Supporting information for

Rapid exosome isolation and *in situ* multiplexed detection of exosomal surface proteins and microRNAs on microfluidic platform

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Microfluidic Chip Fabrication

A soft lithography method is used to obtain a mold on a silicon wafer according to the methods described elsewhere [1, 2]. The detailed processes were as follows. First, the silicon wafer was repeatedly ultrasonically cleaned three times with ethanol and water, dried by nitrogen, and baked at 95 °C for 1 h; the second step was to spin-coating a surface of the silicon wafer with a layer of SU-8 2050 negative photoresist, which was about 50 μm thick. The speed and time of spin-coating were optimized according to the required height of the micro-column. After spinning, the wafer was prebaked at 95 °C for 5 min, and then exposed to UV light through the transparent mask. The third step is to use the printed mask for exposure, then post-baking, development, nitrogen drying and silanization for more than 3 h. The mixture of PDMS prepolymer and curing agent in a weight ratio of 10:1 were poured onto the mold, degassed, and cured at 75 °C in a conventional oven for 2 h. The cured PDMS replica was removed from the mold, subjected to a 90 s oxygen plasma treatment, and bonded with a glass substrate to form the final device. The microfluidic device is further cured at 75 °C for 2 h to reinforce the bonding.

Surface modification of microfluidic chip

The microchannel was modified with CD63-Apt according to the previous reported literature [3]. Firstly, the microchannel was treated with 4% volume concentration of MPTS ethanol solution for 30 min, cleaned the microchannel with ethanol, and Dried at 100 ℃ for 1 h. After the chip was dried, the microchannel was treated with 0.01 μmol/ml GMBS for 30 min, cleaned with ethanol, and dried at 100 ℃ for 1 h. 10 μg/ml streptavidin in PBS solution was injected into the microchannel and incubated for 1 h. After that, the channel was washed with PBS, and 1μM of biotin labeled CD63 aptamer (CD63-Apt, Table S1) was finally added and incubated for 30 min, followed by washing the

unbound CD63-Apt with PBS buffer. The modified microfluidic chip could be stored in the upper layer of the refrigerator for one week. To ensure the saturation modification of the microchannel modified with CD63-Apt, different concentration of the aptamer labeled with FAM fluorescent group was used to modify and the fluorescence images were obtained by inverted fluorescence microscope. The photos were then processed with ImageJ software.

Cell culture and exosomes isolation

The human non-small cell lung cancer (NSCLC) cell lines (A549, H1975) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). A549 and H1975 cells were cultured in DMEM and RPMI1640 respectively, with 10% FBS, 1% penicillin and streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells were passaged every 48 h under sterile conditions. To reduce interference from FBS, cells were washed twice with PBS and cultured in serum-free medium for 48 h prior to harvest of the supernatant containing exosomes.

First of all, cell culture supernatant was ultracentrifuged (2,000 g, 20 min) to remove intact cells, the supernatant was collect and then centrifuged (10,000 g, 30 min) to remove cell debris, and large micro-vesicles. The supernatant was collected and then centrifuged (110,000 g, 70 min) to isolate cell-derived exosomes. In this study, all centrifugation performances were carried out at 4 ℃ unless mentioned. The exosomes were washed in a large volume of PBS to eliminate protein impurity, and then centrifuged again. These nanoscale vesicles were resuspended in 100 μL PBS and stored at -80 ℃ prior to use.

Exosome characterization and quantification

Transmission electron microscopy (TEM). 10 μL of purified exosomes from A549 cells culture supernatant was loaded on a 400 mesh carbon-coated copper grid for 10 min, followed by straining

with 2 % phosphotungstic acid (pH 6.5) for 2 min and excess solution was removed by filter paper. The grid was then allowed to dry completely at room temperature. Transmission electron microscopy (TEM, FEI Tecnai G2 spirit) was then applied to capture the morphology of collected exosomes at 120 kV.

Nanoparticle tracking analysis (NTA). To quantify the concentration and size distribution of exosomes, exosome samples were characterized using NTA (NS300, Malvern Instrument, England). exosome concentrations were adjusted to 10^8–10^9 particles mL^{-1} to achieve optimal counting accuracy.

Western blot analysis. The concentration of total proteins was measured by BCA assay (Sangon Biotech, Shanghai, China). To perform western blots, the exosomes were treated with ice-cold RIPA lysis buffer for 5 min and then boiled for 5 min at 95 ℃. All protein lysates were resolved under non-reducing conditions. Protein lysates were separated by 12 % SDS-PAGE gel electrophoresis, then transferred onto a PVDF membrane (Beyotime, Shanghai, China) followed by blocking with 5 % (w/v) skimmed milk in TBST for 1 h at room temperature. Following incubation with primary antibodies against exosomal marker proteins overnight at 4 ℃, the membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibodies for 60 min at room temperature, and then washed again. The signal was visualized by enhanced chemiluminescence (Thermo Fisher Scientific, United States).

qRT-PCR analysis. RNA was extracted from exosomes using the Exosome RNA Isolation kit (Rengen Biosciences, China) according to the manufacturer's protocol and stored at -80 °C until use. The RNA concentration and purity were evaluated using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNAs (cDNAs) were synthesized by using Mir-X

miRNA First-Strand Synthesis Kit (TaKaRa, Japan). Reverse transcription PCR amplification was performed using an ABI PRISM 7500 instrument (Applied Biosystems, USA) with a TB Green Advantage® qPCR Premix and mRQ 3' Primer (TaKaRa, Japan), which is specific for mature miRNA sequences. U6 small nuclear RNA (snRNA) was used as the internal control.

Feasibility verification and optimization for CHA reaction

The hairpin probes for miRNA-21 and miRNA-200 were annealed before the non-enzymatic amplification experiment. They were then denatured at 95 ℃ for 10 min, followed by a gradient cooling process (1°C/min) to room temperature over one hour, which allowed the probes correctly fold into a complete hairpin structure. The prepared probes were then stored at 4 ℃ until use. In order to verify the feasibility of the designed hairpin probes, all DNA probes were annealed, and then seven mixtures (target miRNA, H1, H2, miRNA+H1, miRNA+H2, H1+H2, miRNA+H1+H2) were prepared for fluorescence spectrum assays. The molar ratio of H1 to H2 was also optimized to obtain optimal performance of CHA reaction system. The fluorescence excitation and emission were monitored at 488 nm and 519 nm. Fluorescence spectrum assays were carried out in a 96-well plate (Costar, Washington, DC, USA) using a microplate reader (Tecan infinite M1000 Pro, Männedorf, Switzerland).

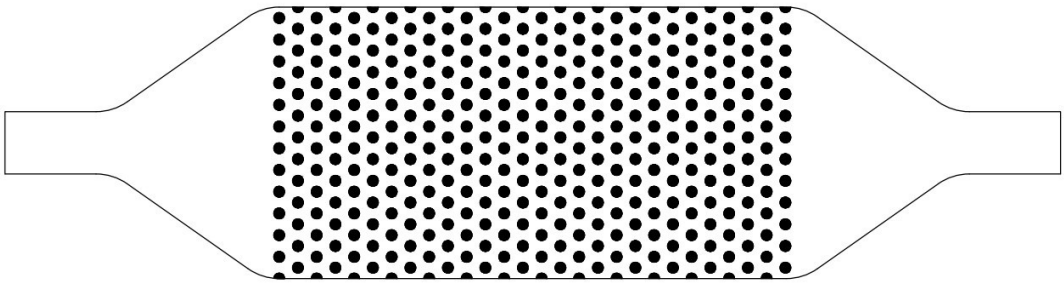

Fig. S1. The designed ship-shaped microfluidic channel with a microcolumn array

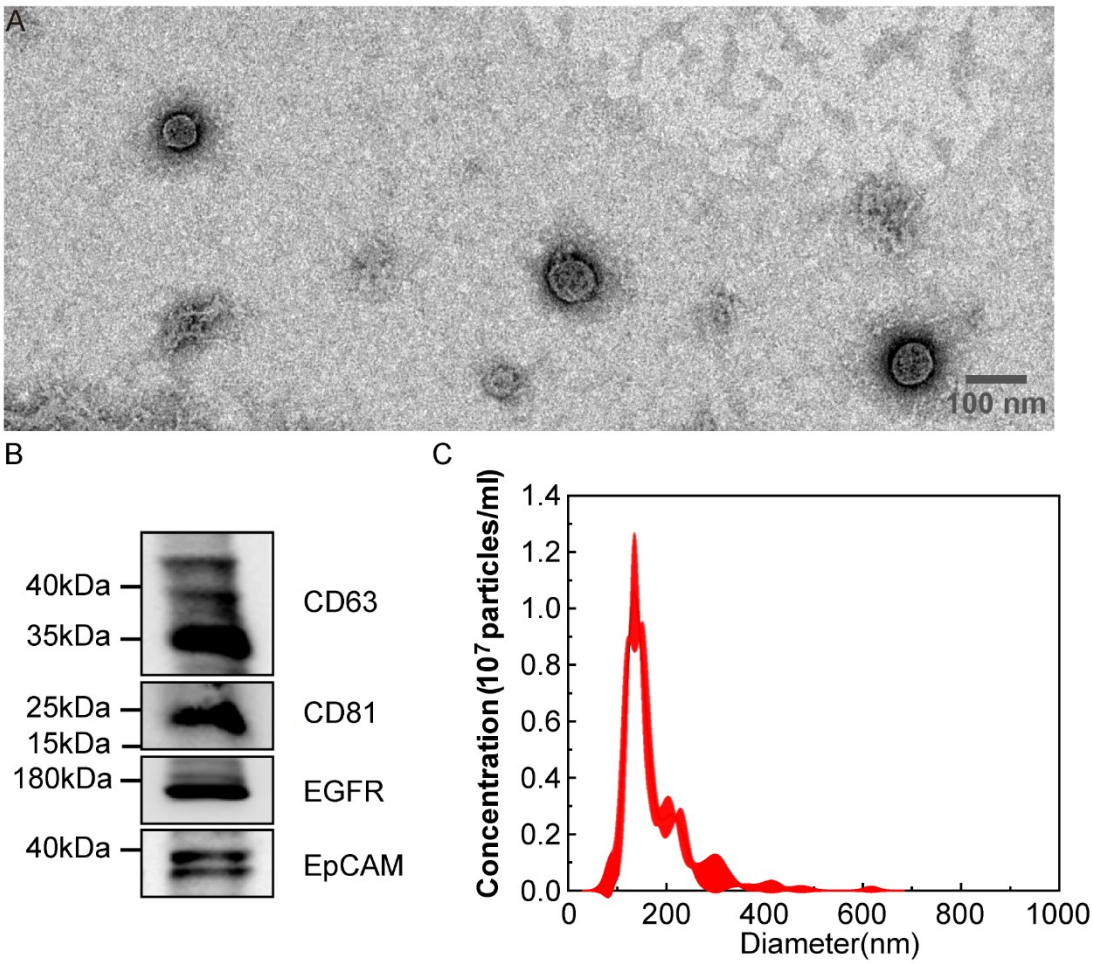

Fig. S2. Exosomes characterization. (A) Exosomes purified were monitored for their morphological characterization under TEM. (B) Western blot analysis of the A549 exosomes. (C) Statistical determine exosomes concentration and size distribution by NTA.

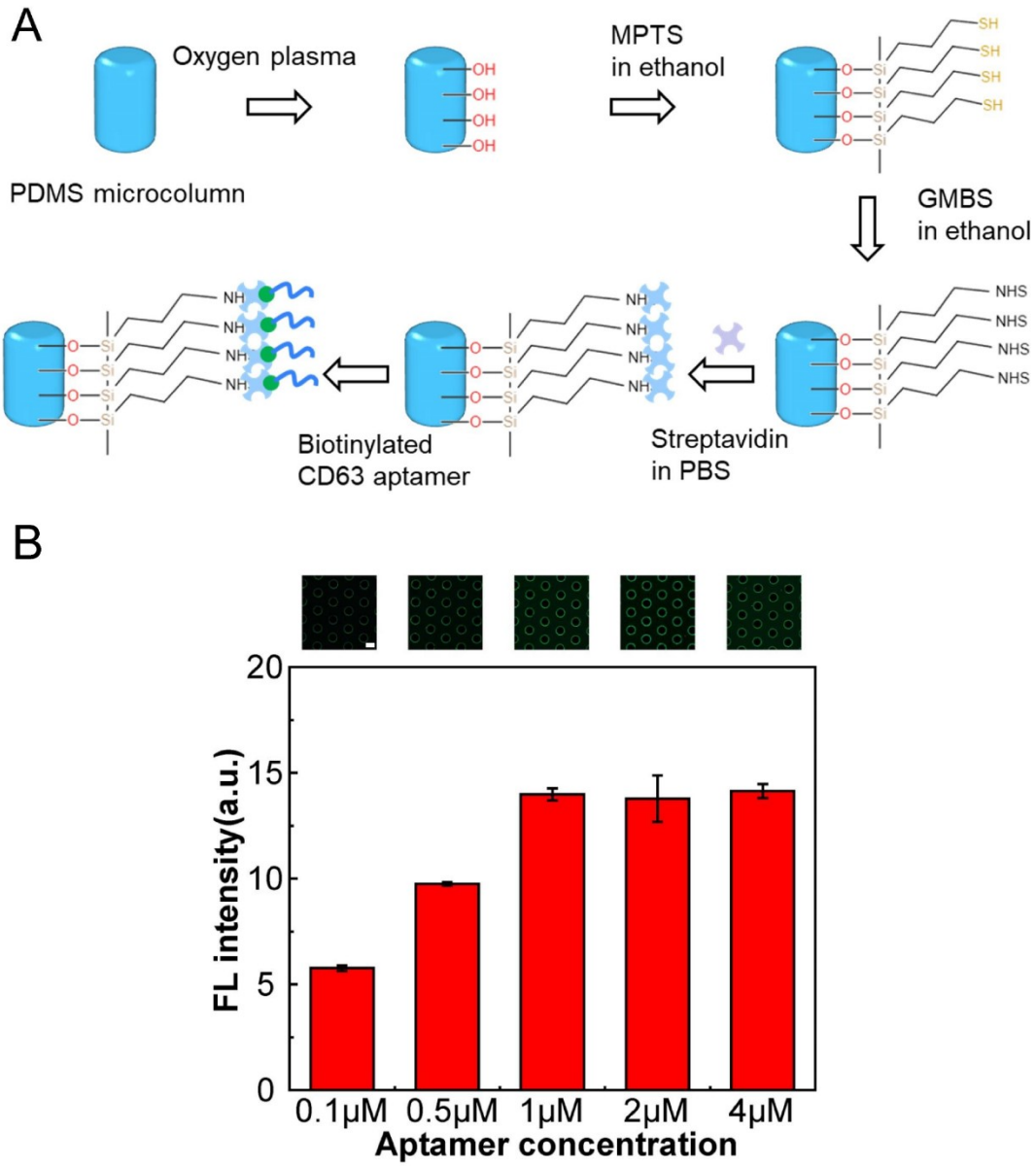

Fig. S3. The schematic of the surface modification on the microfluidic chip. (A) Schematic diagram of surface modification of CD63-Apt on microcolumn. (B) CD63-Apt concentration optimization. Scale bar: 100 μm. The standard error bar represents the variables of three independent experiments.

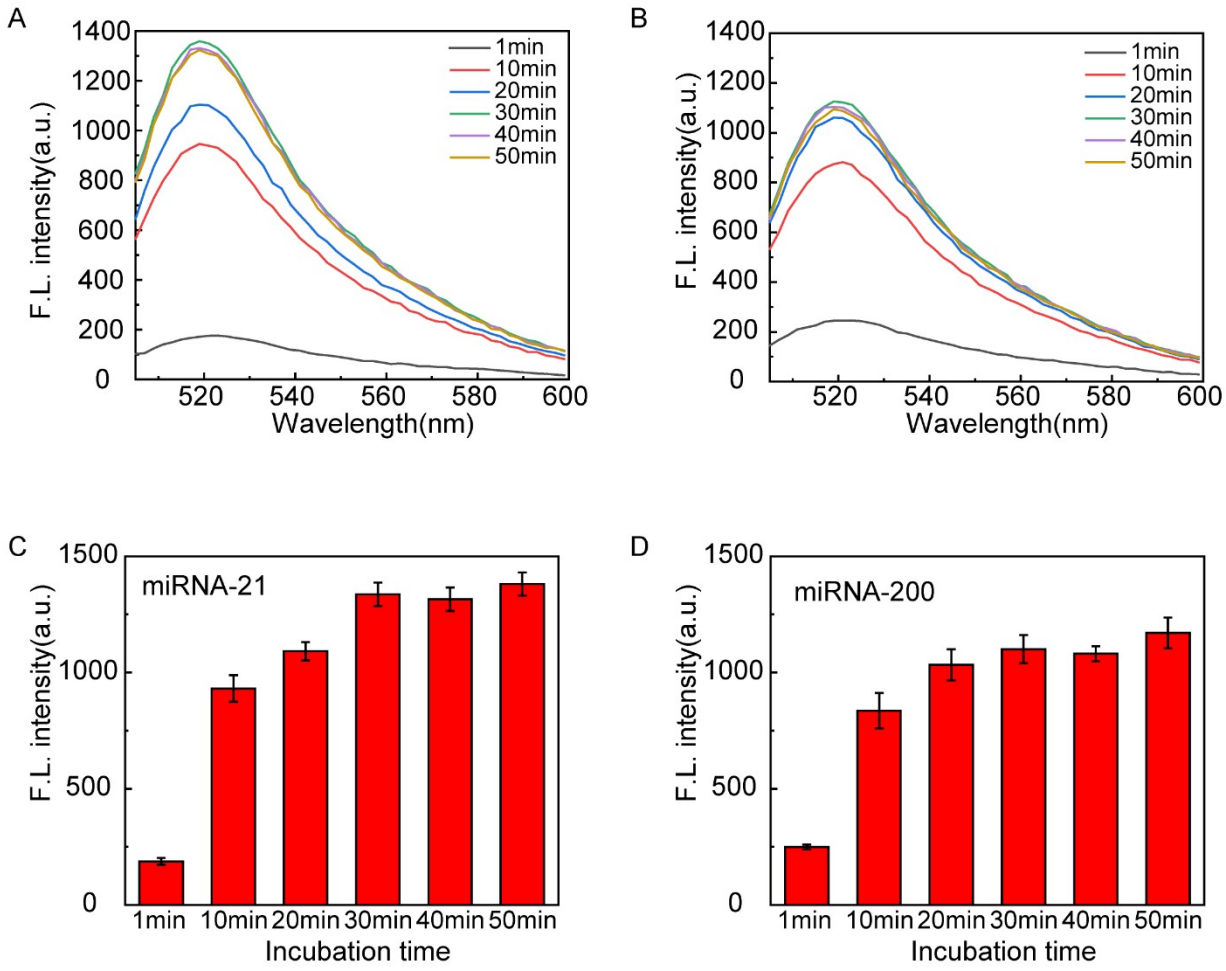

Fig. S4. The relationship between CHA reaction time and fluorescence intensity, the reaction was achieved an equilibrium after incubation for 30 min. Fluorescence spectrum of (A) miRNA-21 and (B) miRNA-200 initiated CHA reaction with various incubation time (1min, 10min, 20min, 30min, 40min, 50min). Fluorescence intensity of (C) miRNA-21 and (D) miRNA-200 initiated CHA reaction with various incubation time. The standard error bars mean the variation of three independent experiments.

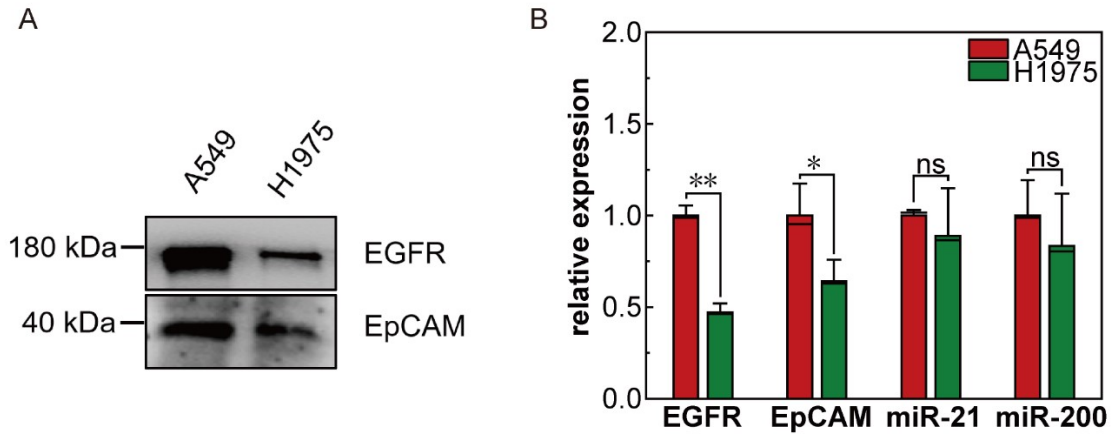

Fig. S5. Characterization of exosomes from A549 and H1975 cells. (A) Western blot analysis of the two exosomal proteins, EGFR and EpCAM. (B) Real-time quantitative PCR analysis was performed to quantify exosomal miRNA-21 and miRNA-200. All the proteins and miRNAs levels were normalized to the expressions derived from A549 cells. The standard error bar represents the variables of three independent experiments.

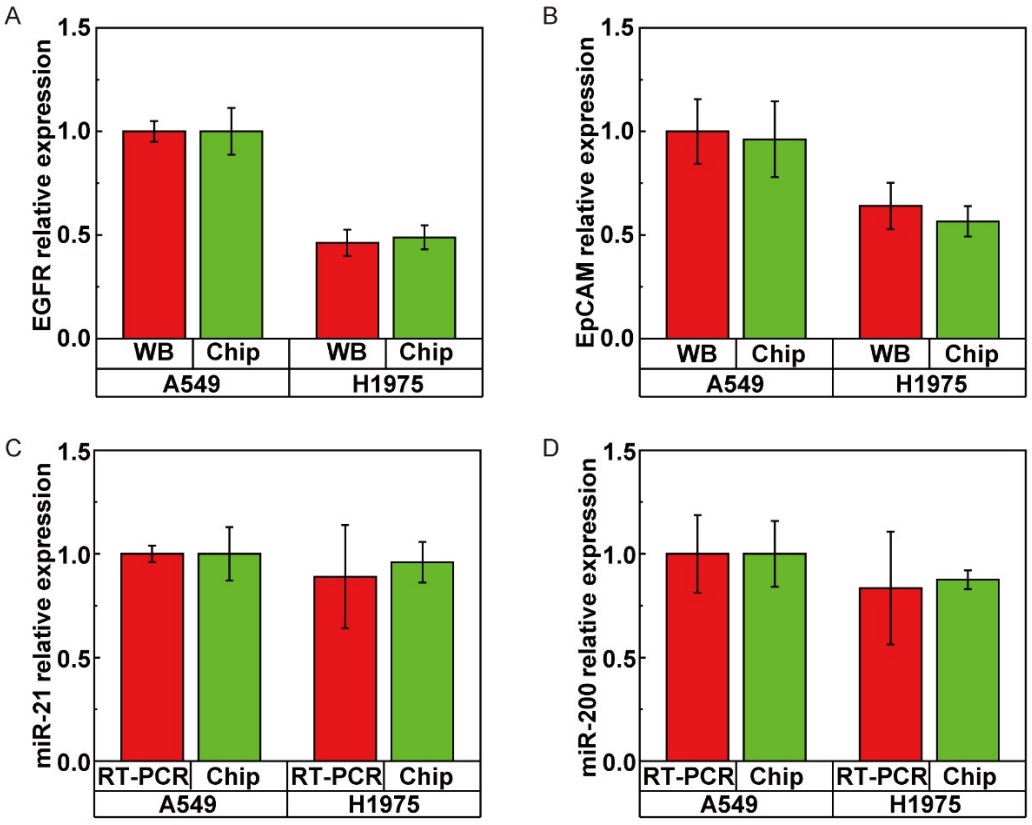

Fig. S6. Comparison of the results obtained by the conventional method for (A) EGFR, (B) EpCAM, (C) miR-21, and (D) miR-200 with those obtained by the microfluidic chip assays. The standard error bar represents the variables of three independent experiments.

Table S1 Sequences of DNA/RNA oligonucleotides used in this work

DNA or RNA	Sequence(5'-3')
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-200	UAAUACUGCCUGGUAAUGAUGA
H1-21	TCAGACTGATGTTCGTAGCTTATCAACATCAGT CTGATAAGCTA
H2-21	FAM-TTCGTAGCTTATCAGACTGATGTTGATAA GCTACGAACATCAGT-Dabcyl
H1-200	GCCTGGTAATGATGATAATACTGCCTGTCATCA TTACCAGGCAGTATTA
H2-200	FAM-ATGATAATACTGCCTGGTAATGATGACAG GCAGATTATCATCATTACC-Dabcyl
CD63-Apt	Biotin-TTTTTTTTTTTTCACCCCACCTCGCTCCCG TGACACTAATGCTA
CD63-Apt-FAM	Biotin-TTTTTTTTTTTTCACCCCACCTCGCTCCCG TGACACTAATGCTA-FAM
EpCAM-Apt-Cy5	Cy5-AGTGACGCAGCATGCGGCACACACTTCTA TCTTTGCGGAACTCCTGCGG
EGFR-Apt-Cy5	Cy5-TACCAGTGCGATGCTCAGTGCCGTTTCTTC TCTTTCGCTTTTTTTGCTTTTGAGCATGCTGACG CATTCGGTTGAC

Table S2 Detection performance of microfluidic based exosome analysis

Analytical method	Detection limit	Operation time	Linear range	Reference
Fluorescent biosensor	10^3 exosomes/μL	4h	NA	[4]
Fluorescent biosensor	28 exosomes/μL	2h	10-10^6 exosomes/μL	[5]
Fluorescent biosensor	21 exosomes/μL	NA	10^2-10^6 exosomes/μL	[6]
ECL biosensor	31 exosomes/μL	6h	50-10^5 exosomes/μL	[7]
Fluorescent biosensor	83 exosomes/μL	5h	10-10^6 exosomes/μL	This work

References

[1] Y. Chen, D. Gao, H. Liu, S. Lin, Y. Jiang, *Anal. Chim. Acta,* 2015, ***898***, 85-92.

[2] Y. Chen, D. Gao, Y. Wang, S. Lin, Y. Jiang, *Anal. Chim. Acta,* 2018, ***1036***, 97-106.

[3] M. Zhou, D. Gao, Z. Yang, C. Zhou, Y. Tan, W. Wang, et al., *Talanta*, 2021, ***222***, 121480.

[4] C. Qian, Y. Xiao, J. Wang, Y. Li, S. Li, B. Wei, et al., *Sens. Actuator B Chem.,* 2021, ***333***, 129559.

[5] S. Zhou, T. Hu, G. Han, Y. Wu, X. Hua, J. Su, et al., *Small*, 2020, ***16***, e2004492.

[6] P. Zhang, X. Zhou, Y. Zeng, *Chem. Sci.,* 2019, ***10***, 5495-5504.

[7] H.X. Zhang, Z.H. Wang, F. Wang, Y.M. Zhang, H.Y. Wang, Y. Liu, *Anal. Chem.,* 2020, ***92***, 5546-5553.