Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information

Ultrahigh-throughput Droplet Microfluidic Device for Single-cell miRNA Detection with Isothermal Amplification

Song Guo, Weikang Nicholas Lin, Yuwei Hu, Guoyun Sun,
Dinh-Tuan Phan and Chia-Hung Chen

Supplementary-1: Oligonucleotide reagent and sample preparation

HPLC-purified custom DNA and RNA oligonucleotides (listed in the table below) were purchased from Integrated DNA Technologies, Inc. (IDT, Inc.). DI water was filtered using a filter with 0.22µm pore size (Minisart, Sartorius) and was used to prepare a stock solution of oligonucleotides. The absorbance of DNA/RNA stock solutions were measured via a UV-Vis spectrophotometer (Nanodrop 2000c, Thermal Scientific) to verify the concentration of oligonucleotides in the tube. All DNA/RNA stock solutions were kept at -20°C until use. Subsequent dilutions of oligonucleotides were performed using the above-mentioned Tris-HCl buffer solution.

Name of oligonucleotide	Sequence
DNA Hairpin 1 a-b-c-d-e-f (H1)	**5'TCA ACA TCA GTC TGA TAA GCT ACC ATG TGT AGA TAG CTT ATC AGA CTC TCG CAT ATA GGA ACC-3'**
DNA Hairpin 2 d*-c*-b*-c (H2)	**5'-TAA GCT ATC TAC ACA TGG TAG CTT ATC AGA CTC CAT GTG TAG A-3'**
DNA with Fluorophore f*-e* (F)	**TAMRA-5'-GGT TCC TAT ATG CGA GAG TCT GA-3'**
DNA with Quencher f (Q)	**5'-CTC GCA TAT AGG AAC C-3'-BHQ2**
Synthetic miRNA 21 target b*-a* (T)	**5'-UAG CUU AUC AGA CUG AUG UUG A-3'**
Mismatched miRNA-21 Control 1	**5'-UAG CUU AUC AGA CUG AUC UUG A-3'**
Mismatched miRNA-21 Control 2	**5'-UAG CUC AUC AGA CUG AUC UUG A-3'**

- BHQ-2: Black Hole Quencher®-2 attached to cytosine residue
- TAMRA: TAMRA fluorophore attached to thymine residue

The buffer solution contained four reagents in the following.

- 1M Tris-HCl: 1.6 ml (40 mM)
- EDTA: 1.488 mg (1 mM)
- $MgCl_2$: 4.75 mg (12.5 mM)
- Deionized water: 38.4 mL

Supplementary-2: Polyacrylamide gel electrophoresis (PAGE)

To verify that the hairpin assay was performing as we hypothesized, PAGE was performed to verify that products with longer lengths than the reagent oligonucleotides were formed. The samples were then loaded into 15% polyacrylamide gel in TAE buffer (a mixture of Tris base, acetic acid and EDTA), run at 90 V for 120 minutes.

Supplementary-3: *In vitro* experiments

To ensure the hairpin assay for target miRNA measurement, the autonomous reactions were performed in a bulk. Oligonucleotide solutions of different concentrations were aliquoted from stock solutions and diluted using the above mentioned Tris-HCl buffer inside DNAse and RNAse free tubes (AITbiotech). The oligonucleotides were first heated to 95°C for 5 minutes, followed by snap cooling in ice for at least one hour before using. 50 µL of H1, 50 µL of H2, 50 µL of T and 150 µL of a mixture containing F and Q, were mixed in a black 96-well plate (Nunc™ F96, Thermo Fisher). The final concentrations of oligonucleotides were in the following: H1 – 400 nM, H2 – 1600 nM, F – 600 nM and Q – 720 nM. Various concentrations of T were applied, such as 0, 1, 5, 10, 20, 50 and 100 nM. The fluorescence intensity of each well was measured using a plate reader (Spark®, TECAN) every 30 seconds for 2 hours. The excitation light (wavelength: 546 nm) was used to generate emission light (wavelength: 575 nm). To verify the specificity of hairpin probe assay to the mRNA21 sequence, we repeated the hairpin assay against oligonucleotides modified from the T RNA with mismatches in the sequence. 50 µL of H1, 50 µL of H2, 50 µL of Mis-1/Mis-2 and 150 µL of a mixture containing F and Q, were mixed in a black 96-well plate. The final concentration of oligonucleotides was set in the following: H1 – 400 nM, H2 – 1600 nM, F – 600 nM, Q – 720 nM and 50 nM of control-1/control-2.

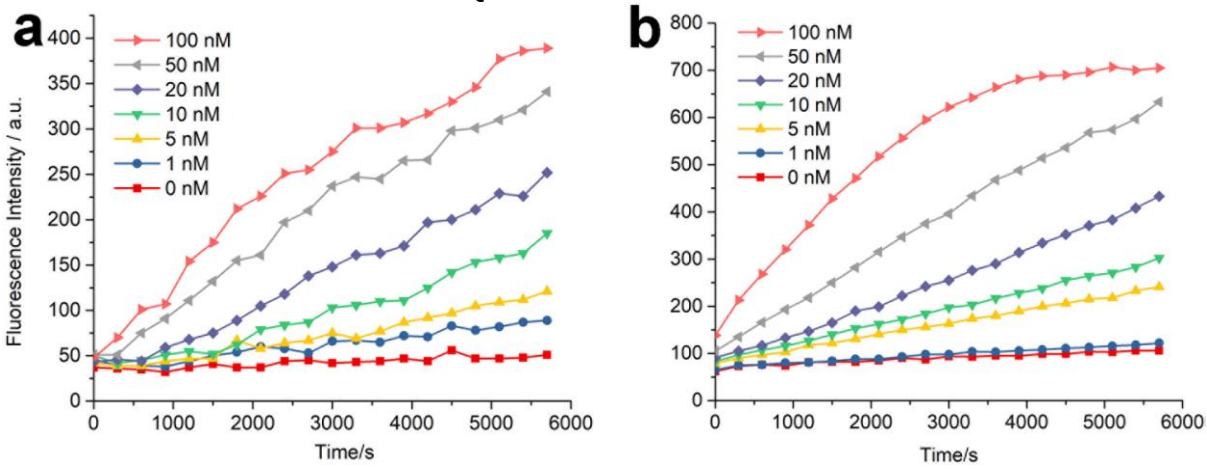

Fig. S1: Fluorescence kinetics of hairpin DNA reaction with various concentrations of target DNA (0, 0.5, 1, 5, 10, 50, 100 nM). A) Concentrations of Hairpin DNAs were as follows: H1-100 nM, H2-400 nM, F-150 nM and Q-180 nM. b) Concentrations of Hairpin DNAs were as follows: H1-200 nM, H2-800 nM, F-300 nM and Q-360 nM.

Supplementary-4: Droplet single-cell microfluidic experiments

1. Cell preparation

Human breast cell line MCF-10A and cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC) and cultured for single-cell experiments. All other cells were maintained at 37°C in a 5% CO_2 - 95% air atmosphere and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (HyClone Laboratories, Logan, UT), penicillin (100 U/ml; Life Technologies, Grand Island, NY), streptomycin (100 µg/ml; Life Technologies) and 2 mM L-glutamine (Life Technologies). When the cells were at 80% confluency, 1mg/ml hoest33342 (Life Technologies) was added to culture medium (volume ratio, Hoechst:medium=1:1000) for 15 minutes. After that, the medium was disposed and PBS buffer was used to wash the cells twice and was disposed. Then, 5 ml trypsin was added to the flask and incubated at 37°C for 5 minutes. Next, all liquid was transferred into a 15 ml tube and centrifuged at 1500 rpm for 5 minutes. After that, the supernatant was disposed and 5 ml fresh medium was added into the tube. The cell numbers were then counted by aliquoting 10 µL of cell solution to mix with 10 µL of 0.4% Trypan blue stain (Life technologies, USA). The mixed solution was then loaded onto a Countess cell counting chamber slide (Invitrogen, USA). Cell viability was maintained above 90% for single-cell experiments to be carried out. Next, the 5 ml cell suspension was centrifuged at 1500 rpm for 5 mins and supernatant was disposed. After that, fresh medium was added to make the concentration of cells at around 6 million per ml. Then 16% OptiPrep Density Gradient Medium was added to prevent sedimentation of cells in a syringe. The final concentration of cells was 5 million per ml.

2. Fabrication of microchips

Microfluidic devices were fabricated in PDMS (polydimethylsiloxane) using soft lithography [Whitesides, G.M., et al]. The droplet experiments were performed by using a flow focusing droplet generator (Figure S2). This droplet generator was fabricated by casting polydimethylsilane (PDMS) on a SU-8 negative photoresist master mould (MicroChem Inc.). The thickness of the SU8 models of two chips (Figure S2) was 40 µm. Replicas were formed from a 1:10 mixture of PDMS curing agent and prepolymer (Sylgard 184, Dow Corning, USA). The mixture was degassed under vacuum, poured onto the SU8 model, and then left to polymerize for 12h at 65 ^{o}C. The PDMS chip was subsequently plasma bonded onto a PDMS-coated glass slide for increased bonding strength. Microfluidic devices were used at least 24 h after their fabrication to allow the PDMS hydrophobic surface to be recovered after the plasma treatment, to facilitate the interaction of water-in-oil droplets produced in microfluidic devices. Polyethylene PE tubes (Scientific Commodities, USA, ID 0.38 mm; OD 1.03 mm) were used in this work for inlet and incubation tube.

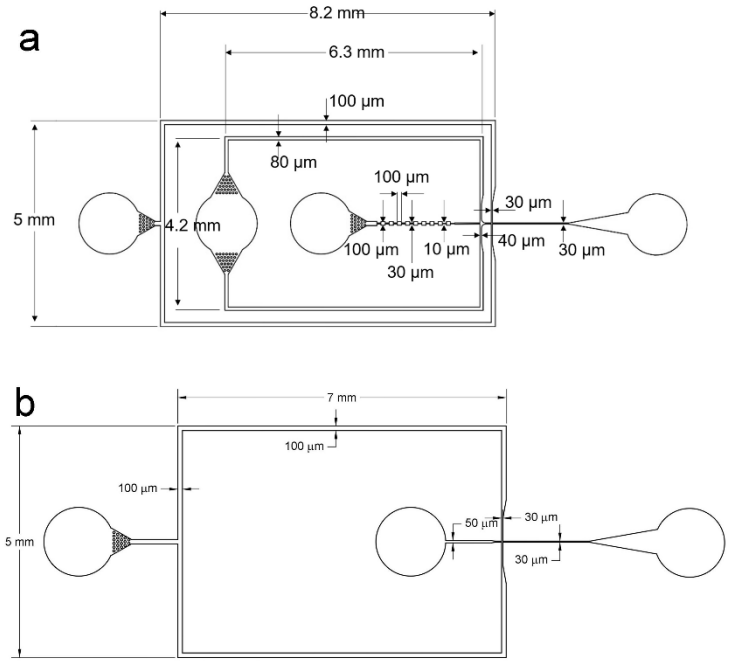

Fig. S2 Design of chips used. (a) Chip 1 used to encapsulated cell with. (b) Chip 2 used for PMT reading.

3. Droplet microfluidic experiments

To assay single cell miRNA-21, individual cells were encapsulated into water-in-oil droplet via microfluidics.

Novec™ HFE-7500 Engineering Fluid (3M) with 5% v/v Picosurf™ 1 (Sphere Fluidics Ltd) commercial surfactant was used as the continuous oil phase. A 150 µL solution comprising of 400 nM of H1, 600 nM of H2, 600 nM of Fluorophore, 720 nM of Quencher, 1.0 % w/v Triton™ X-100 lysis buffer (Sigma-Aldrich), and (0.2 mg/ml) Proteinase K were mixed and used as the reagent mixture for one of the aqueous inlets. Proteinase K was added to prevent nuclease released from the lysed cell from degrading Hairpin DNAs and miRNA 21. Cells used in single-cell encapsulation experiments were first incubated with Hoechst 33342 (Thermo Fisher) for 15 minutes to stain the live cells. After that, cells were then washed with medium, trypsinized, and re-suspended in DMEM. This cell suspension was used as the second aqueous phase in the flow focusing chip.

The oil and two aqueous reagents were filled into syringes and loaded onto syringe pumps (Pump 11 Elite Syringe pumps, Harvard Apparatus). The oil flow rate was 6 µL/min and the aqueous flow rate was 1 µL/min. Droplets formed were observed under a microscope (Leica DMi8, Leica Microsystems). Bright field and fluorescent images of the droplets were captured over several time points at 100x magnification. Fluorescence images for the miRNA 21 hairpin assay were observed through a FITC filter. The fluorescence images of Hoechst 33342 Dye stain were recorded through a DAPI filter. To plot the signal changing, we selected 10 different positions in the observation chip. To indicate the reaction kinetics, the images were taken every 5 minutes by using Leica DFC9000 camera. This process was automatically controlled by Leica microscope software (Leica Application Suite X, version 3.3.3.16958). After that, the images were processed by ImageJ to measure the fluorescence intensity of the target droplets (Schneider, Rasband et al. 2012). The number of expressing droplets in Figure 3c was 43 (1% w/v), 39 (0.5% w/v), 38 (0.2% w/v) respectively.

References

Whitesides, G.M., et al. (1997) *Journal of Materials Chemistry* **7**, 1069.
Schneider, C. A., et al. (2012). *Nature Methods* **9**: 671.

Table S1: Cell encapsulation statistics

Number of cells in droplet (n)	Fraction of droplets with n cells obtained in experiments involving MDA-MB-231 cell line	Fraction of positive droplets with n cells for MDA-MB-231 line	Fraction of droplets with n cells obtained in experiments involving MCF-7 cell line	Fraction of positive droplets with n cells for MCF-7 line	Fraction of droplets with n cells obtained in experiments involving MCF-10A cell line	Fraction of positive droplets with n cells for MCF-10A line	Theoretical value based on Poisson distribution
0	86.5%	-	89.4%	-	88.4%	-	88.2%
1	12.2%	90%	8.5%	73.3%	10.3%	89%	11%
2	0.9%	7%	2.6%	22.4%	0.9%	7%	0.6%
≥4	0.4%	3%	0.5%	4.3%	0.4%	4%	0.2%

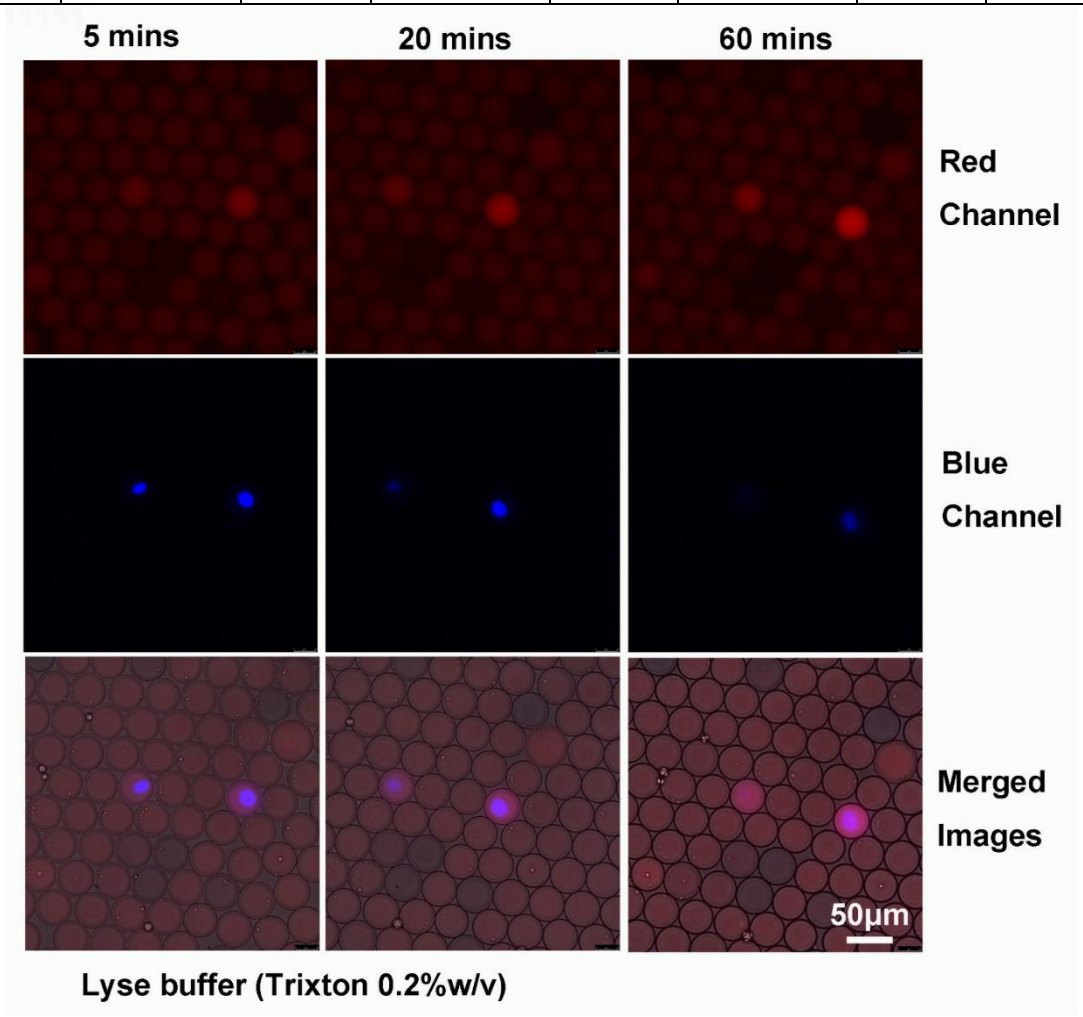

Fig. S3: Microscopy images of single cell miRNA detection in microdroplets. Fluorescence intensity was increased after cell lysis in the droplets. The concentration of Triton was 0.2% w/v.

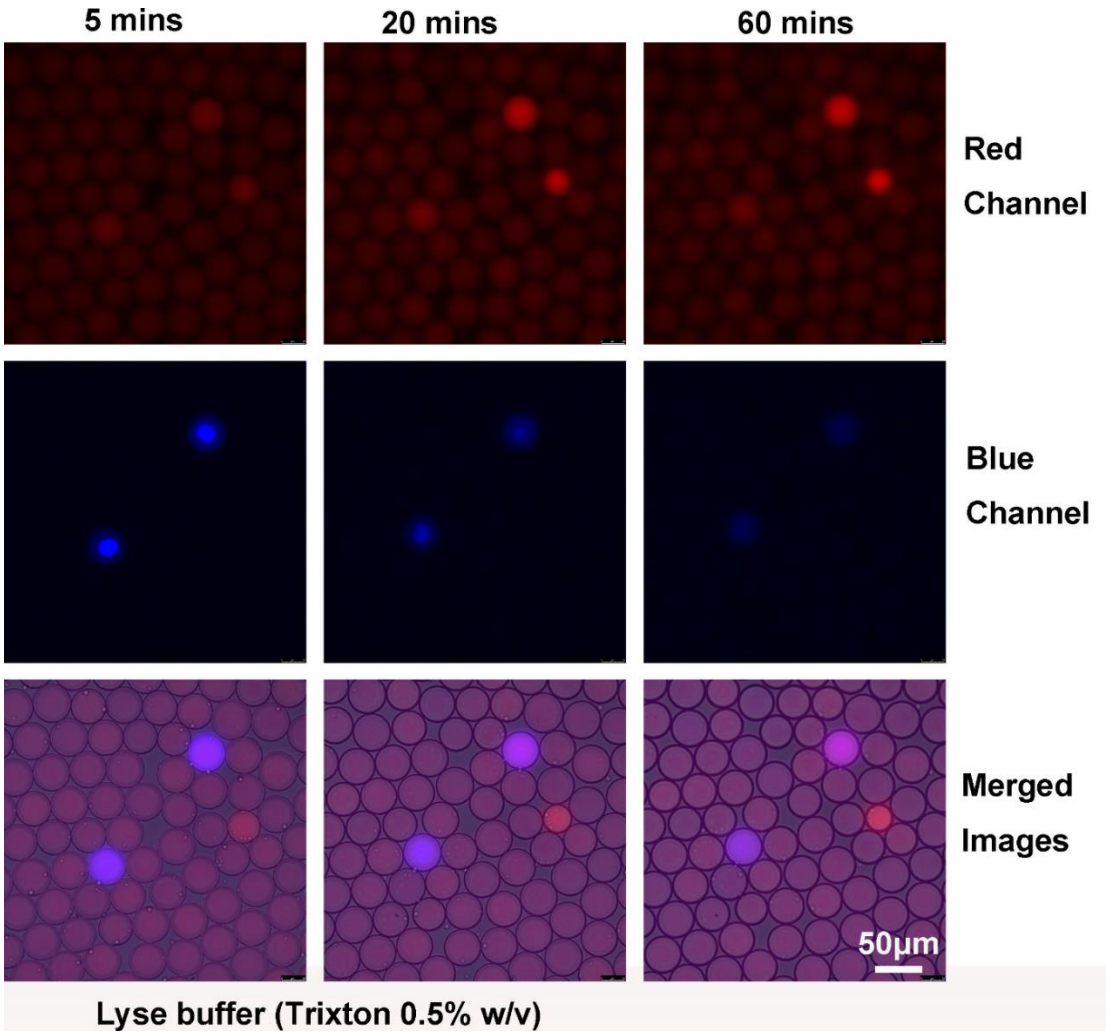

Fig. S4: Microscopy images of single cell miRNA detection in microdroplets. Fluorescence signal of the single cell increased after cell lysis in the droplets. The concentration of Triton was 0.5 % w/v.

Supplementary-5: Continuous flow screening

1. PMT set up

The optical system consisted of an excitation unit and a multiple fluorescence detection unit with four photomultiplier tubes (PMTs) (Hamamatsu, Japan) for signal detections from FRET substrates. A white LED light source (SOLA-SE-II, Lumencor Inc, USA) was used to simultaneously excite the substrate inside droplets. The white light was first passed through a multiband bandpass excitation filter (Blue channel: 473-491 nm and Red channel: 559-568.2 nm) and >90% of light was reflected toward the microfluidic channel to excite the fluorophores in droplets. Emission from the fluorophores was then transmitted to the multiple fluorescence detection unit, which contained two emission filters (Blue channel: 510-540 nm, Red channel: 590-620 nm) with appropriate dichroic mirrors (Blue channel: >495 nm & <565 nm, Red channel: >565 nm & <666 nm) to separate the desired signals from the target droplets.

2. on-line PMT Screening

For PMT reading, the droplets were then collected in a 1.5 meter long polyethylene (PE) tube with an inner diameter of 0.38 mm (Scientific Commodities, USA) (Fig. 4a in main article) before being injected into a flow channel for single-cell screening. The droplets were collected in a 1.5 meter long polyethylene (PE) tube with an inner diameter of 0.38 mm (Scientific Commodities, USA) (Fig. 4a) before being injected into a flow channel for single-cell screening. The tube was coiled and fixed on a glass slide to make sure that most of the tube was in the same horizontal plane. These droplets with cell encapsulations were directly stored in a tube once produced to maintain the temporal order of the droplets in the long tube (Figure S5). The durations of reactions in the droplets therefore was regulated (~ 30 minutes). The outlet of incubation tube was inserted into Chip 2 (Figure S2b, supplementary 4). After incubation, the droplets were injected and flowed through the PMT in Chip 2. MATLAB was used to record digital signals from PMT.

A single PMT reading procedures for each cell lines took 10 minutes. During this, the digital signals were collected by MATLAB. We can count number of the signals from target droplets (show both miRNA signals and nucleus signals) by eliminating the background signals.

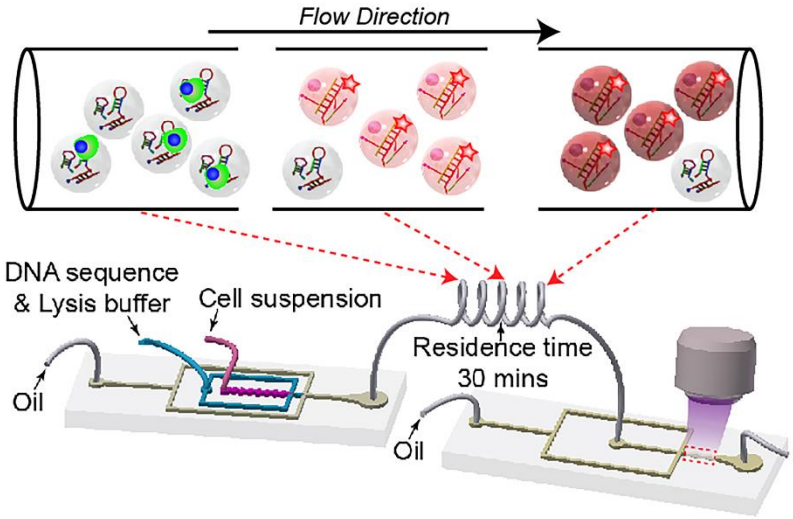

Fig. S5 Illustration of droplet fluorescence signal incubation in the tube at room temperature.

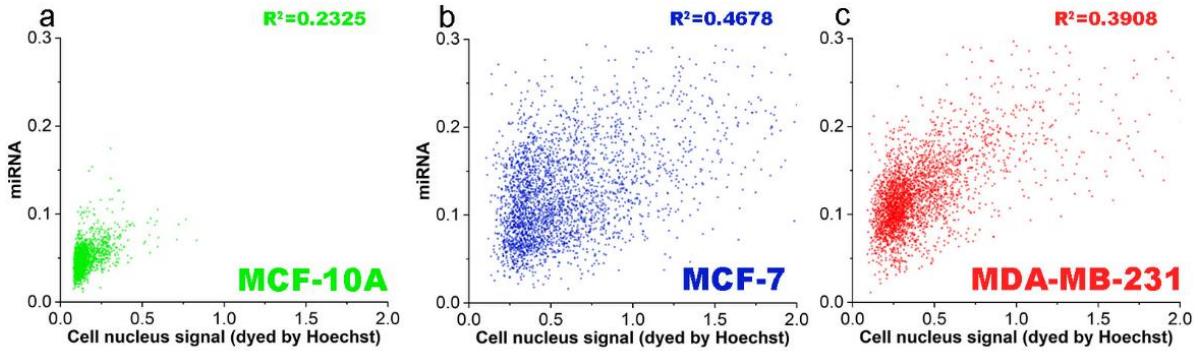

Fig. S6 Plots showing the distribution of MDA-MB-213, MCF-7 and MCF-10A in terms of signal intensity. Each figure represents a single cell. a: MCF-10A, b: MCF-7, c: MDA-MB-231. Inserted R^2 values were obtained from linear fitting the data using Origin 2018.