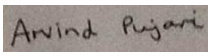
Detection of Ultra Low Concentrations of Exosomes Using Single Molecule Optofluidics

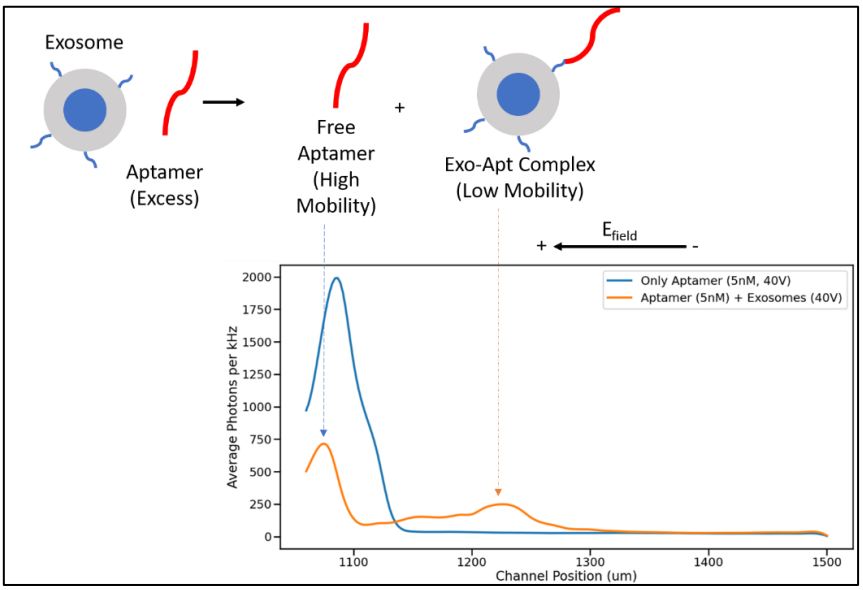
Arvind Pujari, Dr Magda Czekalska, Dr Georg Krainer, Akhila KJ, Walther C. Traberg, Dr Roisin Owens and Dr Tuomas Knowles

Declaration: "*This report is substantially my own work and conforms to the University of Cambridge's guidelines on plagiarism. Where reference has been made to other research this is acknowledged in the text and bibliography.*" 

Abstract

Exosomes are a class of extracellular vesicles with diameters between 30-100nm which have garnered increasing attention due to their possible applications as early-stage cancer biomarkers. In particular, the concentration of specific proteins on the surface of exosomes can give us crucial information to this end. Current characterization techniques (NTA, TEM and ELISA) do not provide adequate information about protein characterization and are limited by their longer time scales. Here, we utilize a single molecule optofluidics setup combined with micro free flow electrophoresis for the sensing of exosomes and the CD63 protein present on their surface. We were able to record the concentration of exosomes in the picomolar regime and detect the number of binding sites per exosome, as well as their mobilities. Thus, we present a robust and scalable technique for the detection of exosomes and the proteins present on them.

Graphical Abstract:



1. Introduction

Extracellular vesicles (EVs) are spherical particles released by cells (both eukaryotic and prokaryotic) enclosed by a phospholipid bilayer [1]. EVs can be further subdivided into exosomes (diameter 30-100nm) and microvesicles (diameter 100-1000nm) [2]. Exosomes have recently garnered a great deal of attention because they contain proteins which can be indicative of the cell type of origin.

In particular, tumour derived exosomes have been studied extensively because they might play a role in facilitating tumorigenesis by regulating angiogenesis, immunity, and metastasis [3]. Additionally, they can serve as biomarkers due to the presence of specific proteins which can signal significant genetic alterations in the cancer cells of origin [4] [5]. Therefore, techniques for the detection and characterization of exosomes have huge potential as a non-invasive oncological diagnostic tool.

Current state of the art techniques for the detection of exosomes include nanoparticle tracking analysis (NTA) [6], qNano techniques [7], and immunocapture based techniques such as enzyme-linked immunosorbent assay (ELISA) [8]. However, these techniques have inherent problems associated with them: NTA and qNano techniques cannot easily differentiate between exosomes and other contaminants such protein aggregates. ELISA involves multiple washing steps to remove excess fluorescent probes, has a large time scale (4 hours) which results in inaccurate measurements due to probe/analyte dissociation across the timescale [9]. Additionally, all these techniques involve several hours of ultracentrifugation which can damage the exosomes and may not be completely successful in removing other cell debris.

There have been several reports on the presence of specific proteins (CD63 and CD9) on the surface of exosomes. In particular, tumour derived exosomes have reported altered expression of the CD63 protein [10] [11]. Thus, the detection and quantification of these proteins could be a useful tool for early cancer diagnosis. Hence, there is an increasing need to develop other techniques for the detection of exosomes and the concentration of proteins on their surface.

Zhang et al. [12] have developed an aptamer-based fluorescence polarization assay for exosome quantification. They use the huge mass/volume ratio of exosomes as a polarization amplifier. Although their timescale of sensing is around 30 minutes, the high value of the dissociation constant of the CD63 aptamer employed here (30-70 nM [13]) means that even lower timescales are needed. There have also been several reports on the potential use of microfluidic devices for the rapid detection of exosomes [14] [15]. Recently, our group has

reported the development of an optofluidic setup combined with micro free-flow electrophoresis for the detection of single molecules called DigitalISA [16]. This provides us with an opportunity to combine aptamer-based quantification of exosomes with a microfluidics approach for their rapid detection (timescale < 1 minute) while using an arbitrarily high concentrations of aptamers.

Our results indicate that this technique can be used for the rapid detection of extremely low concentrations of exosomes (picomolar regime), their mobilities, as well as the number of CD63 proteins present on each exosome. This technique which can be extended to the detection of several other proteins present on exosomes. Although we used samples purified by ultracentrifugation for our analysis, the versatility of our techniques means that it will be possible to detect them at extremely low concentrations, possible from blood plasma too.

2. Methods

2.1 Cell culture and exosome isolation from cancer cell lines

MDA-MB-231 cells were cultured in a T75 flask to reach 75-85% confluency. Cultured MDAs were maintained in 15 mL of Dulbecco's Modified Eagle's Media supplemented with 10% fetal bovine serum (FBS, ThermoFisher) and 1% penicillin–streptomycin (P/S, Invitrogen). The cells were washed with warm PBS and then changed to 15 ml of serum free media (Dulbecco's Modified Eagle's Media supplemented with 1% penicillin–streptomycin) to avoid the isolation of exosomes derived from serum. The cells were cultured in serum free media at 37°C and 5% CO2 for 18-30 hours.

The serum free media was collected from a T75 flask in a falcon tube. The collected media was then centrifuged at 280 g during 12 minutes at 21 C to pellet floating cells. The supernatant was collected (without touching the pellet) and centrifuged one more time at 21 C at 10000 g for 30 minutes to pellet cell debris. The supernatant was ultracentrifuged at 100,000 g and 4°C for 4 hours to collect pure exosomes (seen as a pellet at the bottom of the tube). The supernatant was discarded, and the pellet of exosomes was resuspended in 0.5 mL of 10 mM HEPES buffer.

The isolated exosomes were then mixed in solution phase with a 5nM solution of CD63 aptamer (biomers.net, Germany) and used immediately in the single molecule optofluidic setup.

2.2 Fabrication of PDMS Microfluidic Chips

The microfluidic chip design was printed on acetate transparencies (Micro Lithography Services). The replica mould for device fabrication was done using soft lithography by spinning SU-8 3025 photoresist onto a circular, polished silicon disk to a height of 24 µm. The photoresist was then cured by irradiation under a UV lamp for 1 minute. The feature size of the chip was confirmed to be 25 µm using a profilometer. To make the PDMS devices a 10:1 (w/w) mixture of PDMS (Dow Corning) and curing agent (Sylgard 184, Dow Corning) was poured onto the mould which was then degassed and baked at 65 °C for one hour.

Individual devices were cut using a knife and holes were punched through using biopsy punches. The devices were washed by sonicating in isopropyl alcohol (IPA) for 15 minutes and then bonded to thin glass coverslips after plasma treating both at 40% power for 40 seconds. The devices were then subjected to hydrophilic treatment by plasma treatment for 500 seconds at 80% power. The channels were then wetted by pipetting a small amount of water into the device.

2.3 Micro Free Flow Electrophoresis

The working principle behind the DigitalISA setup which combine micro free flow electrophoresis with single molecule optofluidics is demonstrated in Figure 1 (a) (b) and (d). The sample (exosomes) and the fluorescent probe (CD63 aptamer) are injected into the separation chamber of a microfluidic chip for electrophoretic separation. Under the effect of an electric field the aptamer-exosome complexes and the free aptamer move by differing amounts due to a difference in electrophoretic mobilities. Confocal scanning is performed across the separation chamber and the number of photons emitted are detected by a photodetector. The number of molecules is recorded digitally using a combination of inter-photon time (IPT) and burst-search algorithm.

The design of the electrophoretic chip is shown in Figure 1 (c). It consists of a separation chamber, through which buffer (10mM HEPES solution with 0.05% Tween to prevent the sticking of exosomes to the glass surface) is flowed through at 1000 µL/h. The sample (5 nM CD63 Aptamer + exosomes) is input into the buffer stream at a speed of 10 µL/h to form a defined stream in the middle of the separation chamber while a thin sheet of 3M KCl electrolyte flows on either side of the separation chamber at a speed of 300 µL/h. Electrodes are connected at the end of the electrolyte chambers to provide a voltage.

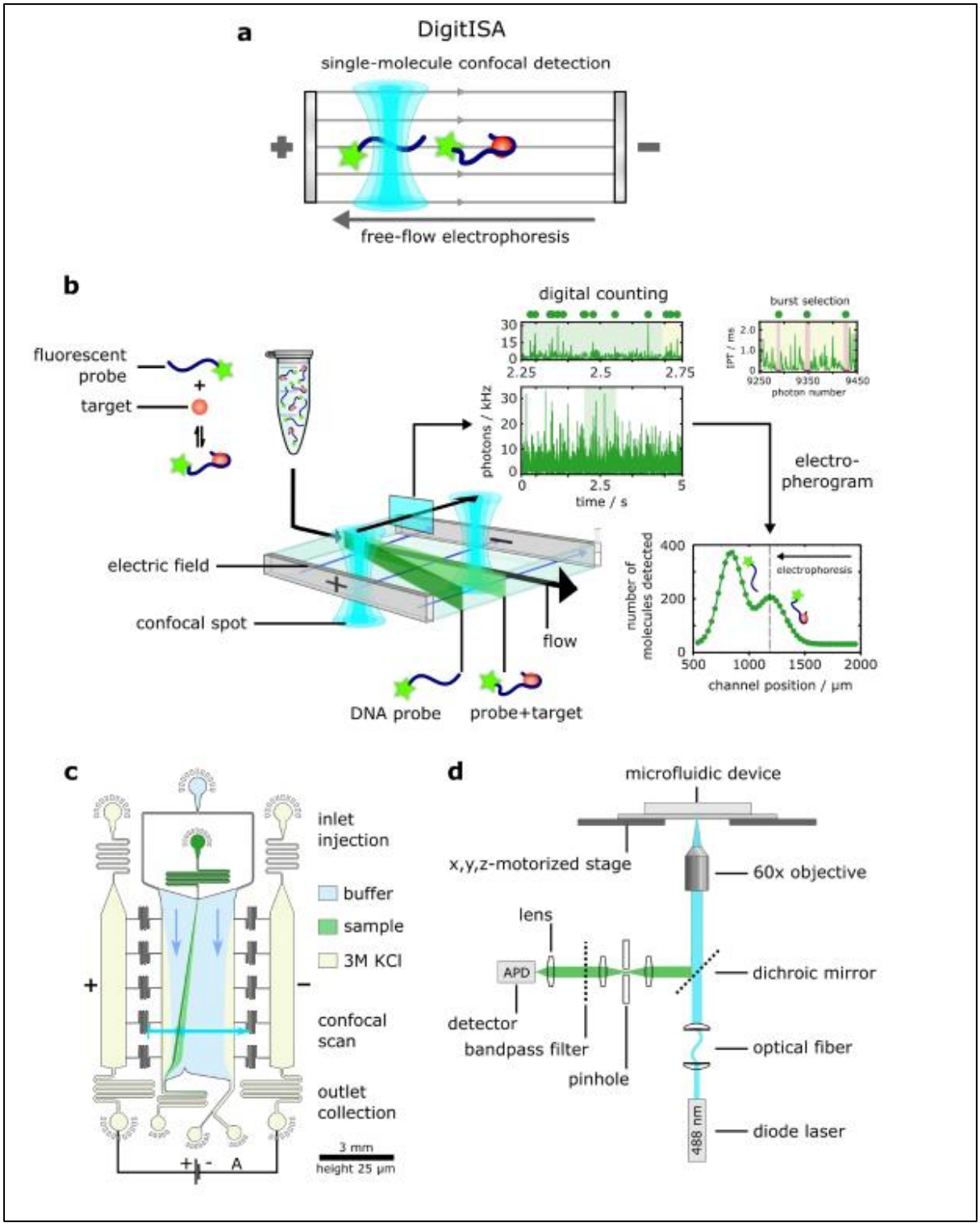


Figure 1. (a) An illustration of the working principle of electrophoretic separation using the DigitalISA setup (b) Due to differing electrophoretic mobilities of the bonded probe and analyte, and the free probe, they are respectively moved by differing amounts under the influence of an electric field. Using the inter photon time (IPT) and a photon burst threshold, single molecules can be differentiated from the background signal, and the number of molecules can be counted. (c) The chip design consists of a separation chamber through which the buffer and sample flow and a thin sheet of electrolyte on either side of the separation

chamber so that a potential difference can be applied across the chamber (d) The optical setup used to record photons emitted from the sample. Image taken from [16].

3. Results

3.1 DLS Measurements

The average size of the exosomes was found to be between 40 and 90 nm using DLS. The samples are found to be highly polydispersed. The aptamer was too small to be detected by DLS.

3.2 Experimental Analysis

A mixture of exosomes (sample diluted 5x from initial, unknown concentration with 10mM HEPES buffer) and aptamer (5nM) were premixed and injected into the separation chamber. The direction of the electric field is from 3000 µm extremity to the 0 µm extremity. The scan is conducted three times to account for statistical anomalies. Both the free aptamer and the aptamer-exosome complex are seen in the electropherogram obtained. When no voltage was applied, both the aptamer and the aptamer-exosome complex eluted at around the 1500 µm position, which is approximately the centre of the device. When a voltage of 40V was applied across the separation chamber, the aptamer-exosome complex is eluted at around between 1150 µm and 1300 µm due their reduced electrophoretic mobilities. The large elution range can be attributed to the polydispersed nature of the sample. The free aptamer, due to its increased electrophoretic mobility is eluted at 1080 µm. A control sample with just aptamer (5 nM) showed only a free probe peak (at 1080 µm) with very little fluorescence detected between 1150 and 1300 µm, where the aptamer-exosome complex has eluted. The results are shown in Figure 2.

This experiment was carried out three times and it was estimated from the burst photon traces that $n_{complex}$=138 ±13 molecules were detected (mean ± standard deviation of three runs). This corresponds to the molecular flux in the regions where the single-molecule time traces were detected.

Hence, the total number of exosome-aptamer complexes in the full device can be estimated by:

$$F_{total} = \frac{n_{complex}}{t} . \frac{h . d_{step}}{\frac{\pi}{4} . z . w} = 80291 \pm 3112 \; molecules/s$$

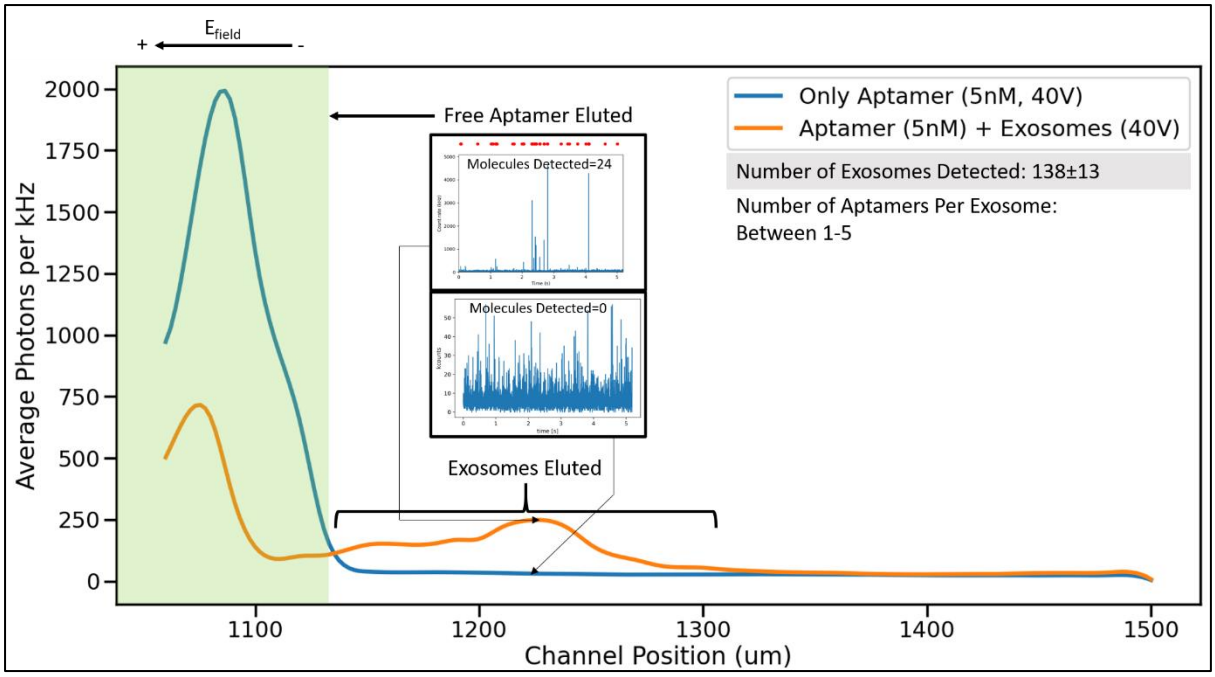


Figure 2. Elution positions of free aptamer (1080 µm) exosomes (1150 µm-1310 µm) and the control sample (1080 µm). To compare the number of molecules between the sample and the control, inter-photon time combined with a burst scan (setting a lower threshold for the detection of a photon) was used.

where *t* is the time-period over which the time traces were recorded (5 seconds), h is the height to which the separation chamber was fabricated (25 µm), d_{step} is the step size at which the single-molecule time traces were recorded (10µm), and *z* and *w* are the width and the height of the confocal detection volume, respectively, describing its cross-section. The latter two parameters were estimated from a fluorescent correlation spectroscopy (FCS) measurement and were determined to be z = 3 µm and w = 0.4 µm for our setup. As the sample was entering the device at a flow rate of Q_{sample} = 10 µL h^{-1}, the concentration of the exosome-aptamer complex can be determined as:

$$c_{complex} = \frac{F_{total}}{N_A . Q_{sample}} = 48.51 \pm 0.54\ pM$$

Where N_A is Avogadro's constant. This corresponds to the concentration of exosomes in the sample since an excess of aptamer was added. To calculate the number of aptamers per exosome, the number of photons corresponding to a single molecule of aptamer in the single molecule regime of a region where only aptamer was expected to be found was calculated (around 40 photons). This was then divided by the total number of photons emitted by an exosome-aptamer complex. Based on this, it was found that although most exosomes emit between 100-500 photons (indicating 1-5 aptamer binding sites per exosome), some exosomes

emit up to 6000 photons. It is unlikely that a single exosome has that many binding sites. What may be happening instead are aptamers binding to aggregates of up to a hundred exosomes, or an aggregate of aptamers binding to a single exosome. Another source of error is that our setup is unable to record aptamers bound to the back of the exosome (i.e. the side away from the laser).

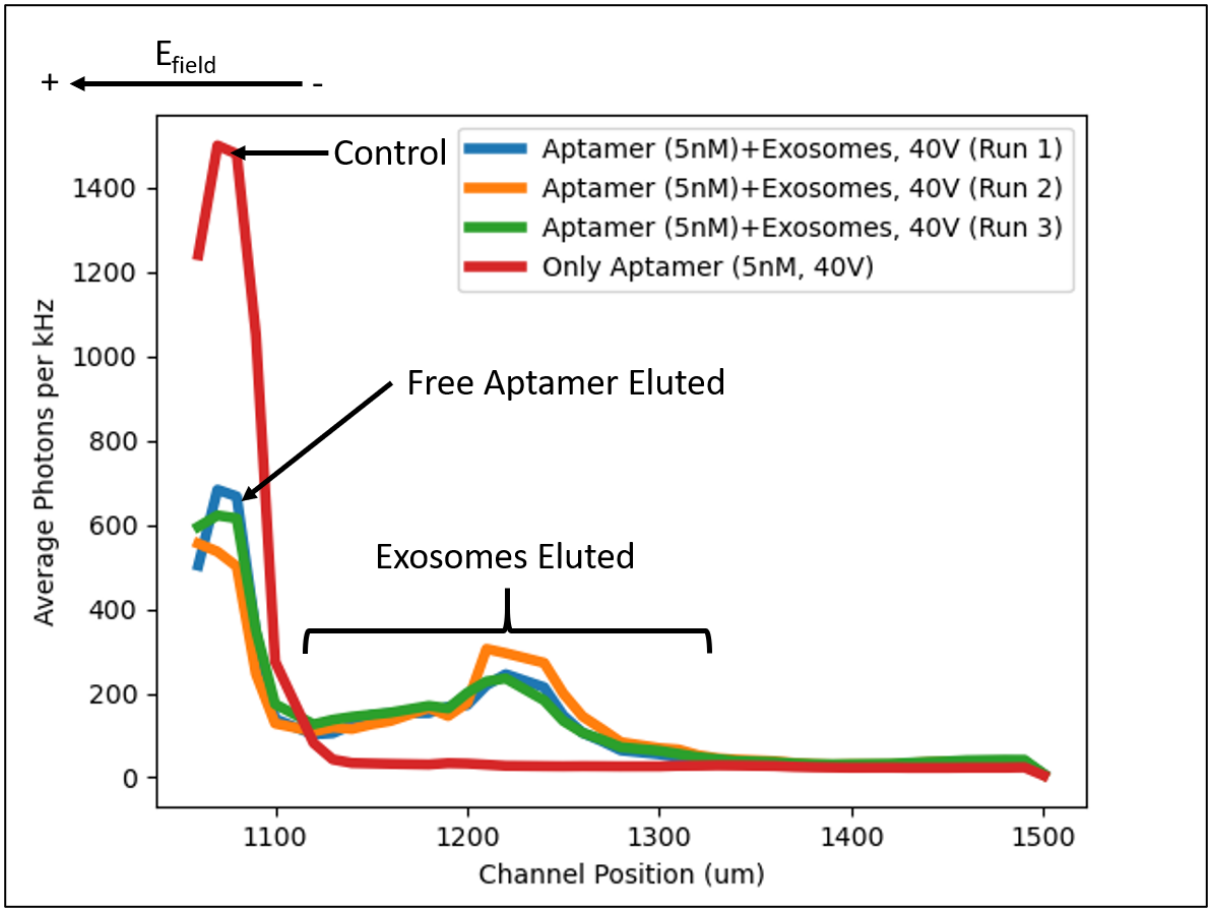


Figure 3. Three replicates of the experiments carried out for the aptamer-exosome complex. A good degree of convergence is seen.

Using a python script, the mobilities of the exosome-aptamer complexes as a function of the number of binding sites was calculated. The results are shown in Figure 4. No direct correlation is visible: we would expect a larger number of binding sites to correspond to a larger exosome, and hence, reduced mobility. However, this is not seen, perhaps because the charge of the exosome changes depending on the number of aptamers bound to it, creating another variable in the mobility.

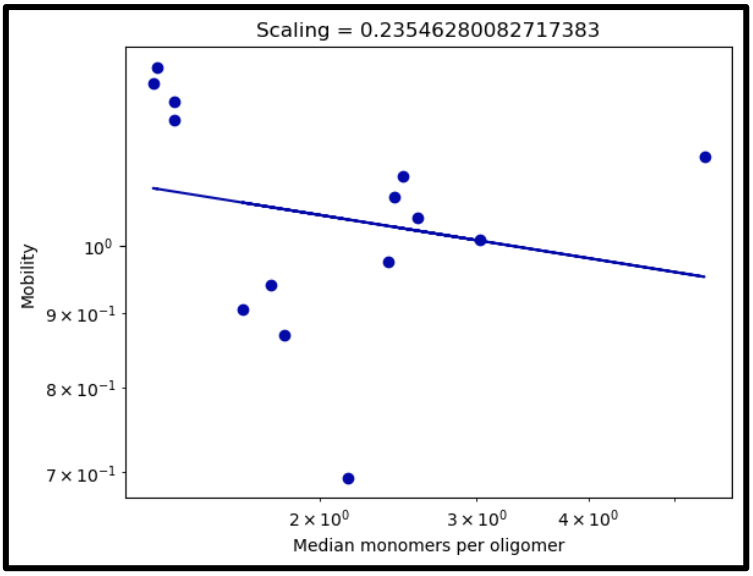


Figure 4. Mobility of exosomes as a function of number of aptamers bound per exosome on a log-log scale. No direct correlation is seen. The regression coefficient for the graph is 0.235 indicating a low degree of correlation.

Protein Concentrations of Exosomes:

To compare different exosome samples, the amount of protein present in each sample (mg/ml) was estimated using the Nanodrop device. The results are summarized as follows:

Age of Sample	*Date cell line was thawed*	*Buffer*	*Run 1 (mg/ml)*	*Run 2 (mg/ml)*	*Run 3 (mg/ml)*	*Average (mg/ml)*
1 day	One week before harvesting	1mM PBS	0.165	0.170	0.155	163.33
> 1 month	One week before harvesting	1M HEPES	0.3285	0.3435	0.3650	0.3513
> 1 month (frozen)	One week before harvesting	1mM PBS	0.2235	0.2190	0.2190	0.2207
> 1 month	One week before harvesting	1mM PBS	0.4385	0.7815	0.5010	0.6123
1 day	Two weeks before harvesting	1mM HEPES	0.585	0.6255	0.6240	0.6012

Table 1. Protein concentrations in different exosome samples measured in (mg/ml) using Nanodrop

The concentration of proteins detected increases significantly with the age of the sample. This could possibly be due to rupture of exosome membranes/other cell debris. Hence, many of our measurements early on in the project have been discarded, since older samples were used.

Freezing seems to be an effective way to preserve the condition of exosomes since not much difference in protein concentration is seen between the frozen and unfrozen samples. It is also clear that waiting for a longer time to harvest exosomes after the cell line is defrosted is beneficial, since a higher concentration of exosomes is seen, which is supplemented by both out results from the optofluidics experiments and results from the Nanodrop. This may be explained by the fact that the increased time gives an opportunity for more exosomes to be released.

4. Conclusion

In summary, we have developed a quick, effective method for the direct sensing of exosomes in solution. The low timescale of our technique (<1 minute) means that dissociation of the aptamer/exosome complex is avoided. We can calculate the concentration of exosome at a picomolar regime. The single molecule nature of our technique also means that we are able to gather crucial information about the number of binding sites per exosome.

Future work on this project will focus on negative controls: i.e using the CD63 aptamer with lipid nanoparticles, or another aptamer (the conjugate of which we do not expect on the exosomes) with the exosomes. There is also much to be learnt about the limit of detection (LOD) of our method and the timescales on which it is effective (i.e. if the experiments are carried out over a few hours, is sensitivity hampered because of the dissociation of the aptamer-exosome complex). Finally, it will be interesting to compare the results between cell-line derived exosome and exosomes harvested from patients. All of these results need to be benchmarked against standard characterization techniques (Western Blot, NTA and TEM) to showcase the effectiveness of our technique. For example, the exosomes can be quantified by particle number (NTA) and/or by total protein amount (BCA). This can be normalized to cell count (number of cells at the time of exosome harvest). Additionally, sensing of ultra-low concentration of exosomes, perhaps directly from plasma may also be possible.

Appendix A: Experimental Learnings

- Exosomes should be suspended in 10mM HEPES and not in PBS because salt content in PBS prevents effective electrophoresis.
- The aptamer stock solution should be prepared in DI water or TE buffer and not HEPES buffer

- The exosomes should be passed through a 220nm filter to remove larger cell debris
- The exosomes should be diluted in HEPES without Tween as Tween may damage the exosomes.
- Exosomes should be used as quickly as possible (within 3-5 days)
- Exosomes should preferable be harvested 2-3 weeks after defrosting the cell line as higher concentrations of exosomes are seen at this point.
- If the sample stream is deflected (not seen at the 15 micrometre mark), or the signal is not strong enough, this probably indicates the presence of an air bubble in the chamber.
- If current is not recorded by the ammeter, this is potentially because the electrodes are not pressing firmly enough into the PDMS chip, or one of the electrolyte streams is not flowing.
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5. References

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