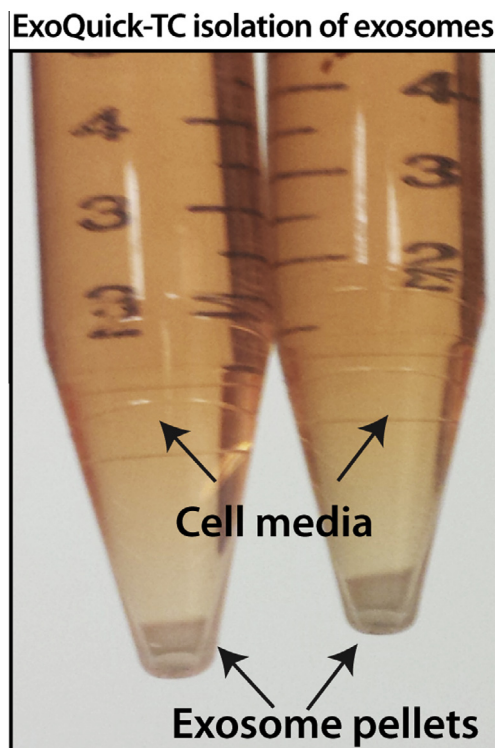


Fig. 1. ExoQuick-TC® precipitated exosome pellet. 1 mL of ExoQuick-TC® (catalog # EXOTC10A-1) was added to 5 mL cell media from HEK293 cells in culture that had been pre-spun (3000×g) to remove cellular debris. Important note: the growth media for the HEK293 cells was exosome-depleted Exo-FBS (catalog # EXO-FBS-50A-1) from System Biosciences to avoid contaminating bovine exosomes present in standard FBS media supplements. The mixture was incubated 12 h at 4 °C and the exosomes recovered with a low-speed spin in a Beckman Allegra 6R (catalog # 36686) centrifuge with a GH-3.8 swinging-bucket rotor spun at 1500×g for 30 min. The pellets appear as a white/beige color and can be easily resuspended in sterile PBS for downstream applications.

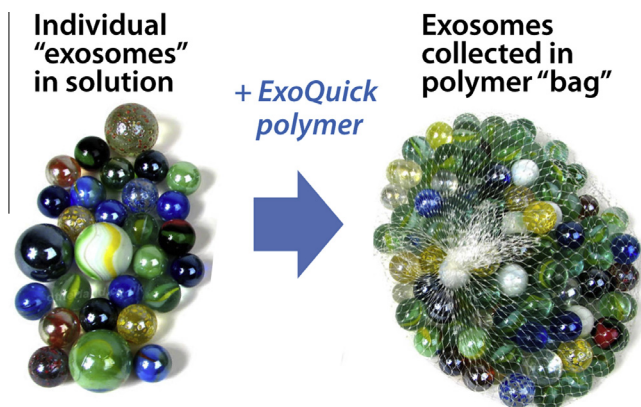


Fig. 2. ExoQuick® polymer nets for rapid exosome isolation. ExoQuick® polymer works to precipitate exosomes by forming a network (analogous to the depicted white mesh bag) and collects all exosomes of a certain size (analogous to the depicted various colored marbles). Once the ExoQuick® mesh is formed, a simple, low-speed centrifugation easily precipitates the exosomes as a pellet. The exosomes are intact, ready for protein, RNA analysis and are bioactive for functional studies.

exosome-sized vesicles. Additionally, the polymer bag dissolves when the supernatant is removed and the exosomes are resuspended in water or PBS. The polymer itself is biochemically inert and does not elicit any immune responses *in vitro* or *in vivo* [24]. The polymer precipitation will isolate all type exosomes, independent of their composition. Depending on the sample source, some non-exosomal material, such as protein aggregates, may co-isolate with the exosomes. The ExoQuick technology offers simple, rapid exosome isolation from any biofluid and can be scaled up for bulk isolation as well as for number of individual isolations needed for any study or clinical application.

2.5. Immuno-affinity purification

Exosomes can originate from any tissue or cell type and end up in a mixed population biofluid, such as serum. Immuno-affinity purification (IP) is a method to selectively capture specific exosomes from such a complex population based upon certain surface markers. This approach employs magnetic beads covalently coated

with streptavidin, which can be coupled in a high-affinity fashion to any biotinylated capture antibody. A schematic is shown in Fig. 3 of how this works. Classic exosomal surface markers such as CD63, CD9, and CD81 are popular capture antibodies used in this approach. Although these markers are found on many exosomes, it should be noted that capturing exosomes with IP techniques results in the selective purification of only exosomes that are positive for the designated surface marker. For example, using a CD9 capture antibody to purify serum exosomes will result in isolation of only CD9⁺ exosomes, a factor worth considering if such a specific isolation may limit experimental findings. SBI has built an integrated system of magnetic beads and exosome antibody sets for capturing exosomes using IP called the Exo-Flow kit. Exosomes can be purified in bulk from large volumes (1.5–50 mL) using magnetic stands. The appropriate amount of antibody-coupled magnetic beads are added to the biofluid containing exosomes and allowed to bind overnight at 4 °C overnight while rotating. The bound exosomes are then easily collected using the multifunctional magnetic stand. The Exo-Flow beads will appear as an orange color which allows for them to be easily tracked during the magnetic separations and washes. The beads adhere strongly after placement on the magnetic stand, allowing for clean separations and washes to be achieved. An example image is shown in Fig. 4A for bulk purification of CD63⁺ exosome purifications from human serum. Captured exosomes can also be eluted, intact and bioactive, for downstream applications using the exosome elution buffer included in the kits. Exosomes can be purified on smaller scales using magnetic beads as well. This method uses a 96-well plate and an accompanying magnetic surface with magnets aligned to each well. The same capture approach is used, but smaller volumes (typically less than 200 μ L starting biofluid) can be applied for successful IP results. A practical example of how this technology can be used to separate exosomes with a certain surface marker is depicted in Fig. 4B to track Alzheimer's disease progression using captured serum exosomes. A summary of each exosome isolation method along with their benefits and drawbacks are summarized in Table 1. Careful consideration as to the type of sample input, volume to be processed and downstream applications should be evaluated before selecting an isolation method.

3. EMV detection and quantitation

3.1. Exosome visualization and detection

Due to their small size, exosomes are difficult to visualize with most microscopy platforms. Historically, various electron microscopy approaches including whole mount electron microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to image exosome samples [25]. An example of an exosome preparation from human monocytic cells cultured in exosome-depleted medium visualized using whole mount transmission electron microscopy is shown in Fig. 5. Before imaging exosomes isolated using ExoQuick or ExoQuick-TC, it is recommended to pass the exosomes through a simple G-25 column to remove any residual polymer that may interfere with EM techniques (GE Healthcare PD SpinTrap G-25 Desalting Columns, Fisher Scientific catalog # 45-001-527). The exosomes flow directly through the column and the polymer remains trapped in the column matrix. Such images revealed exosomes to be “cup-shaped” [25], which came to be accepted as their natural shape *in vivo*; however, this result has since been shown to be an artefact of the preparation protocol for electron microscopy which includes dehydration and fixation of samples (Dr. Jan Olof Lötvald seminar, “Diversity of extracellular vesicles and their cargo in cell to cell communication” Published online March 5, 2015). Electron

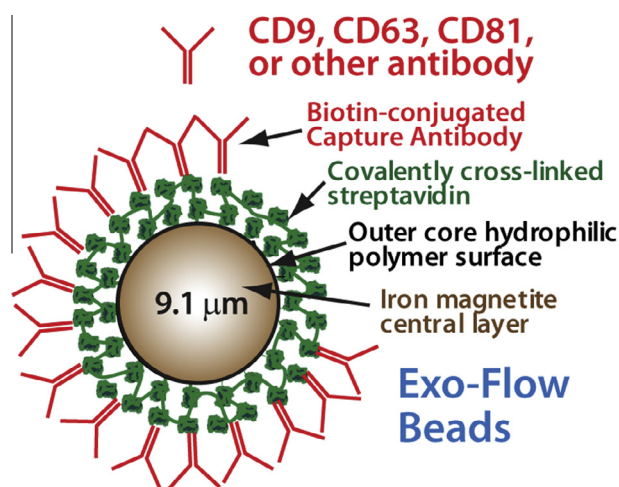


Fig. 3. Exo-Flow magnetic beads for immuno-purifying exosomes. The Exo-Flow magnetic beads (catalog # series EXOFLOWxxxA-1) are colored brown and are covalently coated with streptavidin (green). Various biotinylated exosome capture antibodies of choice can be coupled to the streptavidin-coated magnetic beads are colored in red.

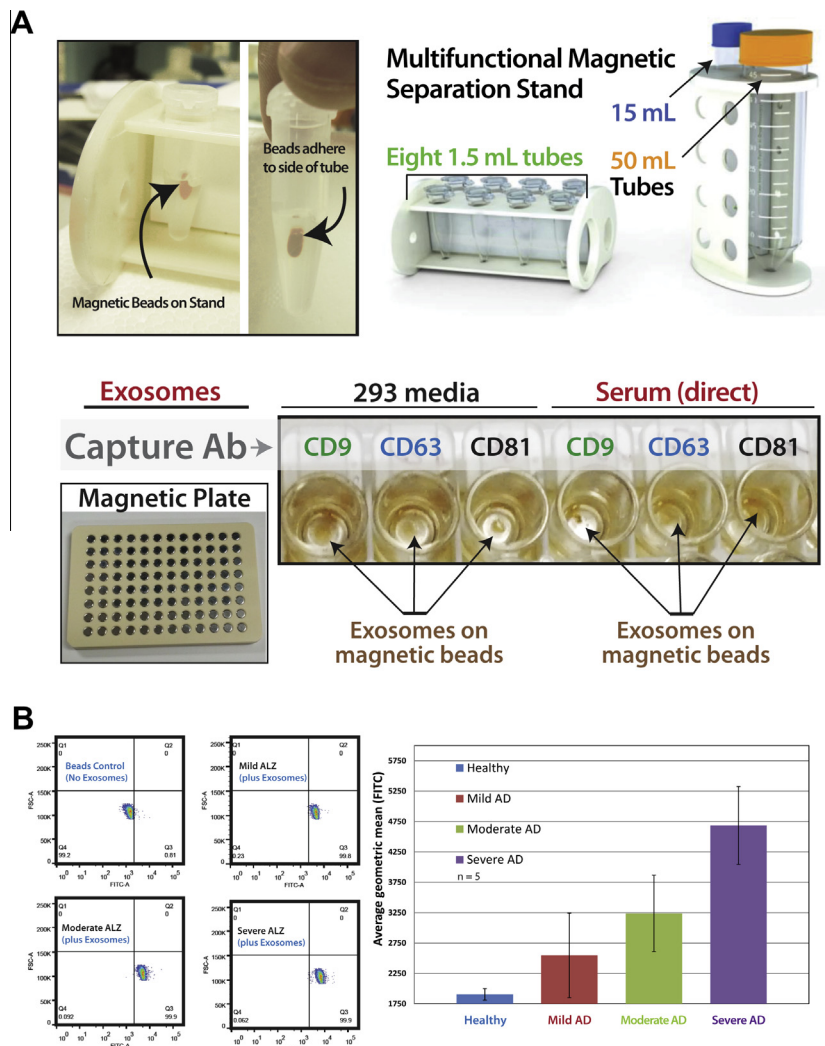


Fig. 4. Exo-Flow magnetic stand for exosome separation and FACS analysis. (A) The Exo-Flow multifunctional magnetic stand (catalog # EXOFLOW700A-1) pulls down the Exo-Flow beads using magnetic force, causing the beads to adhere to the wall of the test tube (left panel). The central and right panels demonstrate how the magnetic stand can be used for 1.5 mL, 15 mL and 50 mL tubes of samples for exosome immunopurification with the Exo-Flow beads. (B) A series of serum samples from patients with varying Alzheimer's disease state were utilized to first capture alpha-2-macroglobulin positive (A2M) exosomes from the patient sera on Exo-Flow magnetic beads and then stained with the universal exosome stain Exo-FITC fluorescent dye. The exosome/beads were then used for flow cytometry analysis using a BD FACS LSRII instrument. The resulting flow data on the left shows the levels of A2M-positive beads and displayed on the FACS plot as forward scatter (FSC-A) on the vertical axis plotted against the fluorescence levels (FITC). A trend in more A2M-positive exosomes can be viewed in the FACS plots when comparing healthy to mild, moderate and severe Alzheimer's disease exosomes. The data trend is also shown in bar graph form to the right of the FACS data.

Table 1
Benefits and drawbacks of various exosome isolation methods.

Method	Time required	Purity & considerations	Scalability
Ultracentrifugation	16–20 h	May pellet ribosomes, debris and rupture EMVs	Low
Ultrafiltration	4–6 h	Proteins may adhere to membrane	Low
Chromatography	6–12 h	Removes most impurities but requires UC at end of protocol	Low
ExoQuick polymer	30 min	Polymer may precipitate protein aggregates	High
Immuno-affinity	16–20 h	Selective capture yield EMVs with only that marker on surface	Medium

microscopy platforms continue to be successfully used to visualize exosomes as they mature within the cellular environment, are released from cells, and are taken up by target cells [26,27]; specific examples are shown in Fig. 5. Such applications are an appropriate use of electron microscopy and continue to advance our understanding of exosome biogenesis and cellular uptake, but the technically challenging and laborious protocol of generating high quality electron microscopy images has proven a challenge

for researchers interested in more basic questions, such as whether their isolated exosomes fall within the standard size range and are intact.

To provide a solution to researchers unequipped to validate exosome size and shape with electron microscopy approaches, particle tracking analyses have been developed that provide a rapid and straightforward protocol generating high quality data that answers basic questions regarding exosome preparations. The

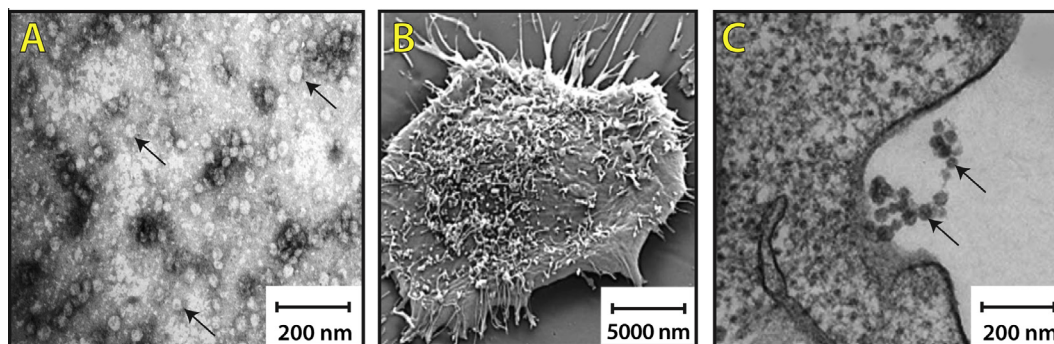


Fig. 5. Visualization of exosomes and exosome biogenesis using electron microscopy. (A) Exosome preparation from human monocyte cells grown in culture visualized using transmission electron microscopy. Exosomes were harvested using ExoQuick-TC[®] from cell media supplemented with Exo-FBS, then fixed in 2% PFA and deposited on Formvar-carbon coated EM grids. After several washes, the sample was stained with saturated uranyl-oxalate solution and embedded in methylcellulose-uranyl acetate solution. Hundreds of exosomes were visualized using an electron microscope FEI Technai 10 at 100,000 \times magnification. Individual exosomes are highlighted with arrows (Gupta and Pulliam, unpublished data). (B) Scanning EM image of exosomes secreted from glioblastoma cell, [27]. (C) Transmission EM image of exosomes released to extracellular space from CD34 positive stem cells [26]. Scale bars are indicated for each image.

two most commonly used particle tracking analysis platforms are NanoSight and Particle Metrix. Both entail resuspension of exosome samples in a particle free solution. A laser beam is passed through the exosome containing solution. The scatter properties of the laser beam as it passes through the exosome particles in suspension are tracked by Brownian motion and subsequently read by a detector, resulting in information as to the size and concentration, but not shape, of the particles in solution. Example data obtained from NanoSight and Particle Metrix is shown in Fig. 6. Both the NanoSight and Particle Metrix instruments and software can be easily utilized by research scientists following minimal training time, making them attractive additions to labs studying exosomes.

An important note is that exosome samples must be highly diluted prior to analysis on particle tracking analysis platforms. Exosomes are coated with many adhesive molecules which bind to each other [22], forming exosome clumps that will skew the size distribution read by the detector and analysis software. Standard serum exosome samples should be diluted in the range of 1:10,000 and standard cell culture supernatant exosome samples should be diluted in the range of 1:2000. The quantifiable range for accurate particle counting metrics is 10^5 – 10^8 particles, necessitating dilution of samples that fall above that range per unit volume input for the measuring apparatus, as most exosome sample preparations do.

3.2. Exosome validation

As with any subcellular organelle, it is important to determine unique markers specific to exosomes to aid in the validation of purified exosome samples. With the growth of the exosome field, such markers have been developed that are considered more or less universal to all exosomes. These markers include CD63, CD9, and CD81, all tetraspanins that are localized to the exosomal surface. Protein markers that are found on the interior of exosomes include Acetyl-CoA Acetylcholinesterase (AChE), Annexins, and TSG101. As with the tetraspanins described above, these proteins are considered to be commonly found within all exosomes but should be validated with individual samples. Examples of proteins commonly found within exosomes as well as their distribution (membrane bound vs. internal) are shown in Fig. 7 (adapted from Ref. [28]). High quality antibodies against these proteins can be used to validate exosome samples as well as utilized as a loading control on a Western blot using lysed exosome preparations.

An alternative approach to validate exosome samples to using Western blots is to utilize antibody arrays. In this technique, many

different antibodies against known exosomal proteins are spotted onto a membrane at defined locations using a dot blotter. A single exosome lysate can be incubated over this array, and after the appropriate washes and detection solution are applied, the abundance of the protein target for each antibody spotted on the array can be detected simultaneously. This approach is useful when validating precious exosome samples for which the quantity is limited, or to obtain a more global view of a variety of exosome markers without having to repeatedly run Western blots.

Aside from validation of the quality of purified exosome samples using known exosomal markers, it is important to determine that exosomes are bioactive after they have been isolated. One measurement of exosomes retaining appropriate biological functions is whether they bind to and deliver their cargo to target cells. One way to ascertain target cell delivery of exosome components is to fluorescently label internal proteins and RNAs, then visualize target cells with a fluorescent microscope after they have been incubated with exosome samples. This strategy is employed by SBI's Exo-Glow kit, in which internal RNAs are labeled red and internal proteins are labeled green. Dye incorporation into exosomal cargo can be visualized using standard fluorescent microscopy on exosomes captured on CD63-coated magnetic beads. Delivery of labeled cargo to target cells can also be monitored easily with fluorescence imaging of live cells. Examples are shown in Fig. 8.

3.3. Exosome quantification

After exosome isolation and validation, many downstream applications require knowledge as to the number of exosomes within a specific sample. To quantitate exosomes, many researchers opt for a standard ELISA approach. Within this method, serial dilutions of a purified protein or peptide known to be specific to exosomes is incubated with an HRP conjugated detection antibody. The HRP signal from each dilution is graphed relative to the protein or peptide input amount, generating a standard curve. Exosome preparations can be quantitated by incubating them with the same HRP conjugated detection antibody and assaying at what point their HRP signal falls relative to the standard curve. Various ELISA kits are available that utilize detection antibodies against the tetraspanins described above: CD63, CD9, and CD81.

SBI has recently developed a kit known as EXOCET that is an alternative to ELISAs to quantify exosomes. It takes advantage of the enzymatic activity of Acetyl-CoA Acetylcholinesterase (AChE) known to be found within all exosomes tested to date [28,29]. The EXOCET kit provides all components to measure AChE activity in colorimetric assay read at OD405. A standard curve that has

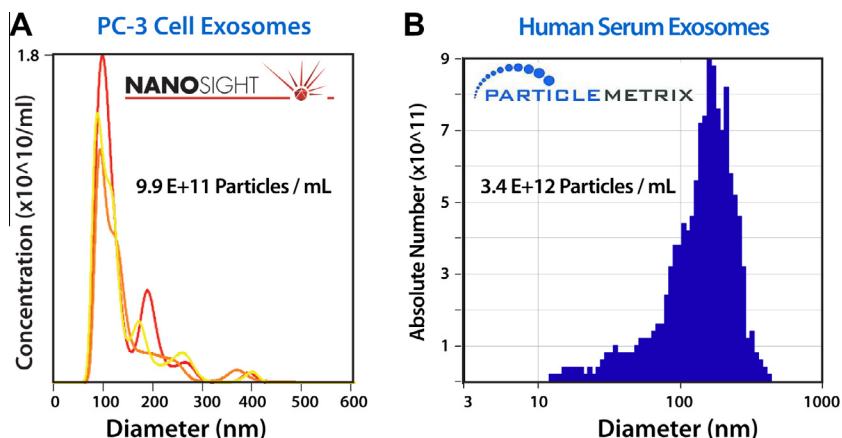


Fig. 6. Exosome detection by particle tracking analyses. (A) NanoSight[®] data obtained for exosomes isolated using ExoQuick-TC[®] (catalog # EXOTC10A-1) from human prostate cancer cell line PC3 grown in Exo-FBS media (catalog # EXO-FBS-50A-1). Exosomes were resuspended at a concentration of $1 \mu\text{g}/\mu\text{L}$ and diluted at 1:2000 before analysis using the NanoSight LM10 instrument. A sharp peak at 100 nm is observed, which falls within the size range expected of exosomes. An additional peak is observed around 200 nm; this additional peak often appears in exosomes isolated from cultured cells, especially cancer cells, and may reflect the presence of larger vesicles such as oncosomes (see Section 1). (B) Particle Metrix data obtained for exosomes from human serum are shown. Exosomes were resuspended at a concentration of $1 \mu\text{g}/\mu\text{L}$ and diluted at 1:10,000 before analysis using the Particle Metrix ZetaView instrument.

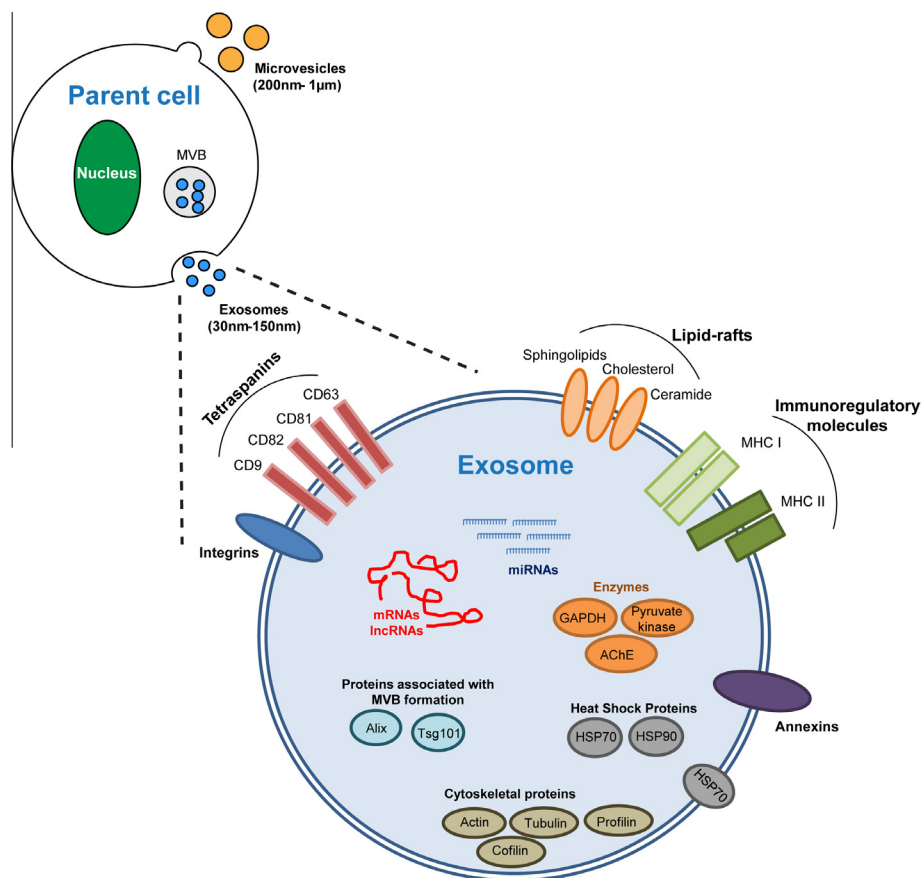


Fig. 7. Proteins commonly found within exosomes. The proteins noted inside the exosome are typically found as internal exosome cargo, whereas those noted as spanning the membrane are presented on the exosomal surface. This graphic is adapted from Gupta and Pulliam [30].

been calibrated to exosome numbers as measured by NanoSight analysis is included in the kit, enabling the user to more accurately measure the absolute number of exosomes in each sample rather than obtaining a relative value as compared to a standard curve generated with serial dilutions of a purified protein. Importantly, this method of exosome quantification is a survey of the entire population and does not take into account some vesicles that

may carry more AChE than others. Using this approach, exosome quantification using EXOCET can be carried out within 20 min, compared to the overnight incubation of exosome sample with a detection antibody required by an ELISA. Additionally, concerns into antibody quality, masking of epitopes due to post-translational modifications on target proteins, and off target binding are not an issue when using AChE activity to quantify

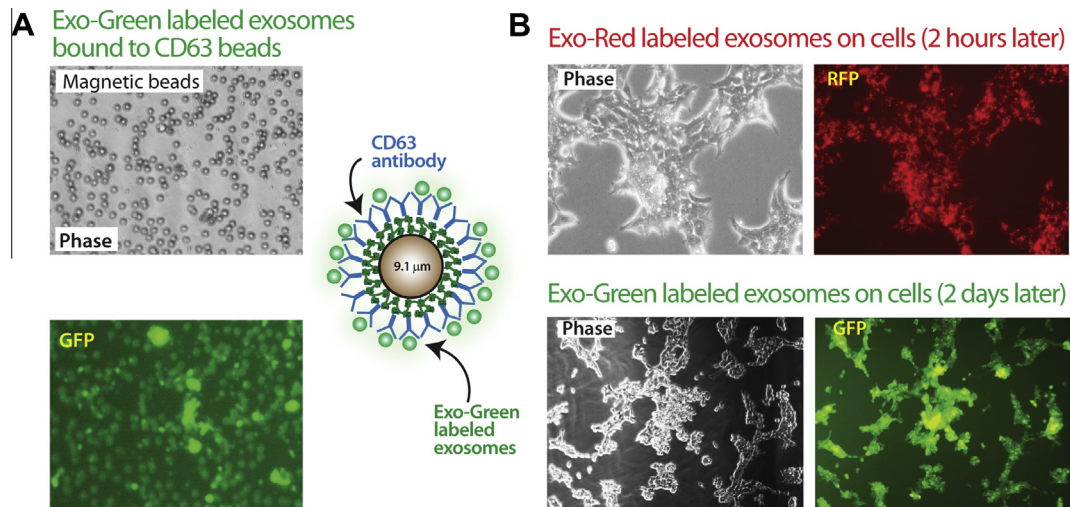


Fig. 8. Analysis of exosomal RNA and protein cargo delivery to target cells. (A) Exosomes from HEK-293 cells were labeled with Exo-Green (catalog # EXOG200A-1, to label internal proteins). Exosomes were then immobilized on CD63 coupled magnetic beads (catalog # EXOFLOW300A-1). A schematic of the magnetic CD63-coupled beads binding labeled exosomes is also shown. After 3 washes in PBS, the beads were visualized on bright field and on a fluorescence microscope with a standard GFP filter set to visualize labeled exosome proteins. (B) Human exosomes labeled with either Exo-Red for internal RNAs (catalog # EXOR100A-1), or Exo-Green dyes (internal exosome proteins) were added to target HEK-293 cells in culture. Target cells were imaged using fluorescence microscopy. RNA delivery was observed as early as two hours shown in red and protein delivery was observed 48 h after addition of labeled exosomes to target cells depicted in green.

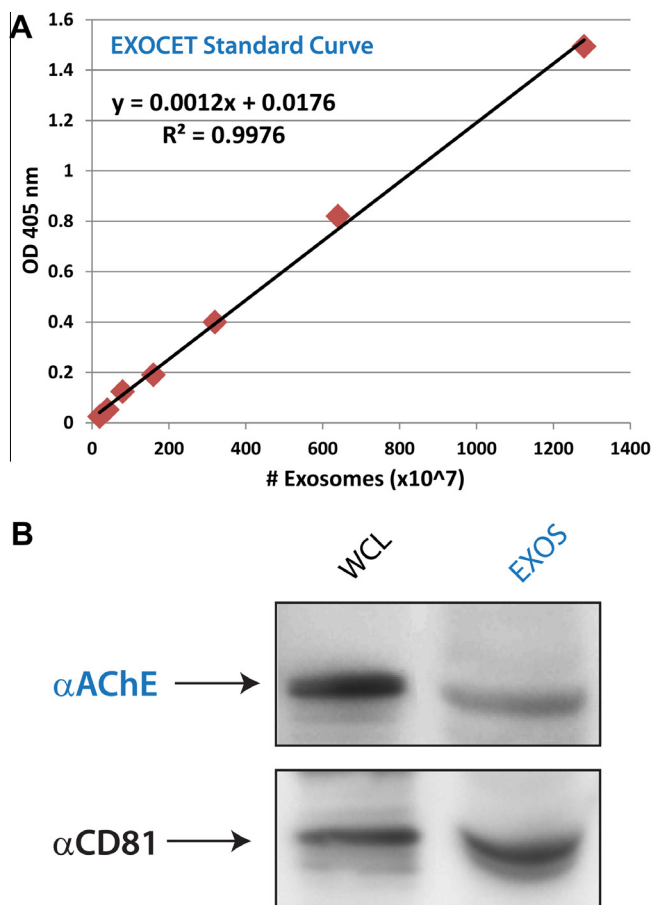


Fig. 9. EXOCET quantification of exosomes from human serum as well as from a culture of human breast cancer cells MDA-MB-231. (A) The standard curve in the EXOCET kit (catalog # EXOCET96A-1) is generated by samples calibrated to known exosome numbers as determined by NanoSight analysis, allowing for a quantification of absolute exosome numbers within a wide linear range. (B) Western blots using an anti-AChE antibody (catalog # EXOAB-ACHE-1) show that AChE is enriched in exosomes (EXOS) and is also present in whole cell lysates (WCL).

exosome samples. An example of data obtained using the EXOCET kit is shown in Fig. 9.

4. Exosome RNA analysis tools

4.1. Exosomal RNA Purification

Exosomes are known to naturally contain RNA transcripts, and the specific RNAs found within exosomes varies based on the secreting cell type. In recent years, much interest has been placed on the presence of miRNAs in exosomes. In one study, viral non-coding regulatory miRNAs were shown to be present and stable within exosomes, indicating that viruses can take advantage of exosomes to deliver their products to and infect naïve cells [30,31]. Growing evidence that the RNA content of these microvesicles plays a role in intercellular communication and the potential for the use of exosomal RNA in diagnostic applications has required the development of tools to analyze RNA content of exosomes and to compare the RNA cargo between different exosome populations. Methods for RNA extraction, quantification, and downstream analyses are described below.

4.2. Exosomal RNA extraction and quantification

Upon isolating exosomes from a given serum or cell media sample using any of the methods described above, RNA can be isolated by use of a lysis reagent to liberate the exosomal RNAs. Column purification kits, such as SBI's SeraMir kit can then be used to purify the RNA from other exosomal contents. For serum or plasma samples collected in heparin tubes, it is recommended to pre-treat the isolated RNA with heparinase (NEB, catalog # P0735S). Heparin contaminants in purified RNA will interfere with downstream analysis by sequestering ions required by certain enzymes for reverse transcription, ligation, etc. After extraction, a Nanodrop spectrophotometer can initially be used to determine the concentration and quality of the RNA preparation. However, due to the low concentration and small sizes of RNAs expected to be isolated from exosome samples compared to total RNA derived from whole cells, a spectrophotometer might not be sufficient to accurately measure the true concentrations and quality of exosome

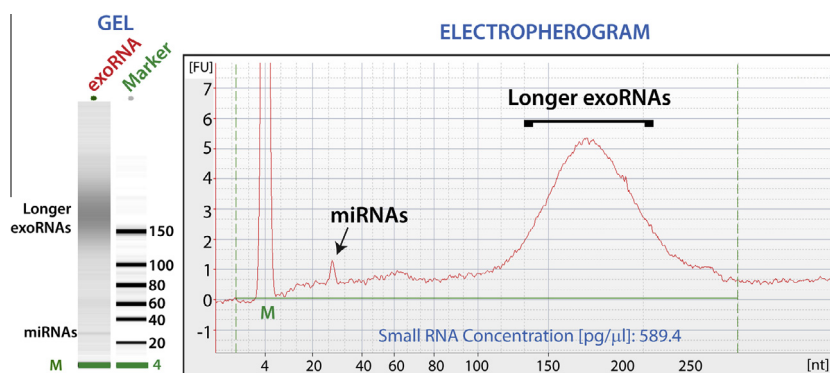


Fig. 10. Agilent Bioanalyzer RNA analysis data. The Agilent Bioanalyzer[®] 2100 system can detect as little as 50 pg/μL RNA sample input to characterize RNA quality and quantify total yield. RNA samples are passed through a specialized, high sensitivity RNA chip (Agilent RNA 6000 Pico Kit) and the Agilent Bioanalyzer software determines the concentration and size range of the isolated RNA fragments. The report generates a gel image of the RNA sizes (on left) and an electropherogram trace graph with the sizes and concentrations of RNA present (on the right). The example shown here is from RNA isolated from human serum exosomes. RNA sizes are indicated in nucleotides (nt).

RNAs. In contrast, the Agilent Bioanalyzer is a highly sensitive instrument that can characterize RNA quality from a minimal amount of sample input. In this system, RNA samples are passed through a chip which is read by the Agilent software to determine the quality, concentration, and size range of an RNA sample. Exosome RNA profiles reveal that the most abundant species are small RNAs, most less than 225 nucleotides (Fig. 10). After RNAs are successfully isolated from exosome samples and passed through a quality control assessment, the exoRNAs can be amplified using a cDNA synthesis kit. Subsequently, a microarray or qPCR analysis can be utilized to compare relative expression of various RNAs of interest between different exosome populations. An example of a microarray experiment is shown in Fig. 11: exosomes were isolated from the serum of colon cancer patients and healthy controls using ExoQuick, and exoRNA was extracted and cDNA was synthesized using SBI's SeraMir Kit (see SBI catalog # RA800A-1). The cDNAs were directly labeled with Cy5 dye and then hybridized in triplicate to an LC Sciences mirBase ver. 16 array chip containing about 1200 different miRNA probes. Of the 1,214 microRNAs analyzed, 79 microRNAs showed a signal intensity >32. Within this set of 79, there was a clear colon versus normal "signature set" of 40 microRNAs that could discriminate normal from colon cancer serum samples with a p -value < 0.01 (Fig. 11).

4.3. Next generation sequencing of exosomal RNA

Microarrays and qPCR are the most common technologies used to analyze known exosomal RNA species. However, there has been a growing interest within the research community to characterizing the whole transcriptome contained within exosomes, with the aim of identifying sequences in an unbiased way rather than testing for the presence of a known target. To this end, next generation sequencing has emerged as a powerful tool to help discover novel exosomal RNA species.

4.4. Preparation of ExoRNA for NGS sample libraries

SBI provides a kit containing all components necessary for preparation of exoRNA libraries compatible with sequencing on Illumina NGS platform. This kit, designed with a partnership with SeqMatic, LLC is termed XRNA and is optimized to use as little as 10 ng of purified exoRNA input. RNA is extracted and analyzed on an Agilent Bioanalyzer as described above, and samples are then input to a library preparation reaction. Within this protocol, each purified exoRNA sample undergoes adapter ligation, cDNA synthesis, and PCR amplification (Fig. 12). At the PCR amplification step, each sample is assigned a specific index primer which allows it

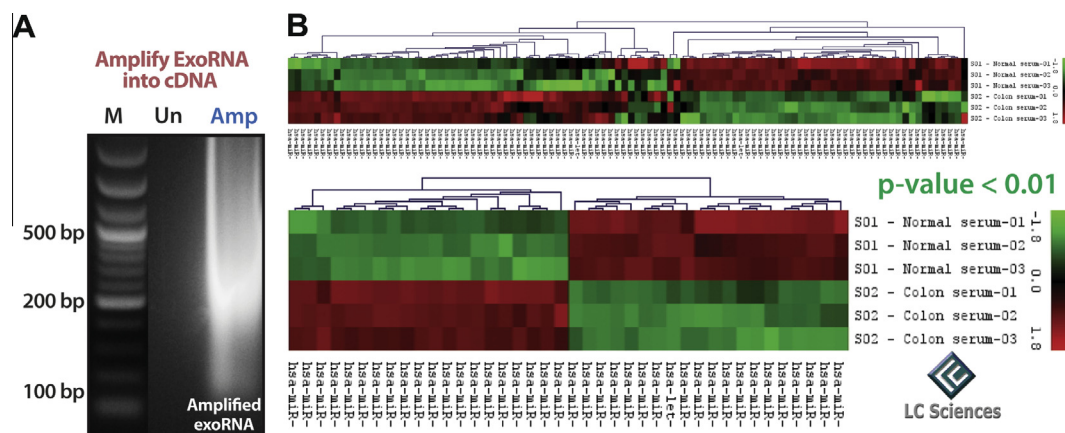


Fig. 11. Microarray analysis of exoRNA samples. Exosome RNA was purified from human normal control vs. colon cancer patient sera. (A) The exoRNA was converted to cDNA and amplified using the SeraMir kit (catalog # RA800A-1). The amplified libraries were visualized on a 2% agarose-TAE ethidium bromide gel. A sample profile is shown with sizes labeled in base pairs (bp). The amplified cDNAs were then fluorescently labeled and hybridized to an LC Sciences mirBase ver. 16 microarray chip containing about 1200 different miRNA probes as a custom service. (B) The miRNAs identified to be more abundant in the colon cancer exosomes are depicted in red color on the heatmaps and the less abundant miRNAs are shown in green. Unsorted data are shown on top heatmap and the lower array heatmap image shows the sorted miRNAs identified as significantly differentially expressed between the two sample sets. This experiment elucidated 79 microRNAs that are capable of stratifying normal serum exosome miRNA from colon cancer serum exosome miRNA samples.

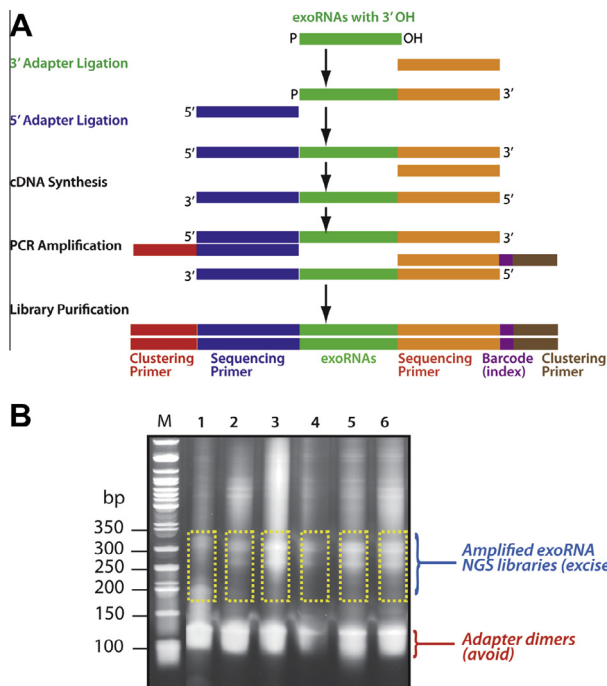


Fig. 12. ExoRNA library preparation protocol. (A) Schematic of the basic workflow of exoRNA next-generation sequencing sample library preparation. Libraries are prepared by 3' and 5' adaptor ligation reactions, cDNA synthesis, primer indexing, and amplification. (B) The amplified, indexed libraries are resolved on a 6% TBE polyacrylamide gel (Bio-Rad) to separate the products based on size and away from any adapter dimers that may have formed during the amplification and the library profiles are visualized using SYBR Gold staining. The desired library band sizes with mobilities between 160 bp and 400 bp are excised and purified for sequencing. The gel shown depicts the NGS library patterns and profiles for 6 different exosome RNA library samples (Lanes 1–6).

to be multiplexed for parallel sequencing in a flow cell along with other samples indexed with different sequences (bar codes). The amplified indexed libraries are then resolved in a polyacrylamide gel from which the desired bands are excised in a streamlined gel purification method (Fig. 12). The recovered amplified libraries are then ready for analysis on the Illumina sequencing platforms: HiSeq, MiSeq, and Genome Analyzer II. After sequencing, reads can be de-multiplexed based upon their unique index sequence and then assigned to their appropriate input samples identities.

4.5. NGS data analysis

The analysis approach for exosome RNA-Seq is different than RNA-Seq using total cellular RNA as input. Within cellular RNA there is a rich complexity of RNA species such as miRNAs, piRNAs, tRNAs, mRNAs, lncRNAs, rRNAs, and other ncRNAs. This huge range of RNA species requires about 300M reads per sample in order to generate substantial sequencing depth. On the other hand, exoRNA diversity only comprises about 1% of cellular RNA and the expected length distribution of exoRNAs (approximately 250 nucleotides or smaller) is significantly less than that of cellular RNAs.

To build an analytical platform to specifically analyze exoRNA NGS data, SBI has partnered with Maverix Biomics (San Mateo, CA). Within the Maverix exoRNA analysis pipeline, sequences are mapped to a reference genome of choice to determine sequence identities and relative abundances of various RNA types such as ncRNAs, antisense transcripts, and miRNAs. These results are summarized in visual form as color-coded pie charts. Examples of such pie charts obtained from analysis of RNA from four different exosome sources are shown in Fig. 13. Differential expression of specific sequences between samples (such as cancer-derived exosomes vs. control exosomes) is displayed in various formats including volcano plots and an interactive heatmap unique to the Maverix

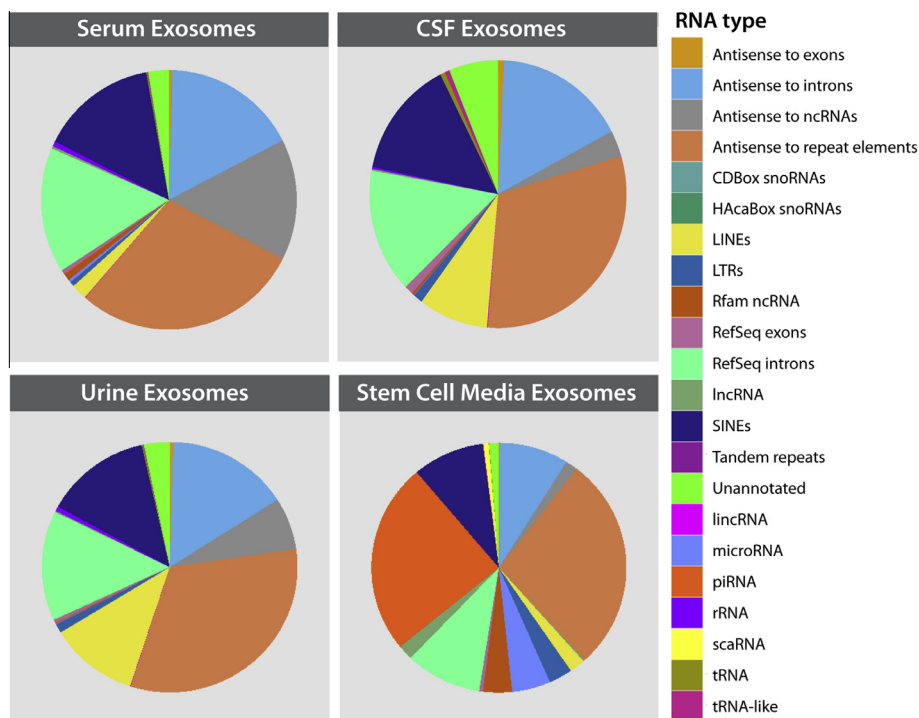


Fig. 13. Color-coded pie charts representing the relative abundances and types of specific RNA classes present in exosome RNA. Raw NGS sequence read data are first trimmed for quality and then the sequences are mapped to a reference genome (human or mouse) for identification. The Maverix analytics platform then reports relative abundance levels of the various ncRNAs, antisense transcripts, miRNAs, and other RNA classes within the exosome RNA sequencing data. The data are summarized visually in color-coded pie charts. Shown here are four examples of exosome RNA NGS sequencing results from human serum, cerebrospinal fluid (CSF), urine and mesenchymal stem cell exosomes from culture media.

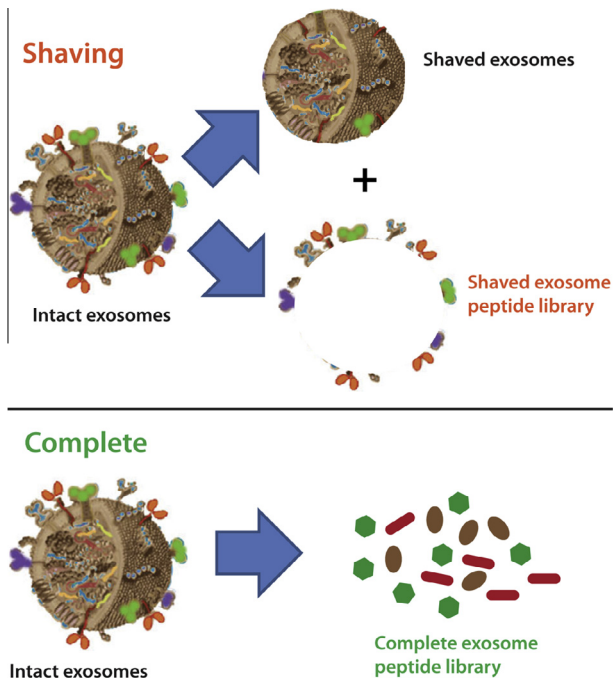


Fig. 14. Approaches to investigate total or surface exosome protein content. Two different methods to study exosome proteomics are depicted. The first, termed “shaving”, is a method to maintain the exosomes intact while gently cleaving away just the surface proteins to create mass spec peptide libraries. The alternate approach utilizes an exosome lysis method to solubilize total protein content prior to trypsin digestion to generate a “complete” exosome peptide library for mass spec analysis. Both of these methods are available in the XPEP mass spec preparation kit (catalog # XPEP100A-1).

platform. All data links to the UCSC Genome Browser, enabling an initial investigation into RNAs of interest using information found within that database. Contact Maverix Biomix for a test drive of these analysis tools.

5. Exosome proteomic analysis tools

In addition to RNA cargo, exosomes also contain specific subsets of proteins that vary based on the cell type from which they are secreted. The importance of these proteins are highlighted by recent research indicating that exosomes act as a communication tool between cells via ligands, delivering receptors and other functional proteins [32]. Using proteomic techniques such as mass-spectrometry on exosomes derived from various body fluids and cell media, several proteins have been identified that play a vital role in biogenesis, cargo sorting and other molecular mechanisms important to exosome functions [33]. Mass-spectrometry proteomic analysis performed on exosomes secreted by cell-specific and disease specific mammalian body fluids or cell media may contribute to biomarker discovery and lead to the development of various diagnostic tools [34]. Here we describe two ways in which proteomic analysis can be performed on exosomes to determine their protein content: “shaving” of exosomes to remove only proteins found on the exosomal surface followed by library preparation and mass spectrometry analysis, and direct lysis to generate peptide libraries comprised of total exosome protein. The shaving method allows for the discovery of exosome surface proteins, which can be a rich source of biomarkers as well as utilized to identify surface markers unique to a particular sample set for downstream antibody-bead purification and FACS experiments. Fig. 14 shows the two different approaches for peptide library preparation from shaving and total lysis methods.

To shave exosomal proteins exposed on the surface, the exosomes are first treated with reducing agents and other enzymes to remove post-translational modifications, then the samples are prepared using the filter-aided sample preparation (FASP) method to remove all the enzymes and buffer exchange components used in this step. Then, the exosomes are incubated with trypsin for 4 h in 37 °C [35]. The XPEP kit developed by SBI includes components necessary for exosome shaving, and has been extensively validated to ensure the removal of only surface bound exosome

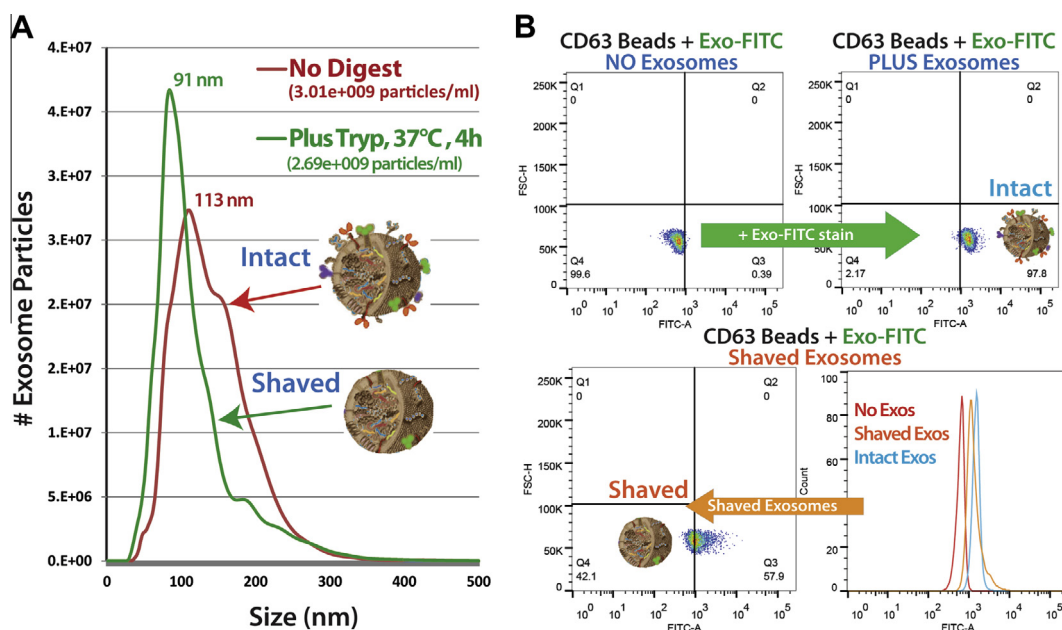


Fig. 15. Exosome shaving removes surface bound proteins. (A) NanoSight® exosome sizing data from both shaved exosomes and intact exosomes show that the shaved exosomes display an average diameter size of 91 nm while the intact exosomes display an average diameter size of 113 nm. These results demonstrate that the shaved exosomes remain intact, but lose their surface protein “coats”. (B) Flow cytometric analysis of intact exosomes captured on CD63 magnetic beads labeled with Exo-FITC stain (catalog # EXOFLOW800A-1) are shown comparing control versus shaved exosome preparations. The observed backward shift in the shaved exosome FACS profile demonstrates that they have lost most of their surface CD63 proteins, rendering them unable to be captured by the CD63 antibody-coupled magnetic beads.

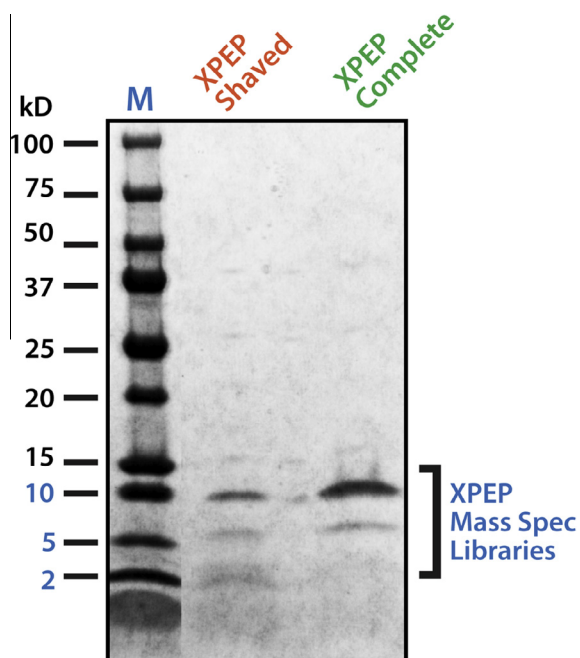


Fig. 16. Size distribution of peptide libraries generated by the XPEP. The size distribution of peptide libraries prepared with SBI's XPEP kit (catalog # XPEP100A-1) protocols by SDS PAGE separation on a gradient 4–20% polyacrylamide gel and Imperial Blue staining is shown. The peptide library fragments generated are in the 2–10 kD fragment range, which is ideal for mass spectrometry analysis.

proteins. Fig. 15 demonstrates a NanoSight comparison between the shaved exosomes and intact exosomes. The shaved exosomes remain intact, showing that the shaving protocol does not result in loss of exosomal structural integrity and that purified surface proteins are not contaminated by internal proteins due to lysis of whole exosomes. Whereas the intact exosomes demonstrate a size

of 113 nm, the shaved exosomes demonstrate a size of 91 nm. This observed condensation of exosome diameter into a more homogeneous population indicates that shaved exosomes experienced a loss of their surface protein “coats.” Fig. 15 shows a flow cytometric analysis of intact exosomes compared to shaved exosomes purified using a CD63 capture antibody coupled to a magnetic bead (SBI's Exo-Flow technology); the observed slight backward shift in signal using shaved exosomes further indicates that surface proteins were removed using the shave protocol.

The complementary method to analyze exosomal protein content is to prepare peptide libraries by complete lysis of the exosome, allowing for exploration of potential biomarkers in the exosomal interior as well as on the surface. The XPEP kit contains a protocol for sample preparation of this type as well, resulting in peptides of appropriate size for mass spectrometry analysis. Separation of peptide libraries generated by the shave or complete lysis protocols on a 4–20% gradient SDS PAGE gel and stained with Imperial Blue (Thermo Fisher) as shown in Fig. 16 demonstrates that this kit yields peptides of the appropriate size for downstream mass spectrometry analysis. Of note, the shaved peptide libraries are typically 5-fold less than the libraries made using the XPEP-Complete protocol, likely reflecting a lower abundance of surface proteins when compared to total exosome protein content. The protein composition of exosomes can vary greatly depending upon the cell source from which it originated. Some typical surface and internal proteins are observed in all exosomes and are often identified in mass spectrometry data. These common proteins include the tetraspanins, MHCs, Rabs, heat shock proteins, GAPDH, keratins, tubulin, actin, vimentin, fibulin, fibronectin, annexins, flotillins, galectin and alpha-enolase [36]. A diagram of proteins routinely found in exosomes is shown in Fig. 7. The standard instrument for mass spectrometric analysis of exosomal proteins is a nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a Thermo Fisher Q Exactive. Peptide identities are determined by spectral analysis and mapping to Mascot databases which are then parsed into the Scaffold proteome software. Another commonly used open source mass spectrometry analysis

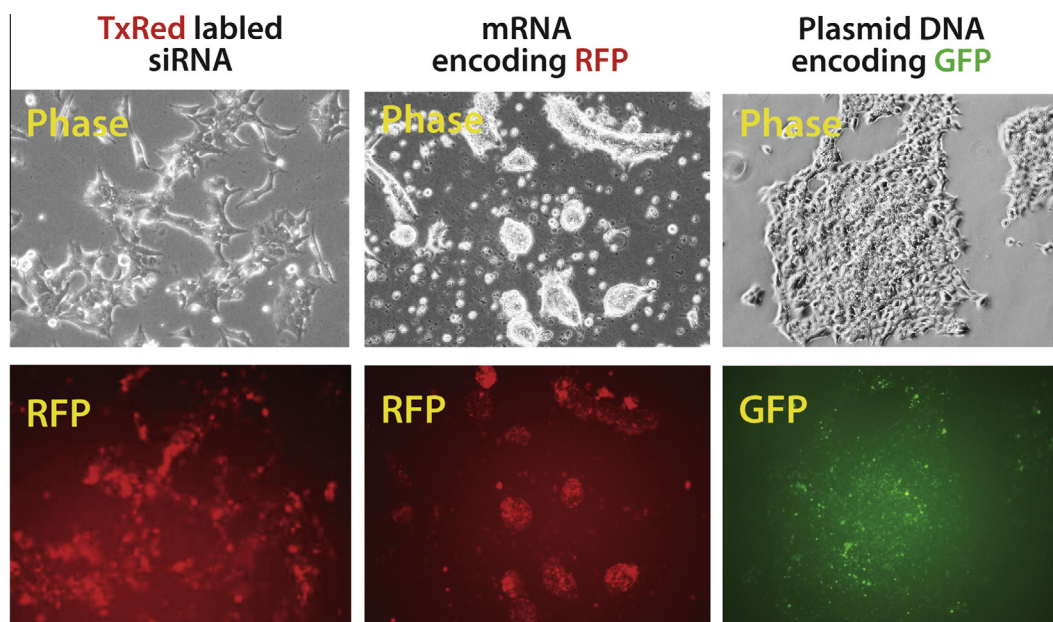


Fig. 17. Exosome mediated delivery of Exo-Fect nucleic acid cargo to target cells. Exosome transfection of a Texas Red (TxRed) labeled siRNA, an unlabeled mRNA encoding RFP, and a DNA plasmid encoding GFP were accomplished using Exo-Fect (catalog # EXFT10A-1). These exosomes were then re-isolated using ExoQuick® to remove unincorporated nucleic acids. These exo-fected exosomes were then added to naïve target HEK293 cells to test for exo-fected cargo delivery. Cells were imaged using fluorescence microscopy. The TxRed labeled siRNA delivery was observed as rapidly as two hours, mRNA expression was observed after 24 h and plasmid DNA expression was observed after 48 h of exosome addition.

Table 2
SBI's pre-made exosomes from various cell lines and biofluids.

<i>Cancer exosomes</i>	<i>Neural exosomes</i>
Breast cancer	Schwann
Lung cancer	Astrocyte
Prostate cancer	Oligodendrocytes
Brain cancer	<i>Stem cell exosomes</i>
Colon cancer	Bone marrow MSC
<i>Immune exosomes</i>	Pre-adipocyte MSC
B-cell (mature)	<i>Other exosomes</i>
B-cell (immature)	HEK293
T-cell human	Human serum
Dendritic cell (mouse)	

software called ProteomeXchange is also available. It is important to note that the method of exosome isolation will influence the peptides identified in any MS analysis. Some methods to obtain more homogenous exosome preparations can be accomplished using Exo-Flow immunoaffinity-based separations.

6. Exosomes as biomarkers

Given that exosomes are packed with cellular information reflective of the biological state of the parent cell, their potential to serve as biomarkers for the diagnosis and prognosis of certain pathological conditions has only begun to be explored. Recent evidence indicates that various body fluids such as plasma [37,38], saliva [39,40], cerebrospinal fluid (CSF) [41], and urine [42,43] provide a rich source of cell-derived exosomes that could be used to determine the onset or progress of various pathological phenomena such as cancer, neurodegeneration, kidney disease and liver injury. The cellular cargo packed into exosomes including proteins, miRNAs, and mRNAs has been shown to provide distinct information about the host cells the vesicles are shed from. For instance, proteomic profiling of exosomes isolated from prostate cancer cell lines as well as clinical samples revealed the enrichment of specific cancer-associated proteins such as XPO1 [44], a nuclear protein known to modulate the nuclear-cytoplasmic export of cellular proteins involved in tumor signaling pathways, and FOLH1, a folate hydrolase the expression of which is highly upregulated in prostate cancer cells [45]. In addition to their diagnostic value, there is also a suggested use of exosomes for monitoring response to treatment by determining the levels of exosomal prostate cancer markers such as prostate serum antigen (PSA) [46]. While extensive clinical studies are required to validate these exosomal proteins as prostate cancer biomarkers, the potential of exosomes to serve as a non-invasive alternative to the current and sometimes unreliable diagnostic tests warrants further investigation.

Nucleic acids such as miRNAs packaged into these nanovesicles have also been demonstrated to serve as meaningful biomarkers for certain pathologies. MicroRNA-122 is a highly liver-specific miRNA that constitutes approximately 70% of the total miRNA content of a hepatocyte [47]. During inflammation and alcoholic liver disease, a significant increase in plasma levels of exosomal miR-122 is observed that correlates to serum aminotransferase levels [48], underscoring its potential as a biomarker for liver injury. Another study published by Bellingham and colleagues demonstrated that prion-infected neurons released exosomes that harbor a distinct miRNA signature compared to uninfected neuronal exosomes [49]. Interestingly, some of the miRNAs that were elevated in prion-infected neuronal exosomes such as miRs- 128a and 342-3p were previously reported to be increased the brain tissue of prion-infected primates and mouse models [50,51]. While the majority of such studies have focused on exosomes harvested from urine and plasma/serum, miRNAs found in salivary exosomes are also being explored for biomarker discovery for conditions that

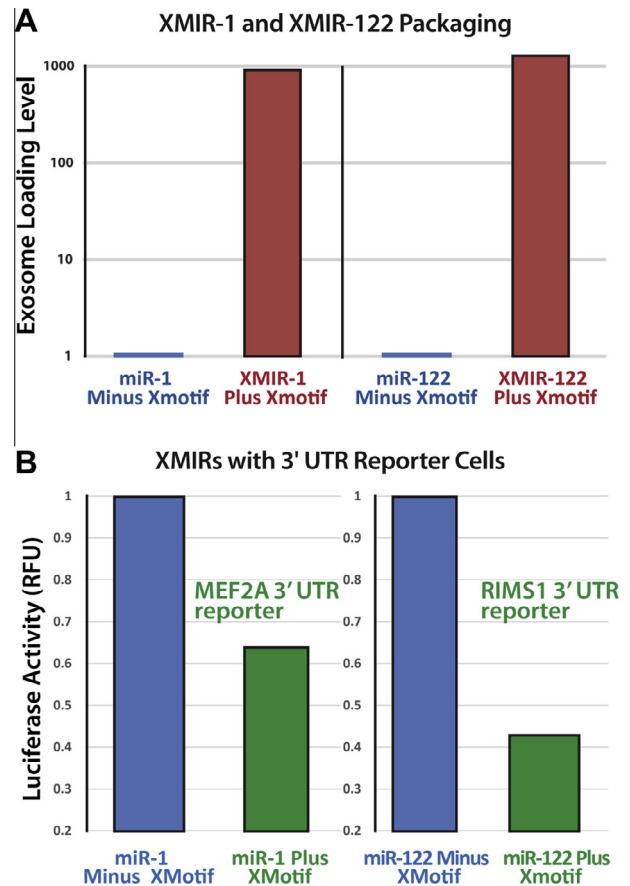


Fig. 18. XMIRs are efficiently loaded as exosomal cargo and are bioactive in target cells. (A) qPCR quantification of XMIR loading into exosomes from HEK-293 cells transfected with miR-122 and miR-1 RNA oligos plus XMotif compared to standard miR-122 and miR-1 RNA oligos without the XMotif sequence are shown as bar graphs. (B) Exosomes from HEK-293 cells transfected with miR-122 and miR-1 oligos plus XMotif and minus the XMotif were added to target HEK-293 cells harboring a luciferase reporter plasmid linked to 3' UTRs known to be targeted by each of these miRNAs (miR-1 targets MEF2A [63] and miR-122 targets RIMS1 [this report]). Regulation of the luciferase 3' UTR reporter by exosome delivery of each miRNA was quantified using a standard luciferase assay.

directly or indirectly affect the salivary gland, such as Sjogrens syndrome [40].

In addition to miRNAs, an increasing line of evidence suggests that mRNA transcripts found in exosomes can be used to identify pathological processes as well. Podocytes, the epithelial cells in the Bowman's capsule in the kidney secrete exosomes that can be isolated from urine. Interestingly, during kidney disease, the exosomal mRNA expression of CD2AP, a gene essential for podocyte function is significantly downregulated and correlates to the severity of renal disease [52]. Such results raise the possibility of using urinary exosomal mRNA expression to monitor renal function and diagnose kidney injury. The recent development of new tools and kits to isolate exosomes from various body fluids will help to advance our current knowledge on exosomes and truly exploit the potential of these fascinating nanovesicles as biomarkers.

7. Nanobiomedicine

7.1. Current examples of exosomes as therapeutic nanoshuttles

Exosomes are beginning to garner much attention for their potential use in the therapeutic arena. They have been shown to

Table 3
SBI products and technologies for exosome research.

Product name	Catalog prefix number	Application
<i>Exosome isolation</i>		
ExoQuick	EXOQ-XXX	Isolation of exosomes from serum, plasma, and ascites fluid
ExoQuick-TC	EXOTC-XXX	Isolation of exosomes from tissue culture media, urine, and cerebrospinal fluid
Exo-FBS	EXO-FBS-XXX	Media supplement with contaminating bovine exosomes removed
Exo-Flow	EXOFLOW-XXX	Immunopurification of specific exosome populations and flow cytometric analysis
<i>Exosome characterization</i>		
ExoAbs	EXOAB-XXX	Markers for Western blot analysis
Exo-Check	EXORAY-X	Profile 8 known exosomal markers simultaneously
Exo-GLOW	EXOR-XXX/EXOG-XXX/ EXOC-XXX	Fluorescently track target cell delivery of exosome protein and/or RNA cargo
<i>Exosome quantification</i>		
ExoELISA	EXOEL-XXX	Quantification of exosomes using cell surface markers
EXOCET	EXOCET-XXX	Quantification of exosomes using CETP enzymatic activity
<i>Biomarker development</i>		
XPEP	XPEP-XXX	Generation of peptide markers from whole exosome lysates or exosomal surface for downstream Mass Spec analysis
XRNA	XRNA-XXX	Generation of RNA libraries from whole exosomes for downstream sequencing on an Illumina platform
SeraMir	RA-XXX	Amplify exosome RNA for downstream qPCR analysis
<i>Exosome engineering</i>		
Exo-Fect	EXFT-XXX	Addition of nucleic acids or chemicals to isolated exosome samples
XMIR/AXMIR Oligos	XMIR-XXX AXMIR-XXX	Transfection ready RNA oligos packaged within producer cells as exosome cargo
XMIR XPress	XMIRXP-XXX	Lentivector cloning plasmid driving expression of small RNA to be packaged within producer cells as exosome cargo
Purified human and mouse cell exosomes	EXOP-XXX	Use as standards in exosome measurements, study of cargo for development of diagnostics, use with ExoFect kit for insertion of nucleic acids or chemicals
Product name	Catalog number	Application
<i>Services</i>		
Mass Spec	CS800A-1	Peptide isolation from whole exosome samples or exosome surfaces followed by mass spec analysis
NGS	CS400A-1	RNA extraction from whole exosome samples followed by Illumina based NGS and data analytics
NanoSight	CSNANO100A-1	NanoSight analysis of exosome samples

naturally promote regeneration of injured heart and neuronal tissues: exosomes from cardiosphere derived cells have been demonstrated to promote a higher degree of tissue repair in mice after a myocardial infarction [24] and incubation of injured neurons with exosomes from Schwann cells results in a more rapid rate of neural repair [53].

Aside from their natural functions in promoting tissue regeneration, much excitement has been generated in the general field of engineering exosomes for use as therapeutic agents. Some of the first studies in which exosomes were manipulated for such purposes used modified exosomes to prime the immune system, inducing it to attack specific types of target cells. In this approach, specific antigens are presented on the exosomal surface by fusing their genetic sequences to the domain of a protein known to be inserted in exosomal membranes, MFG-E8 [54]. Exosomes are subsequently injected into an individual's bloodstream, where they come into contact with immune cells, inducing them to mount an immune response against any cell presenting the same antigen displayed on the injected exosomes [55]. This technique can be utilized to specifically target diseased cells for elimination, such as cancer cells. This approach has been shown to be effective at slowing the growth rate of fibrosarcomas in the mouse model [54] and successful clinical trials were accomplished in humans using patient specific dendritic cell derived exosomes, or "dexosomes" to mount an immune response against melanoma cells and non-small cell lung carcinoma cells [56]. Any disease cell can theoretically be targeted for elimination by the immune system using this approach, with the caveat that they display a specific surface antigen not found on the surface of healthy cells [57].

Display of specific antigens on exosome surface has also been used to target delivery of exosome cargo to specific cell types.

This has most notably been achieved by fusing a rabies viral coat protein (RVG) to Lamp2b, a protein known to localize to exosome membranes. As RVG binds specifically to a protein found within the membrane of neural cells, the investigators were able to deliver a siRNA targeting GAPDH encapsulated within the exosome specifically to brain tissue of mice [58]. A similar strategy was used to target drug delivery to alpha-V integrin positive breast cancer cells: an alpha-V binding peptide was fused to Lamp2b, and the isolated recombinant exosomes were electroporated with the chemotherapeutic drug doxorubicin. Within this study, it was shown that this strategy resulted in drug delivery specifically to alpha-V integrin expressing cells *in vitro* and to tumors *in vivo* [59]. Together, these results show that cell surface fusions of specific antigens can mediate immune system priming against specific cell types and cellular targeting for delivery of exosome cargo, both exciting avenues for the use of exosomes as therapeutic modifiers.

7.2. Looking ahead: tools to realize therapeutic applications of exosomes

Within the above mentioned studies, the siRNAs and chemicals were added to exosomes presenting the designated Lamp-2b fusions by electroporation. A more recently developed technology at SBI termed Exo-Fect uses a chemical transfection reagent to insert nucleic acids into exosomes. This method is gentler than electroporation on exosomes in suspension, resulting in less exosome loss due to lysis or mechanical stress. The "ExoFected" exosomes subsequently deliver cargo to target cells. Exo-Fect has been shown to deliver siRNAs, mRNAs, and plasmid DNAs efficiently to recipient cells as well as certain chemicals, such as cumate, to induce vectors containing the cumate switch inducible

expression technology. Exo-Fect is an attractive technology for delivering nucleic acids for hard to transfect cell types and for studying pathways by which exosomes deliver cargo to target cells. Additionally, delivery of chemicals to recipient cells using Exo-Fect has been shown to be more efficient and less toxic than addition of the same chemicals to cell culture media. Exo-Fect has recently been featured as an article in the journal *The Scientist* as an emerging tool in the growing field of exosome engineering [60]. Examples of nucleic acids as well as chemical delivery to cells using Exo-Fect are shown in Fig. 17. Purified exosomes from a variety of human and mouse cell lines are available through SBI for use with the Exo-Fect kit. These purified exosomes have been validated to be CD63 positive, tested for intactness and size distribution using NanoSight, and shown to be bioactive. Table 2 provides a list of cell lines from which purified exosomes are currently available.

Further exosome engineering tools are now being developed to allow for cell mediated packaging of small RNAs, such as siRNAs and miRNAs. SBI recently released a technology termed XMIRs/AXMIRs to induce cell mediated package of these classes of RNAs into exosomes. Researchers can transfect small RNA oligos that include an “XMotif” sequence into a producer cell line, then isolate exosomes containing high levels of their small RNA of choice as cargo. Using this system, both miRNAs (XMIRs) and anti-miRNAs (AXMIRs) have been shown to be efficiently delivered to and retain bioactivity within target cells (example data is shown in Fig. 18). Any XMIR or AXMIR of choice can also be cloned into a lentivector, allowing for the generation of stable cell lines constantly secreting exosomes packed with the desired small RNA.

Further research is rapidly progressing towards developing tools to package longer RNAs, such as mRNAs and lncRNAs, and proteins as exosome cargo as well as to present specific cell binding proteins and/or ligands on the exosomal surface to mediate cell type specific targeting. These tools can be engineered into the genome of cell lines, allowing for the creation of “designer exosome” producing cellular “factories” that will be a constant source of exosomes containing any cargo of interest that can be targeted for delivery to any cell type of interest. Additionally, such systems will abrogate the need for manipulation of exosomes after their isolation to insert cargo of interest: exosomes can be isolated from the engineered cell lines and be ready to immediately add to target cells for manipulation of biological pathways. Designer exosomes can be utilized as therapeutic shuttles as well as in basic research applications. These technologies are the new frontier in exosome research and as we understand more regarding signals that send RNAs and proteins to exosomes for secretion and what mediates specific cellular targeting, further tools will be developed to allow for a more complete realization of the potential for exosomes to be used as therapeutic vehicles to treat human disease. The exosome research field is still in its infancy with much to be explored. SBI strives to invent and innovate leading edge tools to accelerate discoveries in exosome biology and develop clinical applications. A list of SBI’s technologies and kits for exosome research can be viewed in Table 3. The published citations using these technologies and their applications can be viewed online [61,62].

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