

Supplementary Material

Multiplex Isolation and Profiling of Extracellular Vesicle using microfluidic DICE Device

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Contents

S1. Biotinylation of cells and extracellular vesicles	3
S2. Estimation of biotinylating agent amount for extracellular vesicles	5
S3. Biotinylation of A549 derived extracellular vesicles and NTA analysis	6
S4. Scanning electron microscope analysis of EVs from clinical samples	5
S5. Clinical information of samples.....	8
S6. Reagents	9
S7. References	10

Figures

Fig. S1. Immobilization of biotinylated MDA-MB-231 cancer cells on chip	3
Fig. S2. Immobilization of biotinylated B16FOva cell derived EVs on chip	4
Fig. S3. NTA analysis of biotinylated A549 derived-extracellular vesicles	6
Fig. S4. Scanning electron microscope analysis of EVs from cancer patients	7

Tables

Table S1. The clinical information of patients	8
Table S2. The antibodies and dyes used for this study	9

S1. Biotinylation of cells and extracellular vesicle

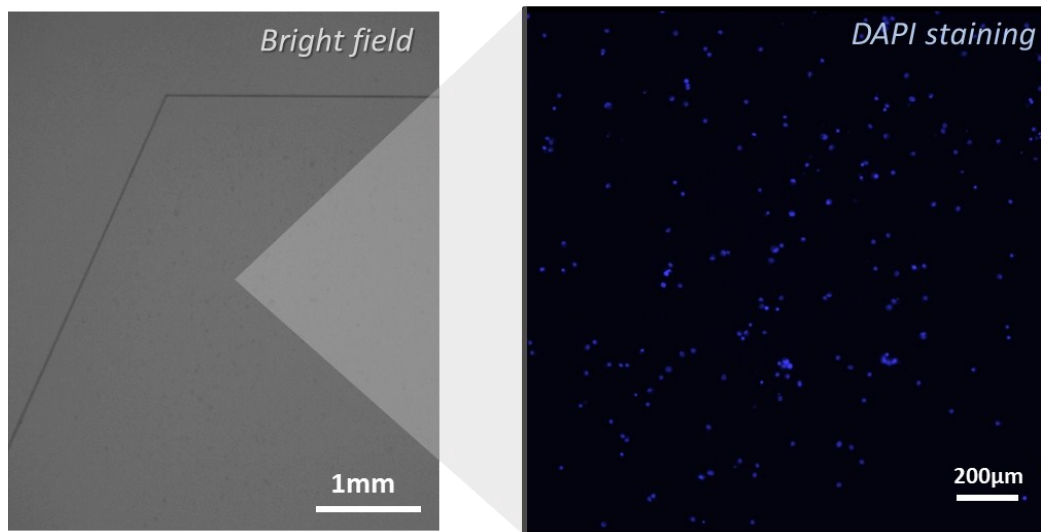


Figure S1. Immobilization of biotinylated MDA-MB-231 cancer cells on chip

Initial tests of the biotinylation and ability of NeutrAvidin to immobilize biotinylated cells/extracellular vesicles were conducted using a small chamber microfluidic device without quadrants. The small chamber device was functionalized using the same procedure as with the DICE device. Because the biotinylation reagent (EZ-link-Sulfo-NHS-LC-Biotin) we used was originally formulated for cell applications, we first attempted biotinylation of breast cancer cells, MDA-MB-231. Cells were biotinylated following the manufacturer's protocol. Roughly 2mM biotin reagent was used for 3×10^6 cells. In order to remove excess biotin reagent, we stopped the reaction using glycine solution, mildly centrifuged and used only the cell pellet.

To confirm the efficacy of the biotinylation and the efficiency of avidin-based cell capture, 10 μL of biotinylated cell stock solution in 90 μL of PBS were injected into the prepared small chamber microfluidic device and incubated for 30 minutes. The unbounded cells were removed by further PBS washes. Cells were then stained with 2.5 μL of DAPI in 100 μL of 1% BSA that was injected into the small chambered device and incubated for 30 minutes. A final PBS wash was used to remove excess DAPI. Each device was imaged using fluorescence

microscopy. The results of biotinylation and immobilization of MDA-MB-231 cells is shown in **Fig. S1**. This result confirmed that biotin/NeutrAvidin capture chemistry can successfully capture cells on microfluidic device and potential of this system for EVs.

We then tested whether the biotin-avidin chemistry would extend itself to EV immobilization on chip. Pre-harvested mouse ovarian cancer cell line (B16FOva) derived EVs were purified and biotinylated using the previously described protocols. 10 μL of biotinylated EVs in 90 μL of PBS were prepared. Lipophilic dye, PKH green, was then applied to the prepared EV sample and incubated for 10 minutes. After this staining, we stopped the reaction with exosome depleted FBS and this mixture was then processed using an ExoSpin column. The flow-through was supplemented with PBS to bring the final EV solution volume to 100 μL . All 100 μL of the sample solution was injected into the small chambered device with/without NeutrAvidin functionalization. After a 30-minute incubation, all devices underwent a PBS wash step to remove unbounded EVs. Results of the biotinylated B16FOva EVs immobilized on chip are shown in **Fig. S2**. The NeutrAvidin functionalized microfluidic device surface successfully immobilized the biotinylated EVs from B16FOva cells, while the device without surface modification captured negligible EVs. This result suggests the capture chemistry we intend to utilize for the DICE device is effective at capturing biotinylated EVs.

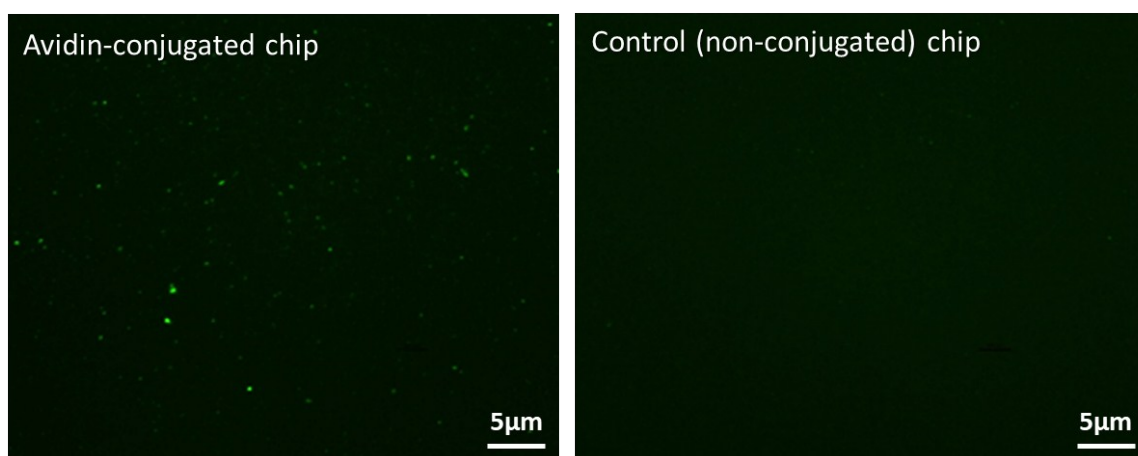


Figure S2. Immobilization of biotinylated B16FOva cell derived EVs on chip

S2. Estimation of biotinylating agent amount for extracellular vesicles

As our biotinylating agent is designed for cell and protein applications, we need to determine an initial concentration of the agent to be used for EV application. Thus far, 333 μ M to 2.2mM range of EZ link agents has been utilized for EV application.^{S1,S2} Here, we used 300 μ M of EZ link solution and this amount is sufficient for EV biotinylation based on our assumptions and calculations below. Since we do not have exact surface protein(mg) quantity on EVs, we hypothesized that our 'Pure EV' has a concentration of 1 μ g of total protein corresponding to 3x10¹⁰ of EVs.^{S3} Also, we used an estimation that 20% of cellular proteins are membrane^{S4} bound, so we applied this same ratio to EVs. We have about 2x10⁹ exosomes per mL of plasma, and thus approximately 0.066 μ g of protein/ mL of plasma sample. Using the 20% estimation, the surface protein concentration is 0.013 μ g of protein/ mL of plasma sample.

The concentration of protein in μ mol = (μ g of protein/mL of sample)/(MW of protein in KDa)

Concentration of protein = 0.013 μ g/ 5 KDa = 0.0026 μ mol

Because the manual of EZ link recommends 20 molar excess of total protein,

Concentration of Biotin= 20* Molar concentration of exosome protein = 0.052 μ mol.

Given the calculated biotin concentration needed for pure EV samples is significantly lower than our experimental concentration, the excess applied biotin should be sufficient to biotinylate our plasma derived EV samples. Further studies regarding an actual portion of EV membrane protein or correlation between protein amounts and EV concentration might be helpful for a more feasible estimation.

S3. Biotinylation of A549 derived exosomes and NTA analysis

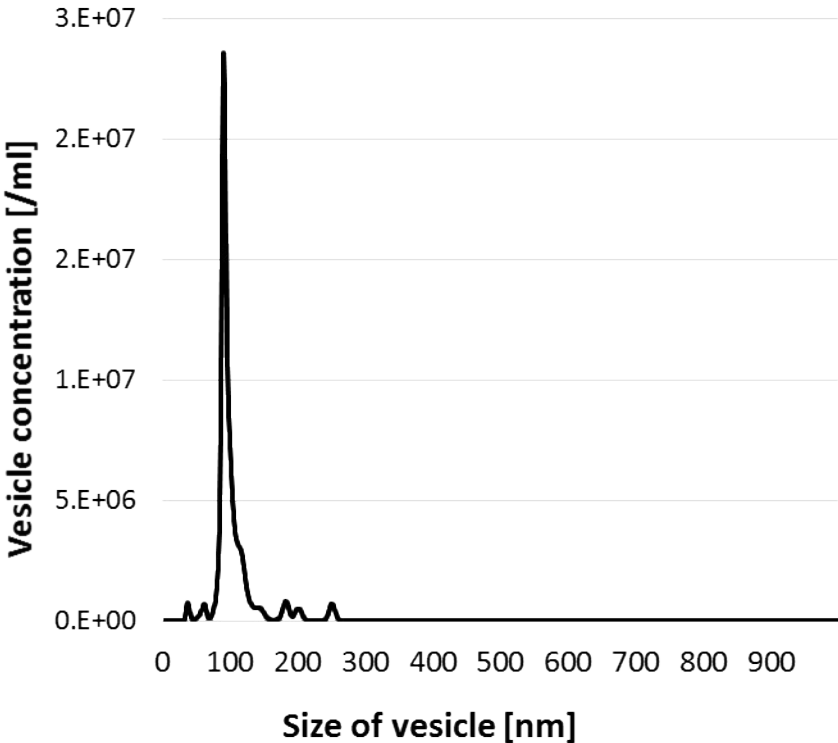


Figure S3. NTA analysis of biotinylated A549 derived-extracellular vesicles

S4. Scanning electron microscope analysis of EVs from clinical samples

By using our setup to isolate and profile EVs, we applied this to plasma samples from melanoma and prostate cancer patients. Comparing to the results with biotinylated A549, more heterogeneous size and irregular shape of EVs were found from the experiments with clinical samples. Because all cells secrete EVs, clinical sample will include EVs from various cell types. At the same time, our protocols and device can be useful for evaluation of the EV shape and size distributions without prior knowledge of the EVs.

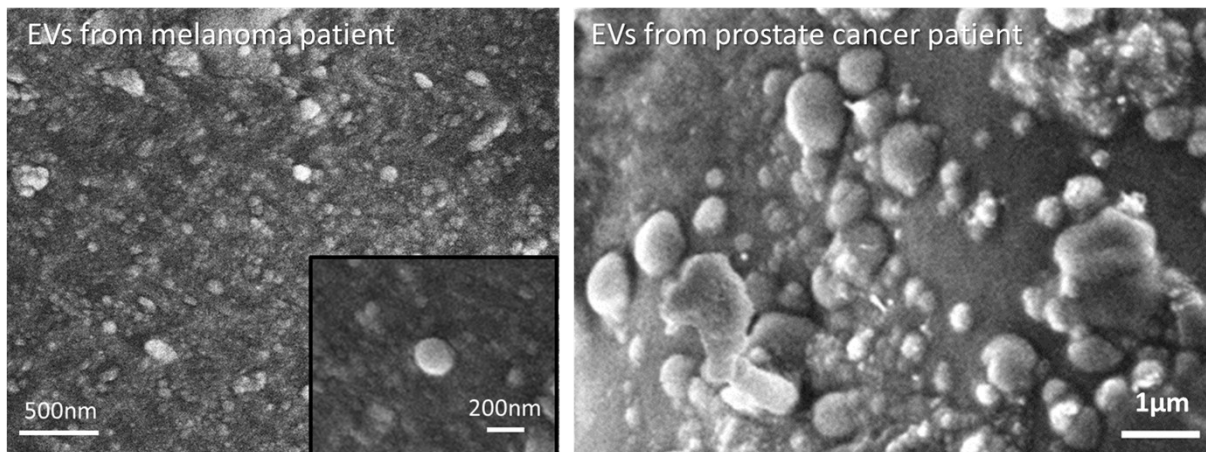


Figure S4. Scanning electron microscope analysis of EVs from cancer patients

S5. Clinical information of samples

Table S1. The clinical information of patients

Cancer Type	Sample ID	Sample description				
		Sex	Age	Stage	Location	Subtype
Lung cancer	<i>LP1</i>	Male	83	IIIA	Lung	Squamous cell
	<i>LP2</i>	Male	60	III	Lung	Squamous cell
	<i>LP3</i>	Female	81	IIIB	Lung	Squamous cell
	<i>LP4</i>	Male	74	IIIB	Lung	Squamous cell
	<i>LP5</i>	Female	81	IIIB	Lung	Squamous cell
Healthy control	<i>HC1</i>	Male	56	NA	NA	NA
	<i>HC2</i>	Female	24	NA	NA	NA
	<i>HC3</i>	Male	26	NA	NA	NA
	<i>HC4</i>	Male	24	NA	NA	NA

S6. Reagents

Table S2. The antibodies used for this study

		Host	Reactivity	Ratio	Catalog number (Company)
Primary	PD-L1	Mouse	Human	1:20	329702(BioLegend)
	Vimentin	Rabbit	Human	1:20	5741(CellSignaling)
	CD9	Rabbit	Human	1:20	13174(CellSignaling)
	EGFR	Rabbit	Human	1:20	4267S (CellSignaling)
Secondary	Alexa Fluor 647	Goat	Rabbit	1:40	A-21235 (Invitrogen)
	Alexa Fluor 488	Goat	Mouse IgG2b	1:40	A-21242 (Invitrogen)

S7. References

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