# Supporting Information

# High-throughput and Ultra-Sensitive Single-cell Profiling of Multiple MicroRNA and Identification of Human Cancer

Lu Li, [a] Meng Lu, [a] Yuanyuan Fan, [a] Lingling Shui, [b] Shuting Xie, [b] Renjie Sheng, [a] Haibin Si, [a] Qingling Li [a] and Yiguo Wang [c] and Bo Tang,*[a]

[a] College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging, Key Laboratory of Molecular and Nano Probes, Ministry of Education Shandong Normal University, Jinan 250014 Shandong (P.R.China).

[b] Guangdong Provincial Key Laboratory of Optical Information Materials and Technology and Institute of Electronic Paper Displays, South China Academy of Advanced Optoelectronics, South China Normal University, Guangzhou 510006 Guangdong (P.R.China).

[c] Shandong Provincial Qianfoshan Hospital, the Frist Hospital Affiliated with Shandong First Medical University, Jinan 250014 Shandong (P.R.China).

* E-mail: tangb@sdnu.edu.cn

# EXPERIMENTAL SECTION

## Materials and Reagents.

All oligonucleotides including miRNAs, probes and primers (sequences shown in Table S1) were synthesized and HPLC purified by Sangon Biotech Co., Ltd. (Shanghai, China). DNTPs were also purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Bst DNA polymerase, nicking endonuclease, lambda exonuclease, NEB buffer2 were purchased from New England Biolabs (Massachusetts, USA). Diethylpyrocarbonate (DEPC)-treated deionized water were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The HFE-7500 containing 2% of PFPE-PEG was purchased from RAN Biotechnologies company (Toronto, USA). Cell lysate IGEPAL CA-630, SDS, Triton x-100 were purchased from Sigma company (Shanghai, China). The human breast adenocarcinoma MCF-7 cells, human embryonic kidneys 293, and human erythroleukemia cell lines HEL, K562 were supplied by cancer research center of Qilu Hospital. Cell culture products, unless mentioned otherwise, were purchased from Hyclone (Logan, Utah). The $Mg^{2+}$ indicator (Mag-Fluo-4 AM) was purchased from Thermo Fisher Scientific (San Jose, CA, USA). RT-qPCR was performed using miScript single cell PCR kit (Qiagen, Germany) and miScript SYBR Green PCR kit (Qiagen, Germany), All steps were performed according to manufacturer's protocol. All the chemicals were of analytical grade and used without further purification. Deionized water was obtained through a Sartorius Arium 611 VF system (Sartorius AG, Germany) to a resistivity of 18.2 MΩ·cm.

## Instrumentation.

Fluorescence spectra measurements were carried out using a Edinburgh FLS 920 Fluorescence Spectrophotometer (Edinburgh, UK). The fluorescence signal from droplets was detected by the self-made laser induced fluorescence detector (LIFD).[1] The videos and bright-field images about droplets were obtained by Leica DFC300FX inverted fluorescence microscope (Oskar-Barnack-Straße, Germany) equipped with a high-speed camera pco.dimax cs1 (Kelheim, Germany). Fluorescence imaging studies were performed with a Leica TCS SP8 confocal laser scanning microscopy (Oskar-Barnack-Straße, Germany) with a 20× objective lens. The syringe pump containing four channels was made by Baoding Longer Precision Pump Co., Ltd. (Baoding, China). qPCR profiling was performed using Roche LightCycler 480 ( Switzerland, Germany).

**Table S1**. The sequences of miRNAs, probes and primer.

| Names | Sequences |
|---|---|
| miRNA let-7a | UGAGGUAGUAGGUUGUAUAGUU |
| miRNA let-7b | UGAGGUAGUAGGUUGU**G**U**G**GUU |
| miRNA let-7c | UGAGGUAGUAGGUUGUAU**G**GUU |
| miR-20 | UAAAGUGCUUAUAGUGCAGGUAG |
| let-7a hairpin Probe | AAGCTGAGGTCTTGGACAAACTATACAACCTACTACCTCATGTCCAAGA |
| miR-20 hairpin Probe | AAGCTGAGGTCTTGGACACTACCTGCACTATAAGCACTTTATGTCCAAGA |
| primer | TCTTGGAC |
| RNA 1 | UAGCUUAUCAGACUGAUGUUGA |
| RNA 2 | UCAACAUCAAAUAUCCGGCACACUCUGAUAAGCUA |

AA and TC in blue were thiosulfate modified. T/U in red were modified with FAM (let-7a Probe/RNA 2) and Cy5 (miR-20 Probe), A in red were modified with DABCYL. The underlined bases indicate recognition site of Nb.BbvCI nicking endonuclease. The bold are the different bases between let-7b, let-7c, and let-7a.

## Cell culture and treatment.

Human breast adenocarcinoma MCF-7 cells and human embryonic kidneys 293 cells were cultured at 37 ℃ in a humidified atmosphere of 5% $CO_2$ and 95% air using DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were passaged every 2−3 days. Before experiment, 0.25% trypsin was used to treat the cells for 30 s and then the trypsin was gently removed. Human erythroleukemia cell lines HEL and K562 cells were cultured at 37 ℃ in a humidified atmosphere of 5% $CO_2$ and 95% air using 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were collected and centrifuged at 1000 rpm for 5 min in culture medium, washed twice with PBS buffer. Then Density gradient solution Optiprep 16% (v/v) (Sigma, USA) was added into the cell suspension to disperse cells. The cell density was determined by a hemacytometer. The cell lysate was obtained after treatment of cells with 10% (v/v) CA630.

## Generation of Droplets.

The PDMS microchip was designed by our group and made by Wenhao Chip Technology Co., Ltd. (Suzhou, China). The structure and size of the chip channels are shown in Fig S1. HFE-7500 with 2% PEG-PFPE used as oil phase was input into the channel from entrance O, the cell suspension and other reagents as one aqueous phase were input into the channel from entrance C and CA630 as the other aqueous phase was input into the channel from entrance L. The oil phase was injected at the rate of 850 μL/h and the two aqueous phases were all injected at the rate of 150 μL/h.

## Quadratic isothermal amplification of miRNA.

Quadratic isothermal amplification of miRNA in tubes. Target miRNAs in 50 μL NEB buffer2 (pH 8.0) consisting of 500 nM probe, 2 μM primer, 16 U Bst DNA polymerase, 15 U Nb.BbvCI nicking endonuclease, 12.5 U lambda exonuclease, 400 μM dNTPs, at 37 ℃ for 30 min.

Quadratic isothermal amplification of miRNA in droplets. Amplification reagents consisting of 4 μM primer, 0.7 U/μL Bst DNA polymerase, 0.6 U/μL Nb.BbvCI nicking endonuclease, 0.5 U/μL lambda exonuclease, 800 μM dNTPs and 0.12 μL/μL lysis buffer CA630 were input into entrance L of the chip. A NEB buffer2 containing 16% (v/v) density gradient solution Optiprep, 1 μM miRNA probe, cell suspension or target miRNAs at different concentrations were input into entrance C of the chip. After the droplets generated in the chip, the cells were lysed, miRNAs were isothermally amplified at 37 ℃.

## Fluorescence Measurements.

After the quadratic isothermal amplification of miRNA in a tube, the fluorescence spectra of the products were obtained on a Cary Eclipse fluorescence spectrophotometer. The fluorescence of Alexa Fluor 405 was collected between 415 and 500 nm by use of the maximal excitation wavelength at 405 nm, the fluorescence of FAM was collected between 500 and 600 nm by use of the maximal excitation wavelength at 488 nm, and the fluorescence of Cy5 was collected between 650 and 730 nm by use of the maximal excitation wavelength at 633 nm.

Laser-induced Fluorescence detection of the droplets in the microchip has been descripted in our previous work. A homemade two-laser excitation and three-channel fluorescence detection system was utilized to obtain the fluorescence signal of the droplets in the chip. A 10 mW diode-pumped solid-state (DPSS) laser operating at 473 nm was used to excite the FITC and a 15 mW DPSS laser operating at 635 nm was used to excite Cy5. The

fluorescent signal was detected using a R928 photomultiplier (PMT, Hamamatsu, Japan). The collected wavelengths for the FITC and Cy5 were 525 ± 15 and 695 ± 15 respectively.

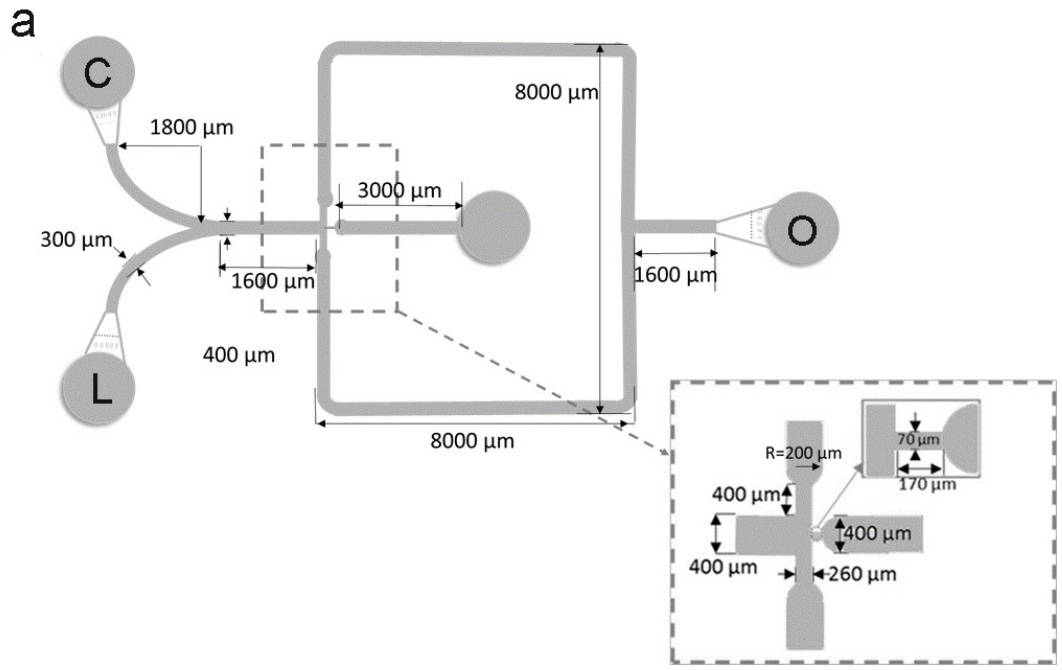
Fluorescence images of the droplets were acquired on a CLSM with different laser transmitters. The fluorescence of Alexa Fluor 405 was collected between 415 and 480 nm by use of the maximal excitation wavelength at 405 nm, the fluorescence of FITC was collected between 500 and 600 nm by use of the maximal excitation wavelength at 488 nm, and the fluorescence of Cy5 was collected between 650 and 730 nm by use of the maximal excitation wavelength at 633 nm.

## Patient sample processing.

The blood samples from patients with pancreatic cancer are received from Shandong Qianfoshan Hospital after obtaining the patient-informed consent. For patient samples, 2 mL peripheral blood samples is processed by red cell lysing reagent and directly processed by the spiral chip reported.[2]

## Reverse transcription and quantitative real-time PCR (RT-qPCR).

The RT-qPCR were carried out according to manufacturer's protocol of miScript single cell PCR kit (Qiagen, Germany). Samples from serial dilution of synthetic miRNAs were used to construct standard curves. CTCs were isolated from the patient's blood using the spiral chip. All miRNA assays were performed in triplicate wells. qPCR profiling was performed using Roche LightCycler 480 ( Switzerland, Germany).



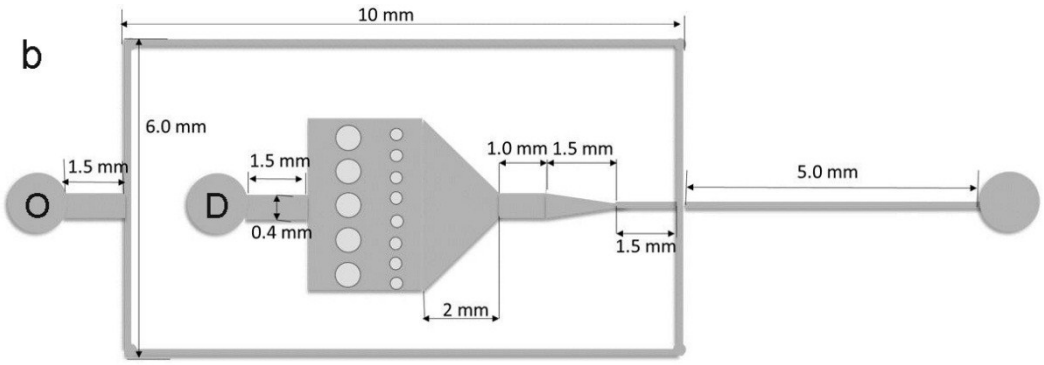



**Figure S1.** The schematic construction and dimension of the droplet microfluidic chip system. a, microchip for high throughput droplets generation; b, microchip for fluorescence detection of droplets. C, the entrance of the cell suspension and other reagents; L, the entrance of lysis CA630 and reaction buffer; O, the entrance of Oil phase; D, the entrance of droplets.

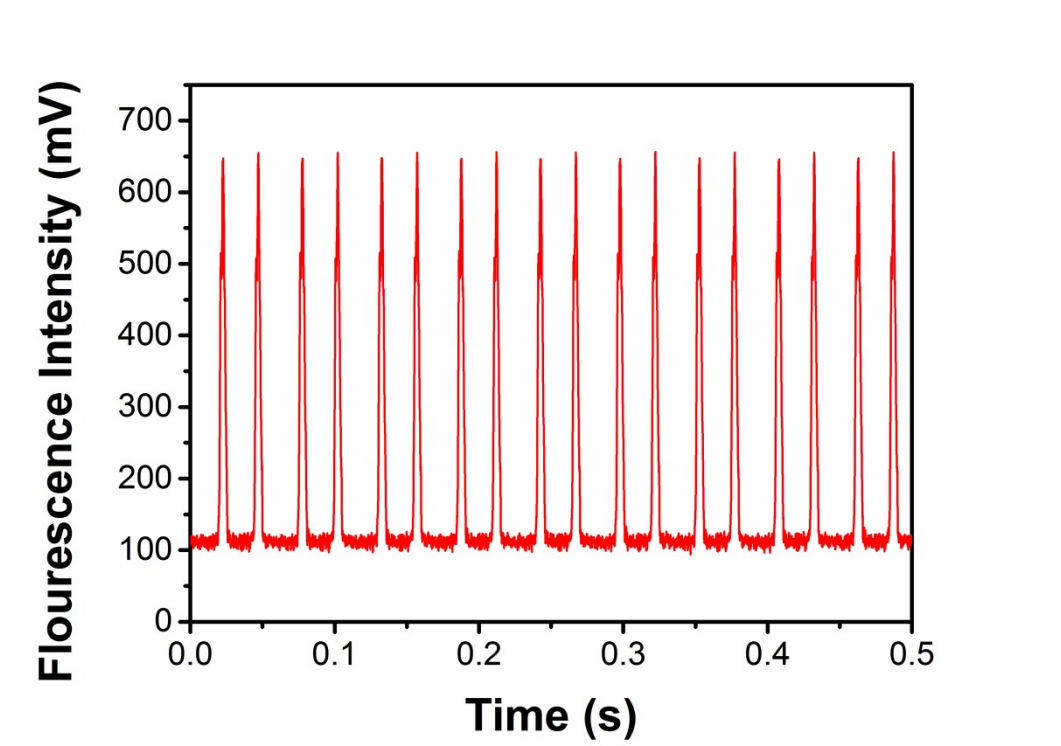



**Figure S2.** Fluorescence signal of FITC in the droplets. The concentration of FITC was 10 μM.

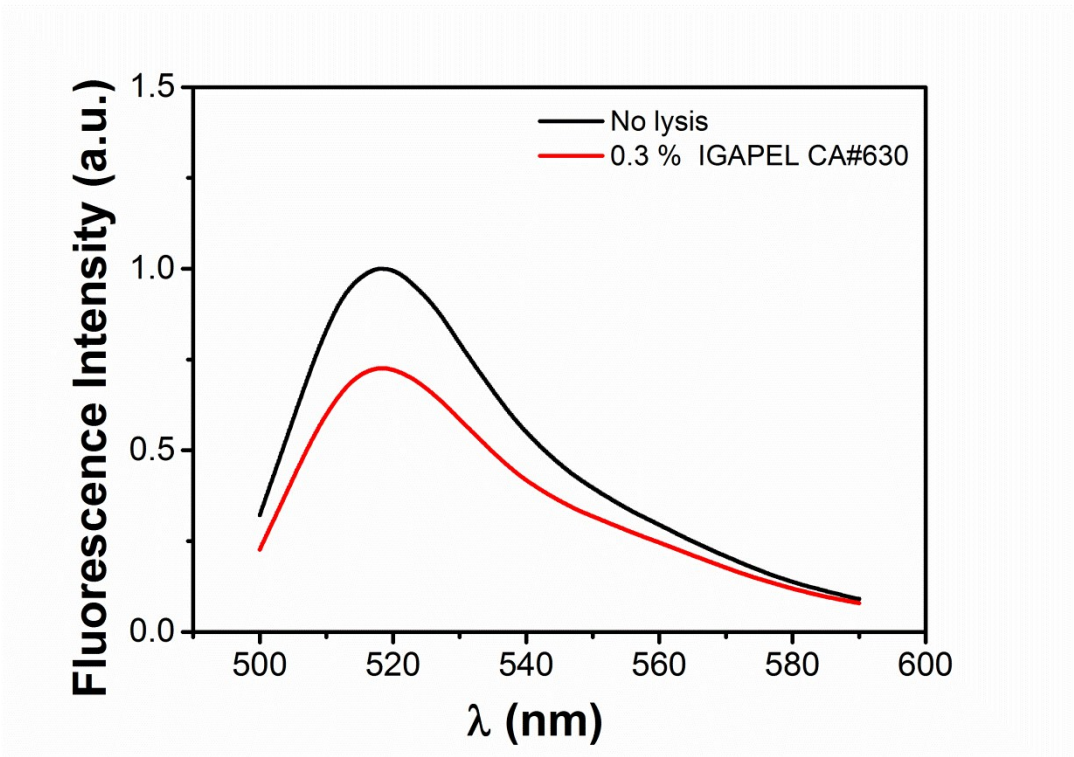



**Figure S3.** Fluorescence intensity of amplification products of miRNA in the presence of cell lysis in tubes. The reaction time was 2 h. MiRNA concentration was 10 nM.

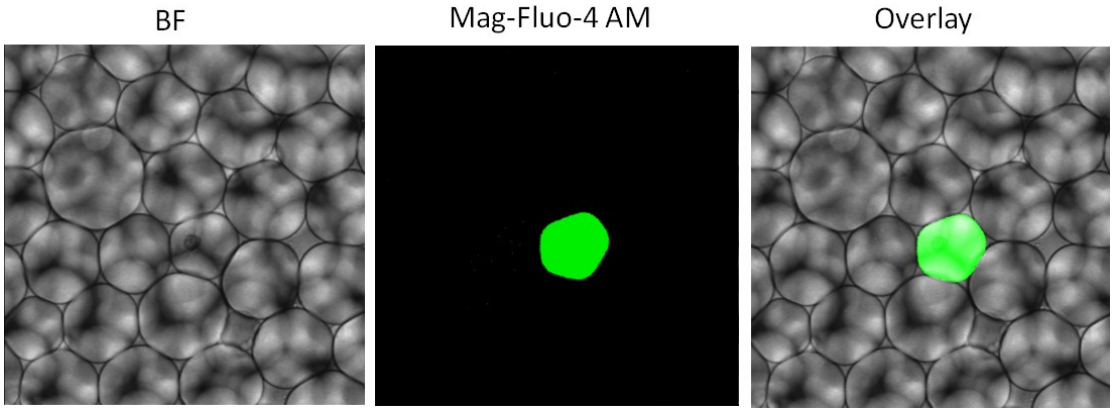



**Figure S4.** Fluorescent images of cells in the droplets after being lysed. In order to demonstrate the efficacy of cell lysis, a commercialized $Mg^{2+}$ probe (Mag-Fluo-4 AM, Thermo Fisher Scientific) was used as the indicator, which could respond to intracellular $Mg^{2+}$ resulting in fluorescence emission with maximum emissions at 515 nm. The MCF-7 cell suspension were first incubated with 5 μM of Mag-Fluo-4 AM for 30 min at 37 °C. The cells were washed with PBS to remove the excess probe after 3 times centrifugation. From the fluorescent images we can observed that intracellular labeled $Mg^{2+}$ flowed out of the cell and the fluorescence was full of the droplets, which illustrate that the lysis buffer is effective and the cell could be lysed in droplet. Only single droplet emit fluorescence and others are silent droplets is due to the low encapsulation rate of single cells (9.05%) rather than the low efficiency of cell lysis. Once one cell is encapsulated in the droplet, the cell could be lysed completely.

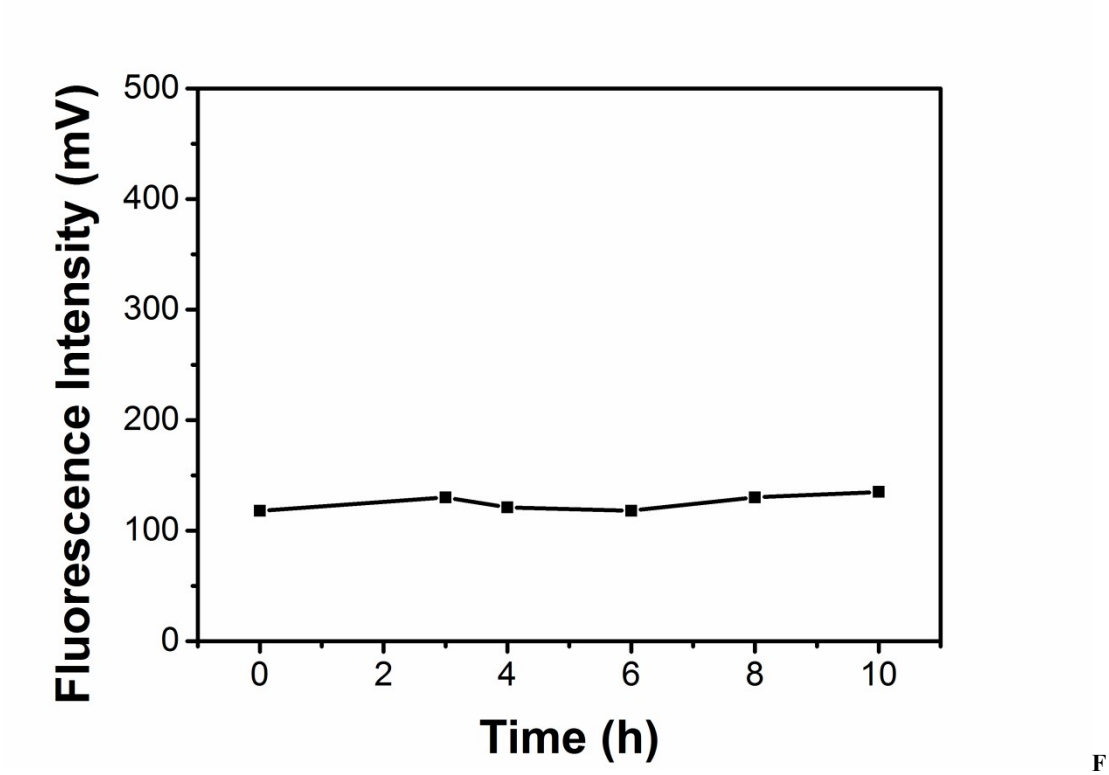



**Figure S5.** Stability verification of miRNAs in droplets.

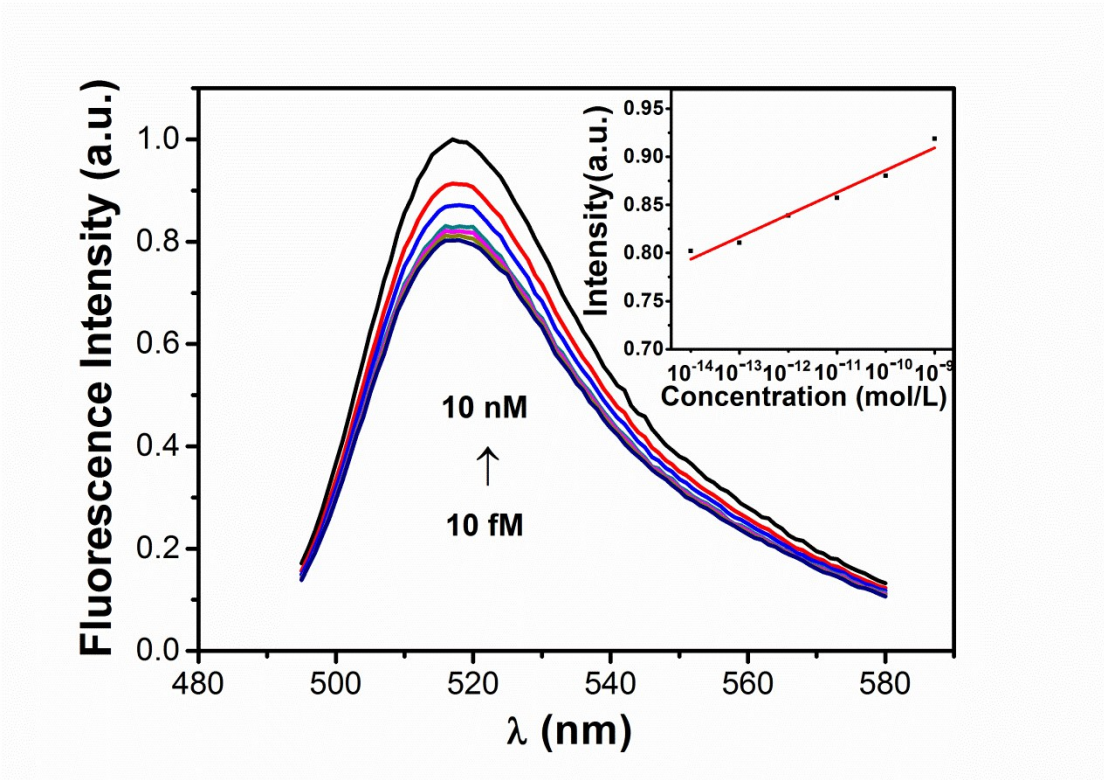



**Figure S6.** Fluorescence intensity of the solution in tubes after a quadratic isothermal amplification of miRNA with different concentrations. The reaction time was 2 h. MiRNA concentrations were 10 fM, 100 fM, 1pM, 10 pM, 100 pM, 1 nM and 10 nM.

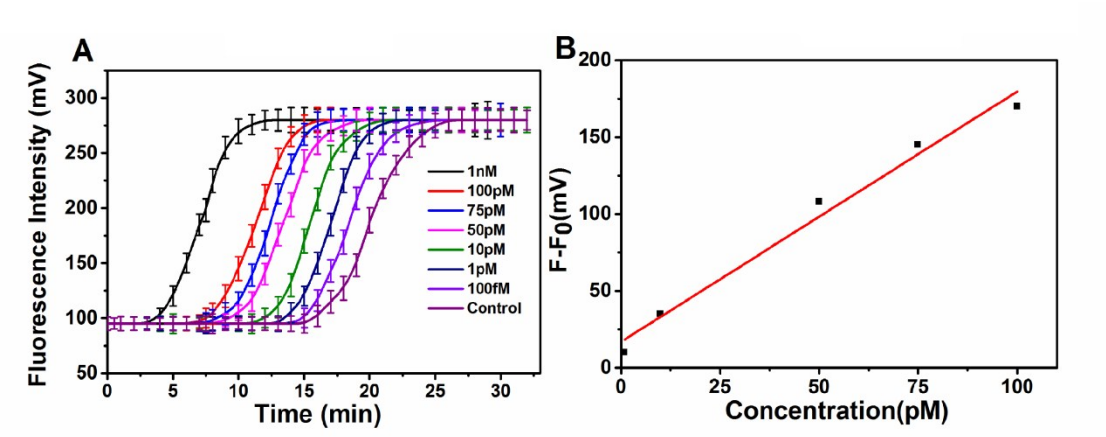



**Figure S7.** (A) Time-dependent fluorescence changes of miRNA 20 using quadratic isothermal amplification in droplets. (B) Linear relationship between the fluorescence intensity at 14 min and different concentrations of miRNA 20.

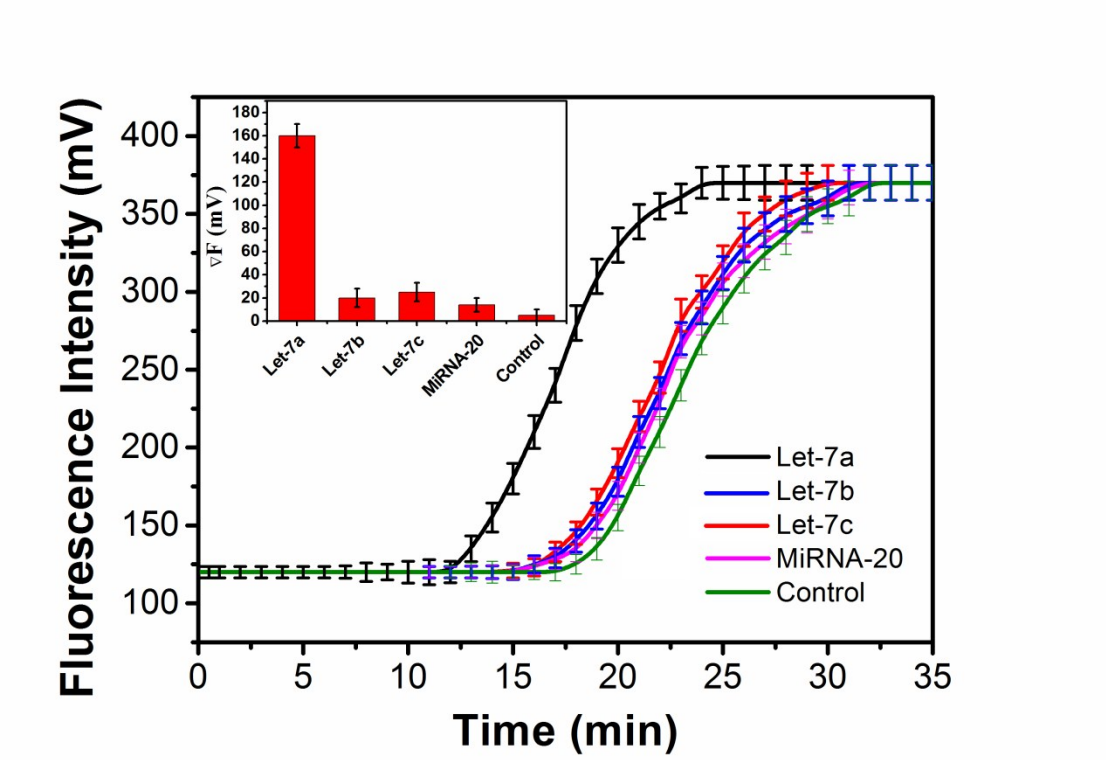



**Figure S8.** Specificity investigation of the miRNA let-7a hairpin probe in the droplets. Error bar were calculated from three independent experiments. MiRNA concentration was 10 pM. The signal intensity in the bar graph was obtained at 20 min.

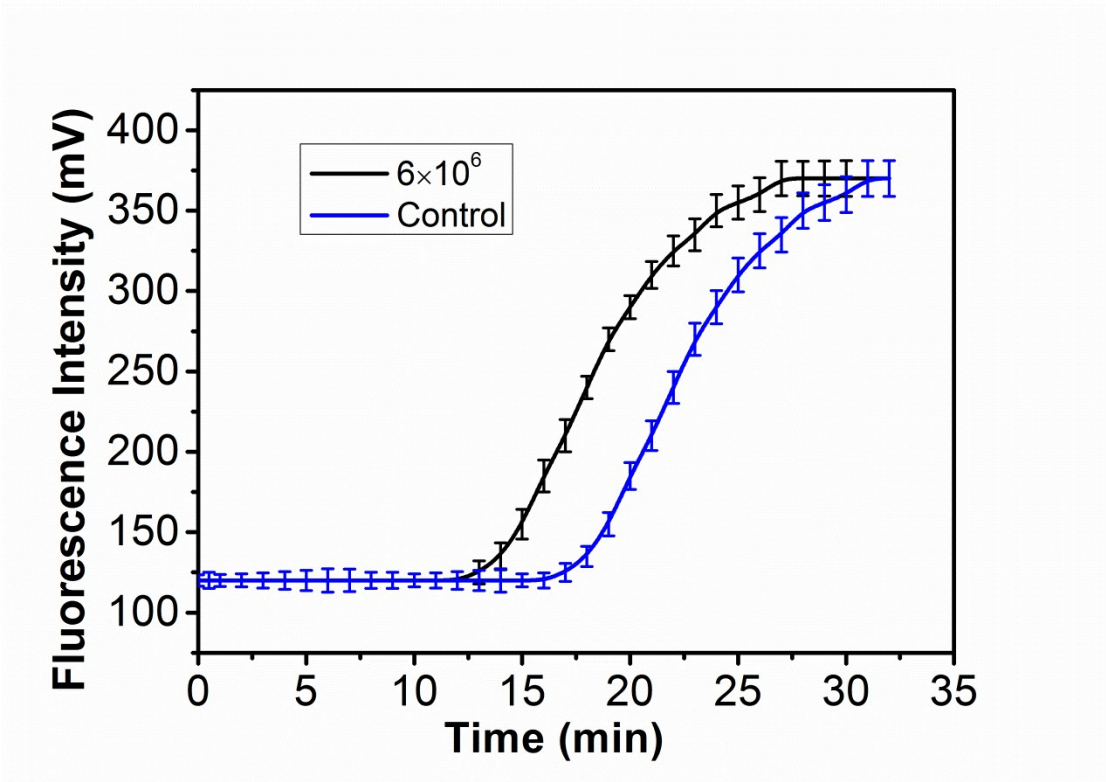



**Figure S9.** Fluorescence signal of miRNA in the cell homogenate containing $6\times10^6$ cells (black line). The control group was PBS without cells (blue line).

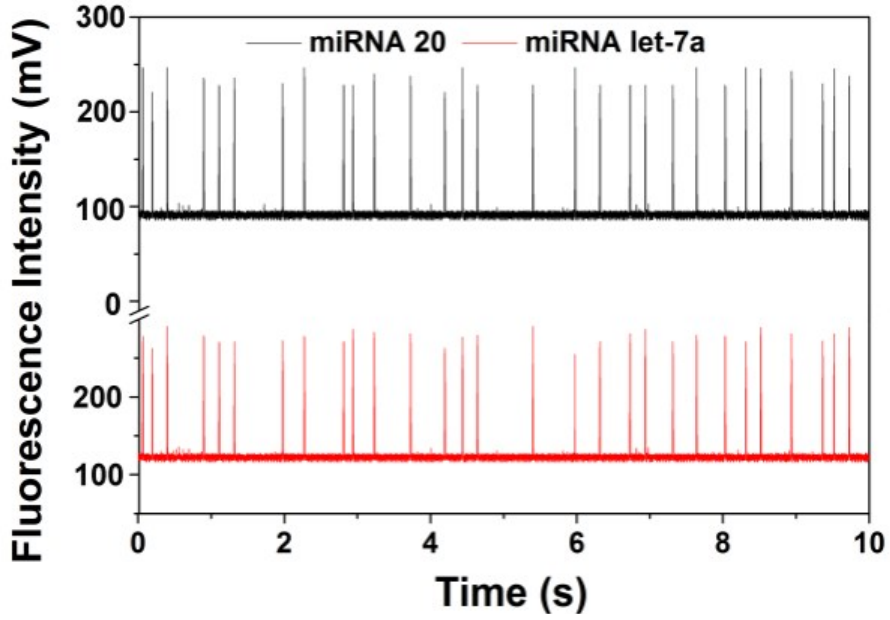



**Figure S10**. Fluorescence signal of miRNA 20 (above line) and miRNA let-7a (below line) in individual cells.

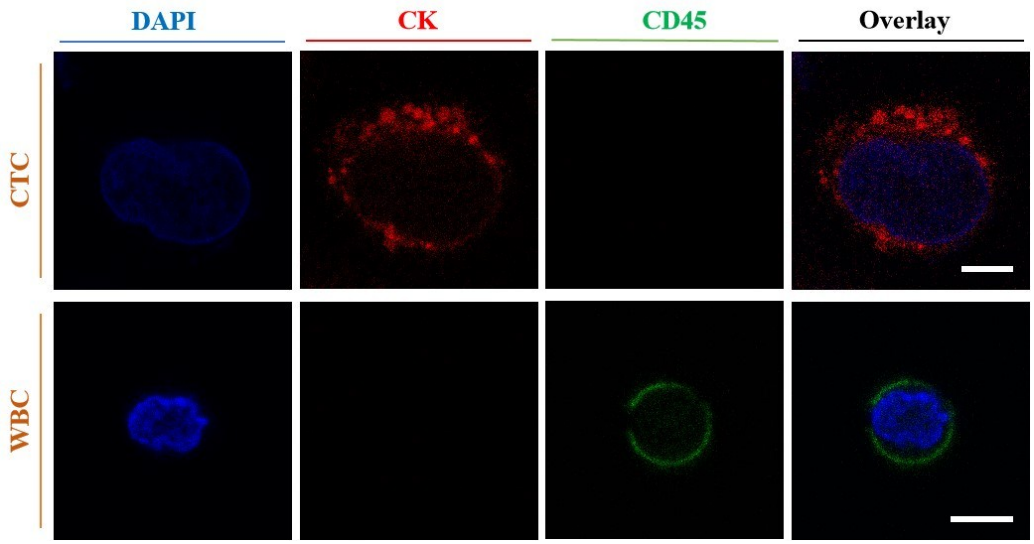



**Figure S11.** Immunofluorescence staining of the captured CTCs cells. Three color immunostaining allows for the identification of CTCs, WBCs using DAPI (nuclear specific, blue), anti-cytokeratin antibody (marker for CTCs, red), and antiCD45 antibody (marker for WBCs, green), Scale bar is 5 μM.

## References

1 Q. Li, P. Chen, Y. Fan, X. Wang, K. Xu, L. Li, B. Tang, *Anal. Chem*., 2016, **88**, 8610-8616.

2 M. E. Warkiani, B. L. Khoo, L. Wu, A. K. P. Tay, A. A. S. Bhagat, J. Han, C. T. Lim, *Nat. Protoc.,* 2015, **11**, 134-148.