Urinary Extracellular Vesicle Diagnostic Tool Development -

Computational Model for Dilution Determination

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Urinary extracellular vesicles (uEVs) are of specific interest as their composition and form can offer insight into the condition of the human body. Current utilization of uEVs as biomarkers is encumbered by time-consuming processing and large sample size requirements. Single Particle Interferometric Reflectance Imaging Scanner (SP-IRIS) (ExoView R100) enables use of unenriched urine samples and requires a significantly smaller volume of samples. The overarching goal of this study is to develop methods for using this platform to create noninvasive diagnostic tools. As urine is a very dynamic biofluid and varies in concentration from sample to sample, a reliable standard for the dilution factor of samples subjected to SP-IRIS must be created. The computational model developed here utilizes the variables of measured serum creatinine, nanoparticle tracking analysis (NTA) particle concentration and dilution factor, and SP-IRIS particle concentration in order to produce the output of SP-IRIS dilution factor.

1. Introduction

Extracellular vesicles (EVs) are membranous particles containing cell cargo with inherent biological information that are shed into the extracellular space. EVs are not bound to a specific location and can travel throughout the body via biofluids, including blood and urine[1]. Urinary extracellular vesicles (uEVs) are promising new kidney disease biomarkers[2,3]. Despite the availability of sensitive instruments for EV analysis, most require extensive processing of EV containing samples. These enrichment methods, such as differential centrifugation or filtering, lead to varied effects on EV properties, highlighting the need for novel methods of analysis using unprocessed samples. In addition, enrichment methods require large volumes and long processing times to complete, limiting their use when less volume is available such as that in existing biobanks, animal studies, and limited patient samples. Following enrichment, characterization of EVs includes western blot analysis, flow cytometry, particle analysis, and omics approaches. Although analysis of urinary EVs by flow cytometry has been proposed to aid in discovery of early markers of kidney damage, lack of specificity to the site of damage and bias towards larger vesicles further highlights the need for new methods development.

Given the lack of non-invasive early detection methods utilizing small volumes, a promising novel approach to harness urinary EVs as a diagnostic tool is single particle interferometric reflectance imaging sensor (SP-IRIS) based on tetraspanin mediated EV capture and detection. The platform requires less than 50 microliters of either unprocessed or processed sample and can be completed, without the need for enrichment or extensive sample processing, within 48 hours of sample collection. The technology, for these reasons, could be cost-effective, and is also non-invasive. However, urine is a dynamic biofluid, as the urine contents, such as the creatinine, vary greatly from patient to patient and even among samples of the same patient based on the diet, health, and hydration of the patient at the time of excretion[4]. This dynamic nature was observed in basic characterization experiments, in which the results elucidated that the techniques utilized and even protocols used within a given method in sample preparation greatly affect the overall outcome and analysis of the sample, such as the EV particle count and overall morphology.

In order to harness the power of SP-IRIS for use of EVs as biomarkers and obtain meaningful and reproducible results, sample dilution is required for optimal saturation of the tetraspanin-coated chips. Currently, there is no standard protocol for determination of sample dilutions prior to their use in SP-IRIS and experiments conducted with uEVs require the use of multiple chips per sample to increase the chance of optimal sample dilution and avoid sample oversaturation and loss of single-particle analysis capability (due to co-localization)[5]. Before a reliable analysis can be conducted using the new EV-harnessing capabilities of the SP-IRIS, sample dilution protocol must be standardized based on available sample parameters and/or known patient data.

Here, we describe the development of a computational model for determination of the optimal urinary sample dilution for use in the SP-IRIS. Our model enables standardized and reliable analysis using the new technology and will hopefully enable subsequent customization of EV capture using this machine to foster earlier diagnoses for kidney disease.

2. Methods

2.1 Urine Sample Collection

Healthy patient samples were collected after proper consent from the patient and approval from the local ethics board were obtained. Patients were instructed to drink variable amounts of water ranging between 1 to 3 liters before going to sleep and were instructed to collect their first void of the day in a sterilized bottle provided by the lab. The samples were put on ice and were immediately processed with the isolation procedure upon arrival.

2.2 Creatinine Measurement

Unenriched, raw urine samples were diluted with a dilution factor (DF) of either 1:100 or 1:50 and placed into 1.5mL eppendorf tubes. A total of 400 µL was created for each DF of each sample. The dilutions of each sample were then separated into three aliquots of 134 µL and placed within a 64 well

plate. 34 µL of picric acid was then loaded into each well containing a sample. The 64 well-plate is then placed within the microplate reader (Synergy HT, BioTek, Winooski, VT, USA). Following the read, 34 uL of 0.75 NaOH are added into each well containing a sample followed by a 20 minute incubation period at RT. The 64 well-plate is then placed once again within the microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

2.3 Urinary Extracellular Vesicle (uEV) Isolation

2.3.1 Differential Ultracentrifugation

Differential Ultracentrifugation (DCF) is a common enrichment technique to conduct EV analysis as it enhances EV purity within a sample. SP-IRIS does not require enrichment of samples; however, in order to maintain simplicity of the experimental model, the samples prepared were enriched to conduct comparative analysis across the various methods. Samples were collected with the intent of further isolation and enrichment and are represented by **Figure 1**. For each sample, 50 mL were taken and centrifugation at a Relative Centrifugal Force (RCF) of 4,600g at max radius 168 mm (5000 rpm) in a TX-400 Sorvall ST16R (Thermo Fisher Scientific, Waltham, MA)) swing bucket rotor (k Factor 9153) was performed for 30 minutes at room temperature (RT). The supernatant of the 4,600g spin (SN 4.6) was then subjected to a second centrifugation step at an RCF of 21,130g (15,000 rpm) for 30 minutes at RT in an Eppendorf microcentrifuge 5424 fixed angle rotor (FA-45-24-11) (Eppendorf, Hamburg, Germany) using 1.5 mL microcentrifuge tubes (Axygen; Corning Inc. Corning, NY)[6]. The supernatant (SN 20) and pellet (P20) of the 21,130g spin were then separated. The pellet was resuspended in 1 mL of 0.1 µm filtered (Minisart ® PES syringe filter code 16553K, Sartorious, Göttingen, Germany) 10 mM HEPES (Stock solution 1 M HEPES pH 7.4) containing 2.5 mM EDTA (Stock solution 0.5 M EDTA pH8.0). The sample was then centrifuged again at 21,130g for 30 minutes at RT. The resultant supernatant and the P20 pellet were once again separated. This process of separation, wash, and centrifugation was repeated until a series of triplicate washes and centrifugation occurred. The resultant pellet (P20 cleared) was compared to that of the P20 pellet obtained before triplicate washing and centrifugation, in order to assess sample preparation and purity.

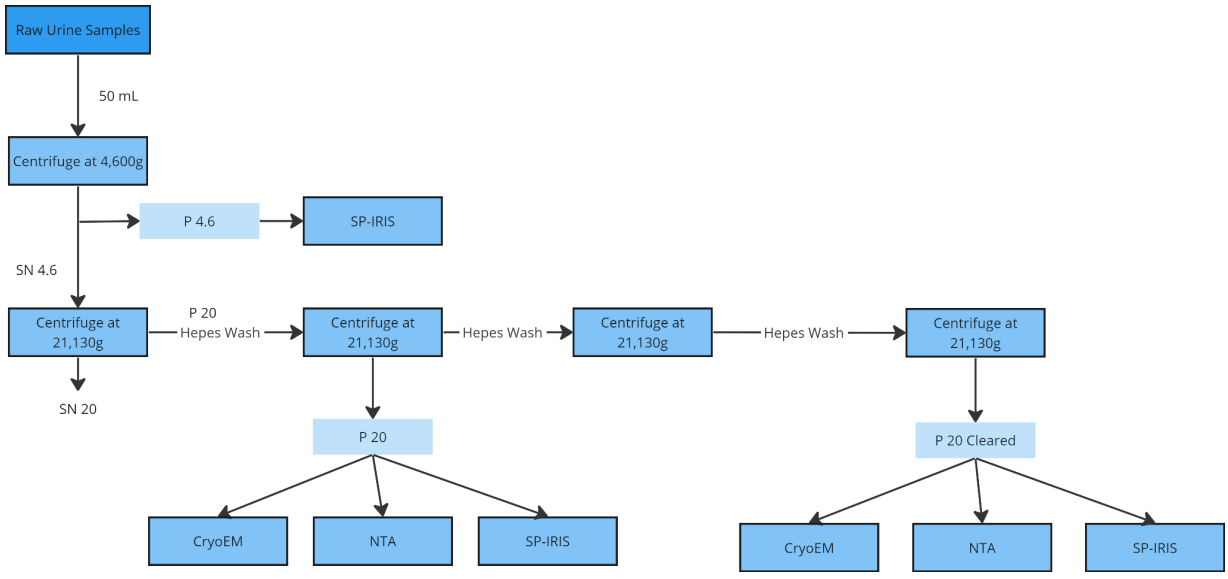


Figure 1: Example of Experimental Protocol of Sample Enrichment and Analysis

2.4 Basic Characterization

2.4.1 Cryogenic electron Microscopy

CryoEM was performed in the molecular electron microscopy core at the University of Virginia (https://med.virginia.edu/molecular-electron-microscopy-core/services/). Low speed centrifuged uEV P20 and P20 cleared pellets were solubilized in 20 µL PBS–0.1 µm and applied to a glow-discharged, perforated carbon-coated grid (2/2-3C C-Flat; Protochips). Low-dose images were collected at a nominal magnification of 29,000 × on the Tecnai F20 Twin transmission electron microscope operating at 120 kV. Digital micrographs were recorded on a Gatan US4000 charge-coupled device camera.

2.4.2 Western Blot Analysis

Pelleted uEVs (P20s corresponding to 25 mL of starting urine) were lysed in 52l of Electrophoresis Solubilization Buffer (ESB) containing 6 M urea, 2 M thiourea, 5% (w/v) sodium dodecyl sulfate (SDS), 40 mM Tris-HCl, pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% (v/v) glycerol, 0.01% bromophenol blue with the addition 50 mM dithiothreitol (DTT). After overnight lysis at room temperature, lysates were pooled and proportional amounts of lysate corresponding to the volume of starting urine were aliquoted and normalized for a constant loading volume (25 µl per lane) by addition of ESB. Polyacrylamide gel electrophoresis was performed with Bio-Rad Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels #4569033, according to the manufacturer's directions. After electrophoresis, proteins were transferred to Amersham Protran 0.45m nitrocellulose membranes at 100 volts for 1 hour at 4ºC in a Bio-Rad mini trans-blot transfer cell in 25mM tris-192mM glycine buffer containing 20% methanol. After transfer, membranes were rinsed in TBS (Tris Buffered Saline), then incubated in Li-cor TBS Odyssey Block overnight at 4ºC on a rocking platform. Immunoblotting with mouse anti-human AQP2 antibody (Santa Cruz Biotechnology #sc-515770) at a 2000-fold dilution in 50% Li-cor TBS Odyssey block, 50% TBS with 0.15% Tween-20 was performed at 4ºC overnight on a rocker. The membrane was then washed at room temperature in TBST (TBS with the addition of 0.15% Tween-20) 5 times, for approximately 15 minutes each with rocking. The blot was incubated with the secondary antibody (Li-cor goat anti-mouse IRDye 800CW #925-32210) at 10,000-fold dilution in 50% Li-cor TBS Odyssey block, 50% TBS with 0.15% Tween-20 for 2 hours at room temperature, followed by 5 washes in TBST, followed by a brief rinse with TBS (no Tween-20) prior to imaging. Imaging was performed using a Li-Cor Odyssey CLx Imager. The membrane was then sequentially immunoblotted for TSG101 using the same technique, with a 3000-fold dilution of primary antibody (Sigma rabbit anti-human TSG101 #T5701) and 10,000-fold dilution of secondary antibody (Li-cor donkey anti-rabbit IRDye 680RD #925-68073).

2.4.3 Nanoparticle Tracking Analysis (NTA)

NTA was conducted utilizing the ZetaViewPMX 120 (Particle Metrix, Germany) equipped with a 488-nm laser and a long wave-pass (LWP) cut-off filter (500 nm) and a sensitive CMOS camera of 640 x 480 pixels. Each experiment was performed in triplicates for the given sample of enriched uEVs with the following specifications and analysis parameters: cell temperature 25°C, sensitivity of 70, shutter speed of 80, and a frame rate of 30 frames per second (fps). Each sample was measured at 11 different positions of the cell and were validated while obtaining at least 1000 valid tracks for each run. For data capture and analysis, Nanoparticle Tracking Analysis Software (ZNTA) v 8.05.04 was used. The automated particle reports of each of the 11 positions were manually examined for the removal of outliers prior to calculation of particle concentration and distribution through the values of mode, median, and mean.

2.4 Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS)

2.4.1 Sample Preparation and Incubation

An overview of the standard protocol used for EV specific biomarker analysis can be seen in **Figure 2**. Chips coated with tetraspanin-specific antibodies anti-CD9, -CD63, and -CD81 (UnChained Labs, Pleasanton, CA, USA Product No.: EV-TETRA-C) were pre-scanned according to the provided manufacturer instructions, and baseline measurements of pre-adhered particles to the tetraspanin microarray chip before sample incubation were taken and stored on the computer. The purified uEV

samples from the 4600G spin were diluted in the Incubation Solution (UnChained Lab, Pleasanton, CA, USA). The incubation was carried out at room temperature in a sealed 24-well plate (provided by the manufacturer) to prevent sample evaporation. After the dilution was made, 50 µL of the solution were loaded onto the pre-scanned chip and incubated overnight.

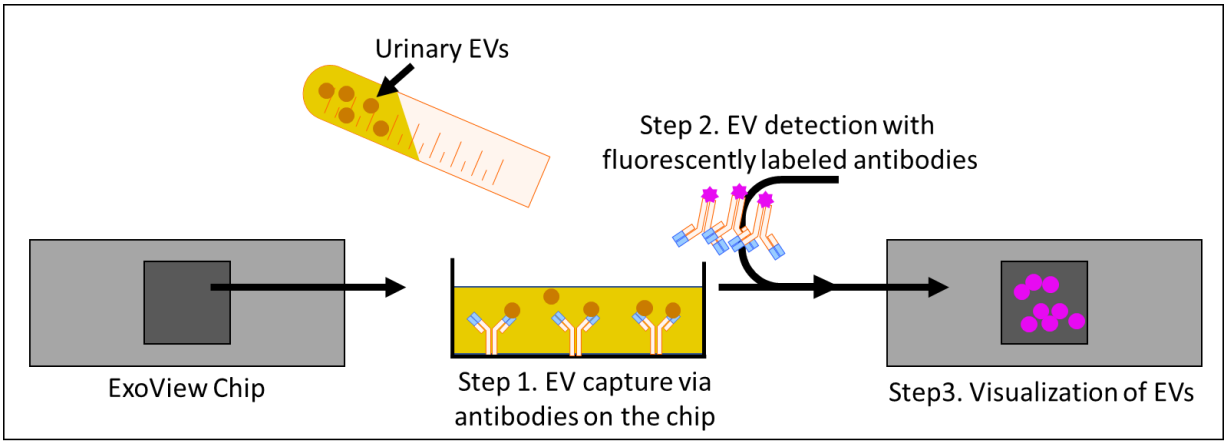


Figure 2: Standard Protocol for SP-IRIS analysis on the ExoView R100 Platform

2.4.2 Chip Washing and Scanning of Samples

Following an incubation period, the chip washing and addition of fluorescently labeled anti-CD9, -CD63 and -CD81 antibodies of the captured uEV were performed according to the manufacturers' instructions. All reagents used were stored at 4°C and were left for 15 minutes to reach room temperature prior to use. 300 µL of Blocking Solution (Unchained Labs, Pleasanton, CA, USA) per chip was prepared in a tube added to each microarray chip. First, the chips in the plate were placed in the chip washer (Exoview CW100), and the chip washer protocol was followed according to manufacturer's instructions. A cocktail of 300 µL of Blocking Solution (Unchained Labs, Pleasanton, CA, USA) per chip containing 0.6 µL per chip of each anti-CD9 (CF® 488A), anti-CD81 (CF® 555), and anti-CD63 (CF® 647) was prepared. The cocktail was vortexed for 5 seconds prior to use, and 250 of the antibody mixture was added to each well containing a chip when prompted by the CW100. The rest of the chip washing procedure from the manufacturer was followed, and the chips were placed on the ramp in the Automated Imager according to the data acquisition protocol provided by the manufacturer. Acquired data were analyzed using the manufacturer provided software.

3. Results

In order to understand how EVs can be harnessed as early biomarkers of kidney diseases such as diabetes, the basic analysis of extracellular vesicles must be understood with the current technology and procedures. The procedures using enriched urine samples through western blots, flow cytometry, ultracentrifugation, nanoparticle tracking analysis, and cryogenic electron microscopy can be harnessed to analyze uEVs. In order to use uEVs for diagnostic purposes in future applications through SP-IRIS analysis, basic characterization of enriched EVs was conducted to better understand their dynamic nature as well as validate physiological aspects including size, shape, protein cargo, and particle concentration. CryoEM, western blot analysis, and NTA were conducted on enriched EVs from healthy patient urine samples. The preliminary results of each of these techniques demonstrated the dynamic nature of urine samples, with variability of results based on sample preparation. The techniques described were carried out on uEV samples that were enriched with DCF and a triplicate wash to rid the samples of protein aggregates.

3.1 Basic Characterization

3.1.1 Cryogenic electron Microscopy (CryoEM)

The resultant images produced from CryoEM displayed variance in uEV concentration dependent on sample preparation. As shown in **Figure 3**, there is an apparent difference in uEV size and concentration between the samples of the P20 pellet without additional clearing and the P20 cleared pellet. Samples that did not go through triplicate washing and centrifugation steps appeared to have contaminants of protein aggregates. Additionally, qualitative analysis of the images obtained from CryoEM revealed that the uEVs are heterogeneous in size and shape as displayed in **Figure 4**. The variation in sample preparation as well as heterogeneity in EVs is concurrent with literature, confirming that the acquisition of samples and enrichment methods used are adequate for further uEV characterization[6].

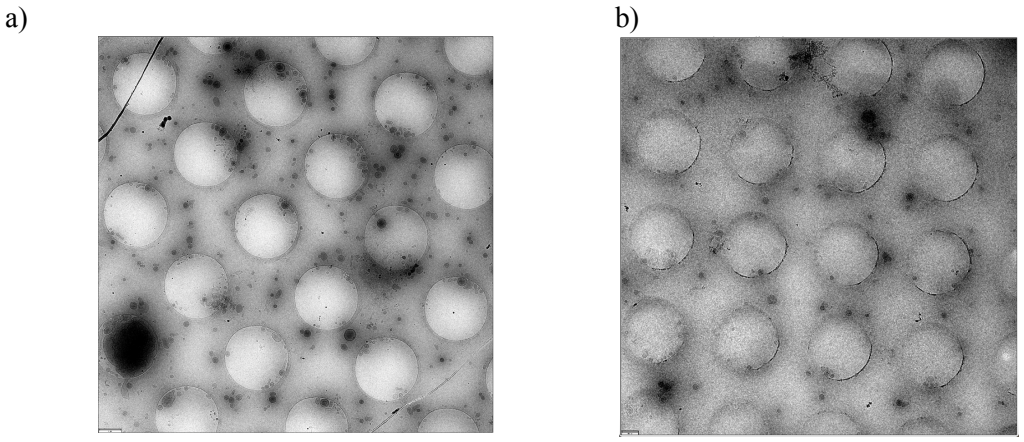


Figure 3: CryoEM imaging plate of a) P20 pellet and b) P20 cleared pellet

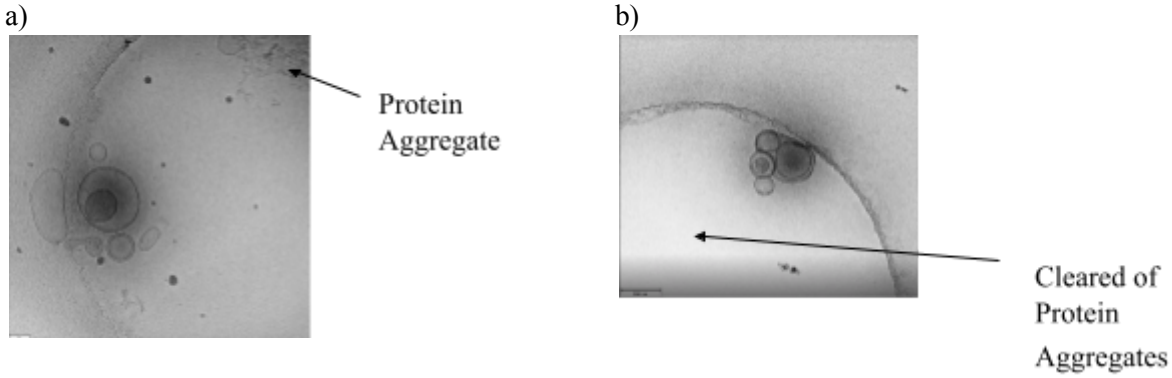


Figure 4: CryoEM Images of a) P20 Pellet b) P20 Cleared Pellet Depleted of Protein Aggregates

3.1.2 Western Blot Analysis

Western blot analysis was conducted in order to analyze the protein cargo present within the enriched uEVs. An example image of the analysis is shown in **Figure 5**. The analysis demonstrates that the protein cargo present in enriched uEV preparations originates from the nephron of the kidney, confirming that the particles captured are uEVs.

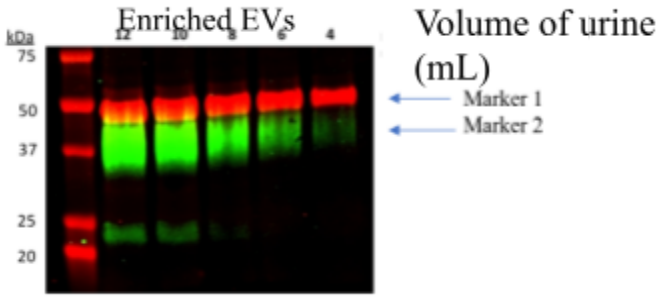


Figure 5: Western Blot Analysis of Healthy Human Urine Sample (Marker 1: TSG101 and Marker 2: AQP2)

3.1.3 Nanoparticle Tracking Analysis (NTA)

Particle count and size distribution of P20 pellets were compared using CryoEM and NTA to understand the accuracy and bias of the respective techniques for the quantification of uEVs in the same sample. The results show that the particle count and size distribution differ between the two techniques (**Figure 6**).

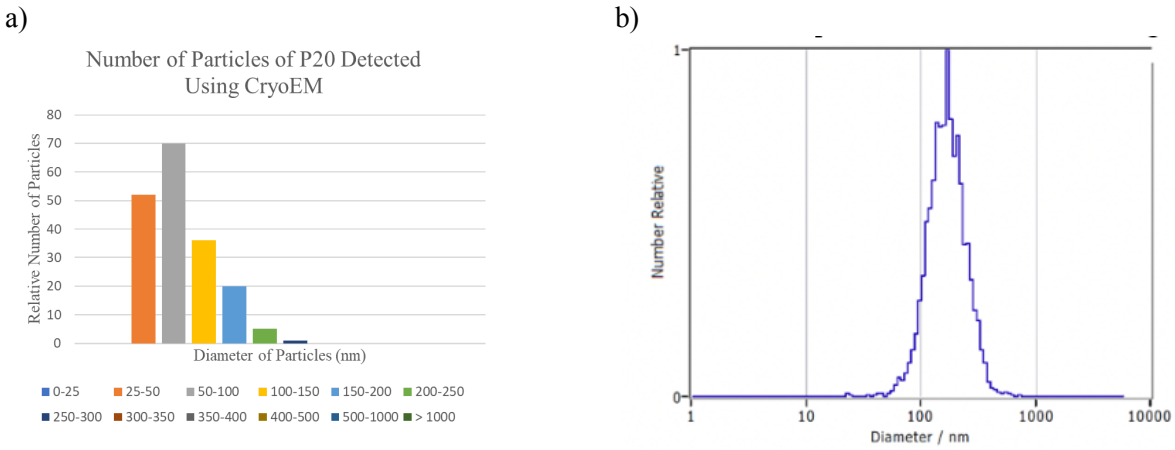
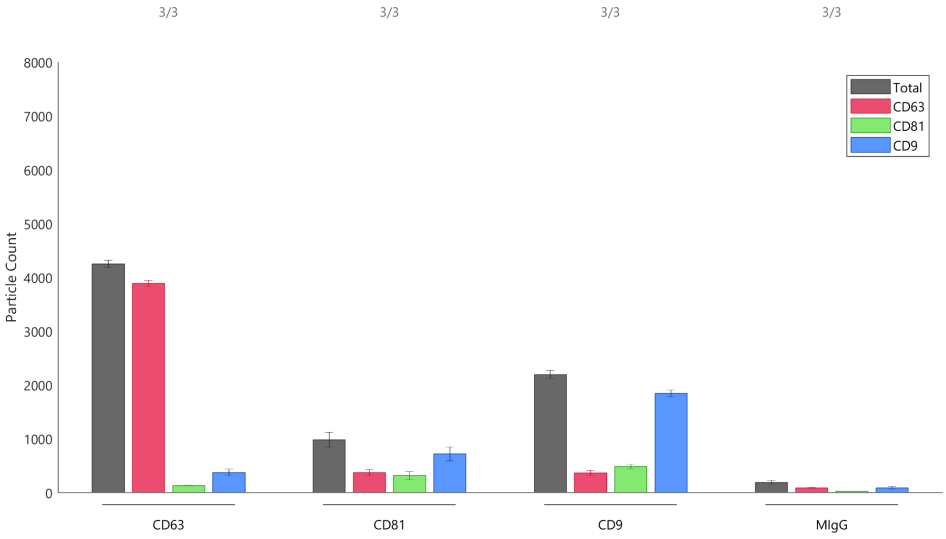


Figure 6: Particle Count and Size Distribution of a) P20 Pellet with CryoEM b) Same P20 sample with NTA using the ZetaViewPMX 120 Platform

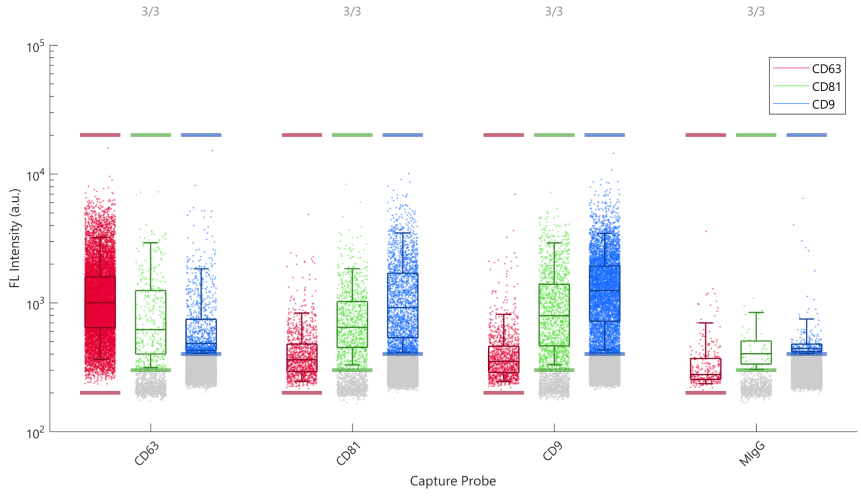
3.2 Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS)

Particle size distribution, concentration, fluorescence, and marker colocalization were also obtained using SP-IRIS. As seen in **Figure 7**, the output of particle counts and fluorescence box plots

generated by SP-IRIS enables quantification of the specific concentration and binding affinity of EVs to each antibody analyzed. Additionally, the platform provides standardization across the various antibodies for non-specific binding via the analysis of IgG as a capture probe. This is particularly useful as it enables for specific biomarker determination via the binding affinity and associated fluorescence of specific uEVs captured. This standard method of detection can be adapted either through the change in the capture antibodies or the fluorescent detection antibodies, in order to identify particular biomarkers of interest that may be related to disease.



a)



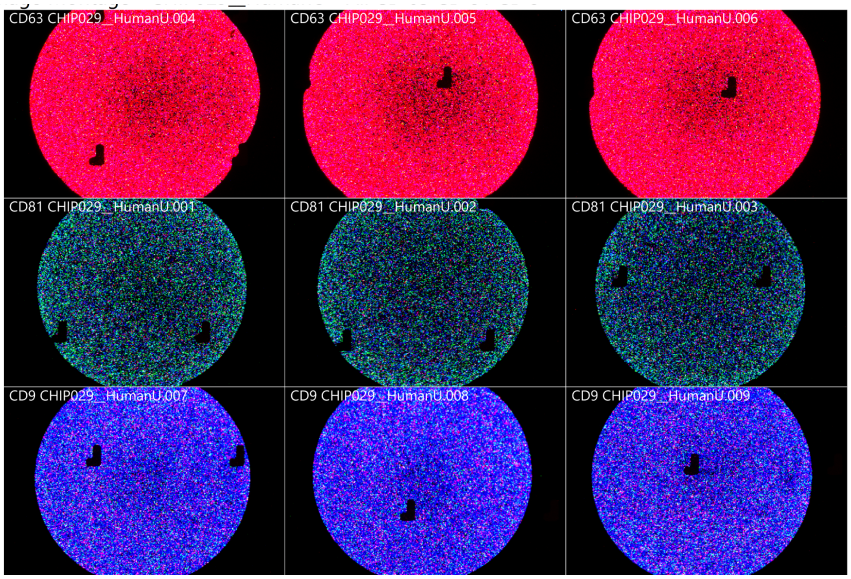
b)

Figure 7: a) Particle Concentration and b) Fluorescent Box Plot of Healthy Human Urine Sample Obtained by SP-IRIS via ExoView R100 Platform of Representative Images.

In **Figure 8**, the outputs of fluorescent imaging of uEVs can be seen. **Figure 8a** displays imaging of an oversaturated sample, whereby the fluorescent signal is hyperpigmented and particle spacing is indeterminate. **Figure 8b** displays imaging of an optimally diluted sample, whereby particle concentration

and placement is apparent and there is variability in fluorescence detected per capture probe. The lack of a standardized protocol or predicted value for associated dilution factors when conducting SP-IRIS is unfavorable. The variability in sample dilution, as seen in Figure 8, renders SP-IRIS unreliable for measurable and actionable data collection using one chip per sample.

a)



b)

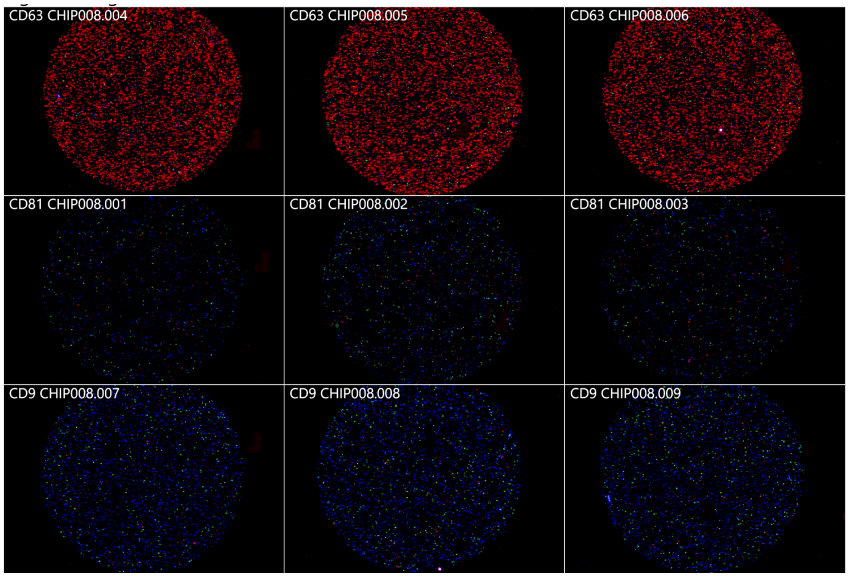


Figure 8: Representative Fluorescent Images of a) Oversaturated Human Urine Sample and b) Optimally Saturated Human Urine Sample Obtained by SP-IRIS via ExoView R100 Platform

3.3 Computational Model Development

In order to develop a standardized protocol for determination of an optimal sample dilution for SP-IRIS analysis, we utilized a computational approach. Six samples from the same healthy patient, with varying volume intake (1-3 liters) prior to each void, were used for the development of the initial series of

equations. For each sample, the following measurements were obtained: serum creatinine, NTA concentration (with the associated dilution factor), and SP-IRIS total particle concentration (with the associated dilution). The associated measurements and sample data can be found in **Table 1**.

Table 1: Samples and Associated Measurement Used for Development of Computational Model for Dilution Factor for SP-IRIS

Chip Number and Volume Intake (L)	Dilution Factor for NTA	Dilution Factor for SP-IRIS	Serum Creatinine (mg/dL)	NTA Concentration (p/mL)	SP-IRIS Concentration (p/mL)
009 1L	4000	50	171	4.10E+11	27163
022 1L	4000	100	171	9.75E+10	22834
023 2L	2000	50	57	4.60E+10	6578
027 2L	2000	100	57	4.10E+11	1989
033 3L	2000	50	45	9.75E+10	2329
035 3L	2000	100	45	4.60E+10	1361

From these measurements, a comprehensive list of potential variables with corresponding symbols and units was developed and can be found in **Table 2**. From these variables, a series of equations was developed, in order to create an output of dilution factor for SP-IRIS.

Table 2: Variables Used in Computational Model

Variables	Symbols	Units
Sample type	S	liters
Dilution Factor for NTA	n	au
Dilution Factor for SP-IRIS	e	au
NTA Concentration	NC	p/mL
SP-IRIS Concentration	EC	p/mL
Creatinine	Cre	mg/dL
Coefficient	coef	
Constant	C	

Equation 1 is based on the relationship between the counting methods of NTA and SP-IRIS, assuming that both produce accurate particle concentrations of a given sample and therefore should have correlation to one another regardless of sample enrichment. Equation 1 incorporates the variables of dilution factor for NTA, particle concentration for NTA and SP-IRIS, as well as an associated coefficient and constant of correlation. The associated coefficients developed from Equation 1 had a standard deviation of 8.98E-05. For this reason, the constant value for Equation 1 was assumed to be a value of zero.

$$EC * n = NC * coef1 + C1 \quad (1)$$

Equation 2 is built on the direct positive correlation between measured serum creatinine and measured SP-IRIS particle concentrations[1]. Equation 2 incorporates the variables of dilution factor for SP-IRIS, measured particle concentration for SP-IRIS, measured serum creatinine, as well as an associated coefficient and constant for correlation. The associated coefficients developed from Equation 2 had a sample standard deviation of 4115. Although this value was significant, in order to preserve the validity of the model and not introduce any confounding variables, the constant value for Equation 2 was assumed to be a value of zero.

$$EC * e = Cre * coef2 + C2 \quad (2)$$

Equation 3 was developed through the use of a system of equations whereby the particle concentrations for SP-IRIS relates Equation 1 and Equation 2. This results in the output of dilution factor for SP-IRIS, given the input of measured serum creatinine, dilution factor for NTA, measured particle concentration for NTA, and the associated coefficients and constants for correlation. The results with reference to the six healthy patient samples yielded the same experimental dilution factors for SP-IRIS as the expected dilution factors for SP-IRIS.

$$e = (Cre * coef_2 + C_2) / ((NC * coef_1 + C_1)/n) \quad (3)$$

4. Discussion

The dynamic nature of urine results in the capture and quantification of uEVs to display highly variable outcomes based on the patient and sample preparation methods. The development of SP-IRIS has the potential to revolutionize personalized, early, and non-invasive diagnostic medicine. With the new light shed on the importance of uEVs, research into these nanoparticles is rapidly growing, necessitating a standardized method for capture and analysis using SP-IRIS. The manufacturer protocol for using unenriched human urine samples was a recommended 1:3 dilution, which does not take into account variability in the dilution of human urine depending on the patient and the liquid volume intake prior to each void. The recommended dilution produced confounding outputs, typically resulting in oversaturation of the chip. Oversaturated samples cannot be utilized for subsequent analysis due to increased colocalization and the presence of non-specific binding, rendering these chips and samples useless. The development of a standard method enables uniform data analysis as well as minimizes the waste of patient samples and chips due to an unusable dilution.

The conventional method for measuring the kidney function of a patient is through the measurement of the serum creatinine of the patient sample[7]. This metric is helpful as an indicator of improper kidney function, but the creatinine levels are late-stage markers of kidney damage as well as highly variable between patients and environmental conditions at the time of the void[8]. Factors such as age, sex, and muscle mass also affect the uEV concentration of the urine, and there is a correlation between uEV counts in samples and the mass of the kidney[9]. The creatinine content in the urine is simple to measure and is a standard metric, so these values were incorporated into the model because the data is readily available, and it has been shown that uEV count and creatinine correlate, which serves as a key link in the development of our computational model. The high variability of uEVs in a given sample demonstrates the need for normalization of the patient samples, as there is no housekeeping standard

protein to quantify uEV counts within the given sample. The known correlation between uEV count and serum creatinine from 24-hour urine collection volumes as well as uEV counts between the NTA and SP-IRIS can be used to accomplish this normalization and were used to create our computational model.

While uEVs contain important biological information, the basic characterization and isolation of uEVs is time-consuming and requires a large sample volume. A challenge faced within uEV research is that not one single tool exists that can analyze all EV features simultaneously and instead completion of several procedures is needed. Additionally, methods such as CryoEM, Western blot, and NTA require enrichment of the samples such as differential centrifugation in order to obtain meaningful results due to the sensitivity of the sample[6]. Furthermore, these methods are often time-consuming and require at minimum 50 mL of a given sample. Comparatively, SP-IRIS requires approximately 5 μL of a given sample with no further enrichment necessary prior to analysis. SP IRIS can also identify several features of EVs simultaneously, such as the phenotype of the EV surface proteome (depending on the specificity of capture and detection antibodies used), as well as EV size and concentration. The multi-capabilities in conjunction with small-volume requirements demonstrate the advantage of SP-IRIS over conventional uEV analysis methods.

4.1 Limitations

The computational model developed requires further testing of a cohort of healthy patient samples in order to validate the linearity of the model, the assumed value of zeros for constants, as well as its robustness. Additionally, the computational model should be tested with a cohort of diseased patient samples to assess if the model still holds true for the assessment of both healthy and diseased sample populations. Furthermore, the computational model only incorporated samples that received enrichment and should be assessed with normalized values of NTA particle concentration, NTA dilution factor, and associated coefficient and constant correlation value for Equation 1, as sampling methods may not have such tools available in order to produce an adequate dilution factor for SP-IRIS.

4.2 Impact of Model

Single-particle analysis is the desired method for examining the uEVs of a patient sample and will yield the most accurate and relevant information. The quantification of uEVs is conducted through the particle count and content measurement and the overall disease state of uEVs can be determined using SP-IRIS once the protocol is standardized for obtaining the optimal results. The creation of our computational model is based on the important known links between the serum creatinine and uEV particle count, as well as the known particle counts from both NTA and SP-IRIS of the same sample. The model was developed using the algebraic logic of creating a system of equations using these relationships to result in three equations for three unknown variables that could be solved because of the relationships established between the equations.

The analysis of uEV will eventually enable examination of the cells that the uEVs were released from and will allow earlier diagnosis of disease, prior to development of systemic symptoms (such as elevated serum creatinine). Early detection of kidney injury will allow faster response to correct the disease that causes nephropathy, avoiding the loss of time and difficulty of therapeutic approaches once kidney failure is apparent and damage is permanent. The noninvasive and small-volume nature of the protocol and use of SP-IRIS will also allow for greater accessibility for patient nephrology care, which could help to combat the pervasive inequities and disparities plaguing the United States.

4.3 Future Work

The future of this model can next be applied to diseased patient samples, such as a cohort of patients with type 1 diabetes (T1D). The developed model will hopefully be applicable to diseased patient samples, and the experimental results from this study would, at minimum, elucidate necessary dilutions for the use of samples in the ExoviewR100. Once the computational model is validated through both healthy and diseased samples, focus can be turned to the development of a customized chip to capture kidney cell type-specific uEVs to analyze the disease state of the given sample. The development of the computational model unlocks one important piece of the greater puzzle to optimally analyze uEVs using ExoviewR100, and this simple and non-invasive method for processing patient samples should serve to standardize and optimize protocol in all work with uEVs using SP-IRIS.

5. Conclusion

The technological capabilities that have been created through the development of the SP-IRIS technique are vast and promising, and the standardization of optimal protocol must be developed first for the most impactful contributions to be made with this technique. The correlation made between particle count and serum creatinine provides a straightforward and validated method to obtain analytical information from a small-volume patient sample through the capture of extracellular vesicles. The computational model will allow for easier experimentation, as other researchers will not need to spend time and effort seeking out an optimal dilution for analysis in the machine, and the results determined in the field will be more reliable due to a standardization of protocol within use of the ExoviewR100 for uEVs. The next steps for the development of this model are the subsequent validation of this model with diseased patient samples, and the chips can then be customized to target and capture disease-indicative extracellular vesicles to serve as a diagnostic device. The potential for this new technology to serve as an early diagnostic tool is game-changing for the medical field and will lead to an increased quality of medical care and overall life for patients.

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