

*Electronic Supplementary Information for*

**Probing cytoplasmic and nuclear microRNAs in single living cells  
via plasmonic affinity sandwich assay**

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# MATERIALS AND METHODS

## 1. Reagents and Materials

All oligonucleotides used in this study were custom-made from Takara Company (Nanjing, China). 4-Aminothiophenol (PATP) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100, chloroauric acid ( $\text{HAuCl}_4$ ), silver nitrate ( $\text{AgNO}_3$ ), trisodium citrate, NaOH,  $\text{KHCO}_3$ , and anhydrous ethanol obtained from Nanjing Reagent Company (Nanjing, China). The hybridization chain reaction (HCR) buffer was Tris buffer (20 mM, pH 7.6) containing 100 mM NaCl. All chemicals employed were of analytical grade and used without further purification. Human cervical cancer cells (HeLa), human mammary cancer cells (MCF-7), human normal breast cells (MCF-10A), Dulbecco's modified eagle medium (DMEM, containing 4.5 mg/mL glucose, 80 U/mL penicillin and 0.08 mg/mL streptomycin) and 1x phosphate-buffered saline (PBS) solution were purchased from Keygen Biotech (Nanjing, China). Water used in all experiments was purified with a Milli-Q Advantage A10 system (Millipore, Milford, MA, USA). Borosilicate glass capillaries of 0.58 mm i.d. and 1.0 mm o.d. were obtained from DL Naturegene Life Sciences (Shanghai, China).

## 2. Instrumentation

Plasmon-enhanced Raman scattering detection was carried out on a Renishaw InVia Reflex confocal microscope (Renishaw, UK) equipped with a high-resolution grating with 1800 grooves/mm, additional band-pass filter optics, and a CCD camera. Spectra were acquired using a 633 nm excitation laser line (1 s integration time and 1 accumulation). The laser was focused onto the sample by using a  $\times 50$  objective (N.A. 0.75), providing a spatial resolution of ca.  $1 \mu\text{m}^2$ . All measurements were carried out using a He-Ne laser ( $\lambda_0 = 633 \text{ nm}$ ; laser power at spot, 17 mW). Wavelength calibration was performed by measuring silicon wafers through a  $\times 50$  objective, evaluating the first-order phonon band of Si at  $520 \text{ cm}^{-1}$ . The microprobes used in this work were fabricated by tapering 1.0 mm core-diameter borosilicate glass capillaries using a P-2000 pipette puller (Sutter Instrument, Novato, CA, USA),

producing probes with a diameter of 1-2  $\mu\text{m}$  (the pores of the capillaries were sealed off at the tip end of prepared probes). A three-dimensional manipulator (Eppendorf, Germany) equipped on an inverted microscope (Olympus, Japan) was used to precisely insert the extraction microprobe into specific regions of single living cells under investigation. Transmission electron microscopic (TEM) characterization was carried out on a JEM-1011 system (JEOL, Tokyo, Japan). The UV absorbance measurement was performed on a NanoDrop 2000/2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

### **3. Fabrication of extraction microprobes**

The preparation procedure of nucleic acid-based extraction microprobes is shown in Fig. S1A, which included two major steps: 1) preparation of Au-coated starting microprobes, and 2) preparation of nucleic acid-functionalized extraction microprobes. The detailed procedures are described below.

#### ***3.1. Preparation of Au-coated starting microprobes***

For preparation of starting microprobes, a gold layer was prepared onto the surface of bare microprobes according to a previously reported method.<sup>1</sup> Briefly, bare microprobes were immersed in a mixture solution containing 12 mM  $\text{HAuCl}_4$ , 0.5 M  $\text{KHCO}_3$  and 25 mM glucose for 4-5 h at 35 °C (air bath) until an obvious gold layer appeared on the microprobe surface. Then, the Au-coated microprobes were washed with water and anhydrous ethanol 3-4 times each, and then dried by air at room temperature for further use.

#### ***3.2. Preparation of nucleic acid-modified extraction microprobes***

For preparation of nucleic acid-functionalized extraction microprobes, the Au-coated microprobes were immersed in a 20- $\mu\text{L}$  solution of 10  $\mu\text{M}$  extraction sequence that is complementary to half of target miRNAs for 3 h at room temperature, followed by rinse with phosphate buffer (10 mM, pH 7.4) for three times and dried at room

temperature, then stored at 4 °C for further use.

#### **4. Preparation of Raman nanotags**

For preparation of Raman nanotags, silver nanoparticles (AgNPs) were first prepared as described by Lee and Meisel.<sup>2</sup> Briefly, AgNO<sub>3</sub> (36 mg) was dissolved in 200 mL water and brought to boil under continuous stirring. Then, 4 mL of 1% (w/v) trisodium citrate was added. The mixture was boiled with stirring for about 1 h and then cooled down to room temperature. The obtained AgNPs solution was stored at 4 °C before use. The obtained AgNPs were then modified with labeling sequence and Raman reporter according to the procedure shown in Fig. S1B. Briefly, a volume of 5 µL of 10 µM labeling sequence solution for miR-21 and 10 µL of 100 µM Raman reporter (PATP) solution were added to 1 mL of solution of bare AgNPs and the mixed solution was gently stirred for 1 h at room temperature, the obtained Raman nanotags were stored at 4 °C for further use.

#### **5. The procedure of PASA**

The procedure of PASA included four major steps: 1) *In-vivo* extraction, for which a nucleic acid-modified extraction microprobe was carefully manipulated to insert into a single living cell of interest for 3 min, 2) Washing, for which the extraction microprobe was taken out of the cell and washed with phosphate buffer (10 mM, pH 7.4) for three times, 3) Labeling, for which the captured miRNA molecules on the extraction microprobe were labeled by dipping into 5 µL of Raman nanotags at 37 °C for 3 min, 4) Detection, for which the extraction microprobe was first washed with phosphate buffer (10 mM, pH 7.4) for three times, dried, and then scanned under the Raman spectrograph from the tip with a step of 1 µm.

#### **6. Finite-difference time-domain method (FDTD) simulation**

The near-field enhancement properties of AgNP and Au layer/glass were simulated by using the finite-difference time-domain (FDTD) method for solving Maxwell's

equations. The software FDTD Solutions (Version 8.6, Lumerical, Vancouver, Canada,) was employed. The modeled nanostructures are illustrated in Fig. 2. A silver nanosphere with diameter of 60 nm was modeled, and its refractive index was taken from the material database of Palik (provided by FDTD Solutions, Lumerical). A gold thinlayer (thickness, 100 nm) was modeled with refractive index from the material database of Johnson and Christy (provided by FDTD Solutions, Lumerical). The incident light injected backward y direction, and polarized along x-axis. A non-uniform mesh was used in simulating regions. When profiling the electric field distribution, the incident light wavelength was set at 633 nm as the same as the laser wavelength used for the PASA detection. In all cases, a mesh step of  $dx=dy=dz=0.5$  nm was used. Standard Fourier transform was carried out to visualize the electromagnetic field distributions in the x-y plane at the pre-set wavelength.

## **7. Investigation of background signals**

Considering that amino and thiol can bind with noble metals and thus may produce some background signals, background signals due to extraction sequence-modified microprobes and labeling sequence-functionalized AgNPs (without the presence of PATP) were investigated.

### ***7.1. Background signal of extraction sequence-modified microprobes***

The Au-coated starting microprobe was immersed in a 20  $\mu$ L volume of extraction sequence (10  $\mu$ M) solution for 3 h at room temperature, followed by rinse with phosphate buffer (10 mM, pH 7.4) for three times and dried at room temperature, then directly detected by the Raman spectrograph. The measurement was performed three times in parallel. The results are shown in Fig. 3B.

### ***7.2. Background signal of labeling sequence-modified AgNPs***

A 5- $\mu$ L volume of 10  $\mu$ M labeling sequence was added to 1 mL of bare AgNPs solution and then the mixed solution was gently stirred for 1 h at room temperature. A

2- $\mu$ L volume of labeling sequence-modified AgNPs solution was added on a piece of gold substrate for Raman detection. For comparison, Raman signal of the same volume of solution of bare AgNPs, Raman nanotags (with the presence of PATP) were collected, respectively, and the results are shown in Fig. 3D.

### **8. Optimization of the composition of the Raman nanotags**

The composition of the Raman nanotags, particularly the ratio between the labeling sequence and PATP, was optimized. For this optimization, miR-21 was used as the target; the concentration of the labeling sequence for miR-21 and PATP was set at 10  $\mu$ M and 100  $\mu$ M, respectively; while the volume ratio between the solution of the labeling sequence and the solution of PATP was set at 1:1, 1:2, 1:3 and 1:4. Other conditions were the same as those described in the section 4. Signals of the prepared Raman nanotags under two detection fashions were collected, i.e., 1) plasmonic detection, on a gold-coated substrate (without the presence of the extraction sequence); and 2) PASA detection, on a target miRNA-bond extraction sequence-modified gold-coated substrate. The corresponding results are shown in Fig. 4A.

### **9. Characterization of surface-plasmon resonance properties of Raman nanotags**

A 5- $\mu$ L volume of 10  $\mu$ M labeling sequence and 10  $\mu$ L of 100  $\mu$ M PATP were added to 1 mL of bare AgNPs solution and the mixed solution were gently stirred for 1 h at room temperature. The obtained solution was used as Raman nanotags for the characterization. A 2- $\mu$ L volume of the Raman nanotags was used for UV-vis spectrum measurement. For comparison, UV-vis absorption spectra of the same volume of AgNPs solution, PATP solution, labeling sequence solution and AgNPs modified with labeling sequence (without Raman reporter) were also measured. The corresponding results are shown in Fig. 4B.

## **10. Raman intensity-time course of target miRNAs in the plasmonic affinity sandwich assay**

Raman intensity-time course of miRNAs detection at different concentrations of miR-21 was investigated. The concentration of miR-21 was set at 10  $\mu$ M, 10 nM and 10 pM. The extraction microprobes were dipped in miR-21 solutions of different concentrations at 37 °C for 3 min, 30 min, 2 h, 6 h and 12 h, respectively. Each extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times. Then, captured miR-21 sequences were labeled with 5  $\mu$ L Raman nanotags at 37 °C for 3 min. After that, the extraction microprobes were washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and detected by the Raman spectrograph. The related results are shown in Fig. S2.

## **11. Cross-reactivity test**

The cross-reactivity of the PASA approach towards miRNAs bearing different mismatches was investigated. The nucleic acid-modified extraction microprobes were dipped in a 0.5-mL volume of 10  $\mu$ M concentration of miR-21 solution or the same concentration of mismatched sequences: miR-21A (a mismatch), miR-21B (a mismatch), miR-21C (two mismatches) and miR-21D (four mismatches) for 12 h at 37 °C. Each extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times. Then, captured molecules were labeled with 5  $\mu$ L Raman nanotags for 12 h at 37 °C. After labeling, each extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and detected by the Raman spectrograph. The corresponding results are shown in Fig. 5A.

## **12. Binding constant measurement**

Binding constant is an important parameter for the use of extraction microprobe in miRNAs assay. The binding constant of extraction microprobe was estimated through measuring Raman signal for the target miRNA molecules captured by the extraction microprobe from a series of target miRNA standard solutions shown in Fig. 5B. Each

extraction microprobe was dipped into a 20- $\mu$ L volume of the standard solutions of different concentrations (from 10  $\mu$ M to 1 pM) for 12 h at 37 °C. After extraction, each extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times. Then, captured miR-21 molecules were labeled with 5  $\mu$ L Raman nanotags for 12 h at 37 °C. After labeling, the microprobes were washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and detected by the Raman spectrograph.

Binding constant was measured according to the logistic function<sup>3</sup> as given below:

$$Y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

where  $Y$  is the signal intensity,  $x$  the concentration;  $a$  the signal intensity when  $x = 0$ ,  $d$  the signal intensity for “infinite” concentration;  $c$  the concentration resulting in halfway between  $a$  and  $d$ , which gives the  $K_d$  value; and  $b$  a “slope factor” that determines the steepness of curve.

### **13. Analysis of miRNAs in single cells via PASA**

#### **13.1 Cell culture conditions**

Different cell lines including HeLa, MCF-7 and MCF-10A were seeded in DMEM medium with 10% fetal bovine serum for 2 to 3 days (37 °C, 5% CO<sub>2</sub>, 95% humidity). The cell culture medium was removed and the cells remained on the cell culture dishes were washed with 1 $\times$  PBS buffer for two times, then the cells were kept in fresh fetal bovine serum for further assay.

#### **13.2 Preparation of single-cell lysate**

Cultured cells were grown on a 100-mm plate until 50% confluency was reached. They were detached using trypsin, followed by rinse with 1 $\times$  PBS buffer for twice, and then the cells were kept in 1 $\times$  PBS buffer for further sampling. A target single cell was selected by a pico-litter pipette through a three-dimensional manipulator equipped on an inverted microscope. Then the obtained single cell was dissolved in different



volumes (20  $\mu$ L, 2  $\mu$ L and 0.2  $\mu$ L) of modified RIPA buffer (Triton-X 100: 1%, NaCl: 150 mM, and Tris-HCl: 50 mM, pH 7.4). After that, the single cell was kept on ice for 30 min, and the debris was pelleted by centrifugation at 10,000 rpm for 10 min and the supernatant was obtained for further use.

### **13.3 Analysis of miRNAs in single living cells via PASA**

Analysis of miRNAs in single living cells with subcellular resolution via PASA was investigated through measurement of the target miRNAs in the nucleus and cytoplasm within single living cells. A nucleic acid-modified extraction probe was carefully manipulated to penetrate both plasma membrane and nuclear membrane within a single living cell to allow target miRNAs to be extracted for 3 min. The extraction microprobe was then taken out of the cell and washed with phosphate buffer (10 mM, pH 7.4) for three times. After that, the captured miRNA molecules on the extraction microprobe were labeled by dipping into 5  $\mu$ L of Raman nanotags at 37  $^{\circ}$ C for 3 min. Finally, the extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and scanned under the Raman spectrograph from the tip with a step of 1  $\mu$ m.

### **13.4 Analysis of cytoplasmic miRNAs in single living cells**

For the analysis of cytoplasmic miRNAs in single living cells, a nucleic acid-modified extraction microprobe was precisely manipulated to penetrate the plasma membrane of a target single living cell, go further until the tip close to the cell membrane on the opposite end, avoiding touch with the nucleus. After insertion, the extraction microprobe was kept in the cytoplasm for 3 min for extracting target miRNA molecules. After that, the extraction microprobe was taken out of the cell and washed with phosphate buffer (10 mM, pH 7.4) for three times, then captured miRNA molecules by the extraction microprobe were labeled by dipping into 5  $\mu$ L of Raman nanotags at 37  $^{\circ}$ C for 3 min. Finally, the extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and detected by the Raman

spectrograph.

### **13.5 Analysis of total miRNAs in single-cell lysate**

Plasmonic affinity sandwich assay of miRNAs in the lysate of a single cell was carried out under otherwise identical conditions except that nucleic acid-functionalized extraction microprobes were inserted into the lysate of a single cell rather than a single living cell. Single HeLa cells were dissolved in 20  $\mu\text{L}$ , 2  $\mu\text{L}$  and 0.2  $\mu\text{L}$  lysis buffer, respectively.

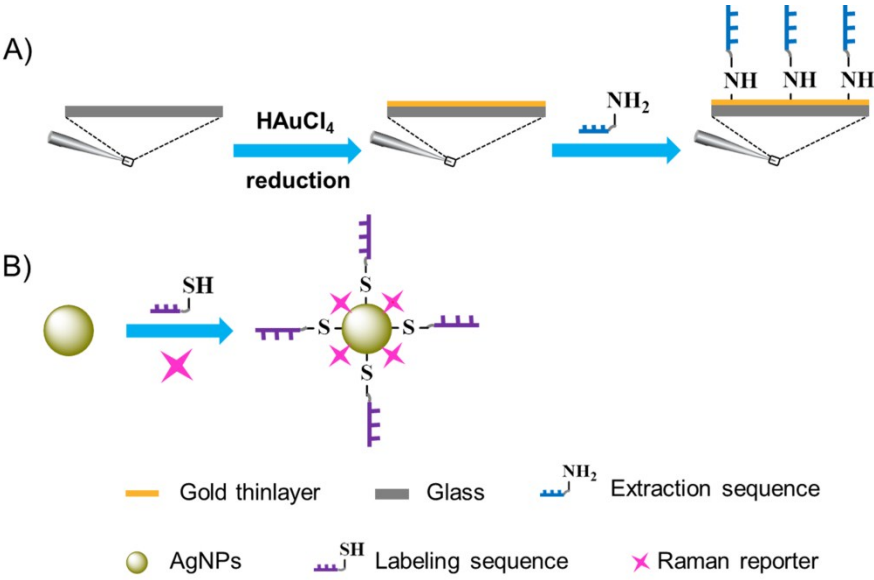
### **13.6 PASA of miR-21 in single living MCF-10A cells spiked with known miR-21 concentration**

To validate the detectability of the PASA approach for low-copy-number miRNAs in single living cells, a volume of 100 fL of  $1.0 \times 10^{-9}$  M,  $1 \times 10^{-10}$  M miR-21 standard solution (about 60 and 6 molecules, respectively) was injected into single living MCF-10A cells using the microinjector. After 5 min of diffusion, a nucleic acid-functionalized extraction microprobe was precisely inserted into the miR-21-spiked single living cells. After insertion, the microprobe was kept in the cells for 3 min for target extraction. Then, the microprobe was taken out of the cells and washed with phosphate buffer (10 mM, pH 7.4) for three times, miRNA molecules captured by the extraction microprobe were labeled by dipping into 5  $\mu\text{L}$  of Raman nanotags at 37  $^{\circ}\text{C}$  for 3 min. Finally, the extraction microprobe was washed with phosphate buffer (10 mM, pH 7.4) for three times, dried and detected by the Raman spectrograph.

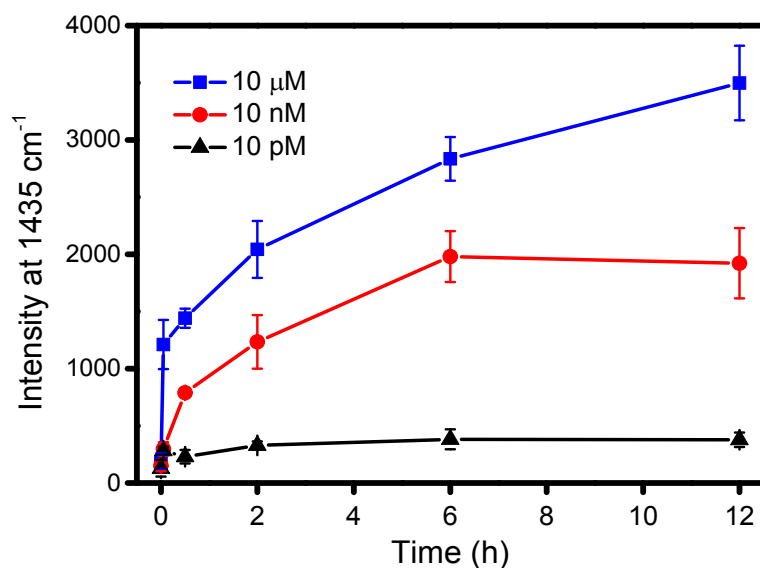
### **References:**

- (1) F. Zhou, M. M. Wang, L. Yuan, Z. P. Cheng, Z. Q. Wu, H. Chen, *Analyst*, 2012, **137**, 1779-1784.
- (2) P. C. Lee, D. Meisel, *J. Phys. Chem.*, 1982, **86**, 3391-3395.
- (3) A. Delean, P. J. Munson, D. Rodbard, *Am. J. Phys.*, 1978, **235**, E97-E102.

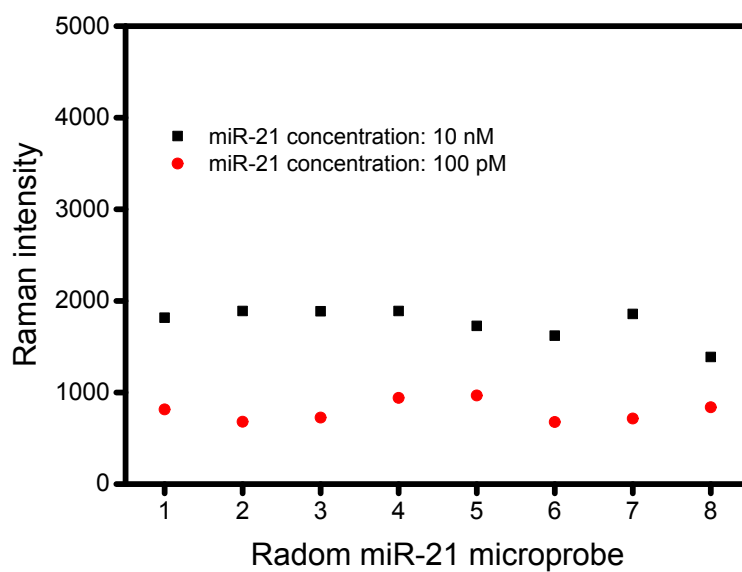
# SUPPLEMENTARY DATA



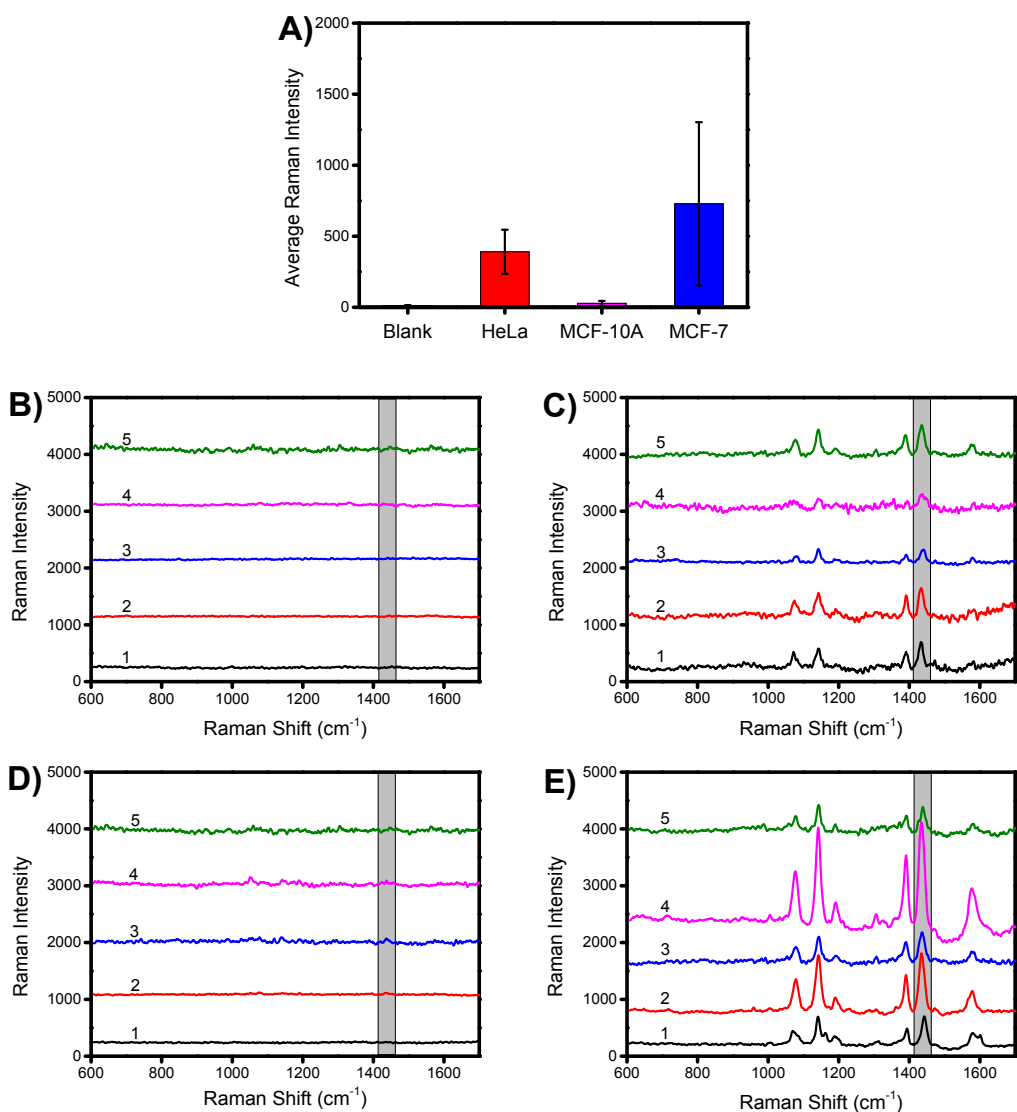
**Fig. S1** Schematic of the fabrication procedures of A) extraction microprobe and B) Raman nanotags.



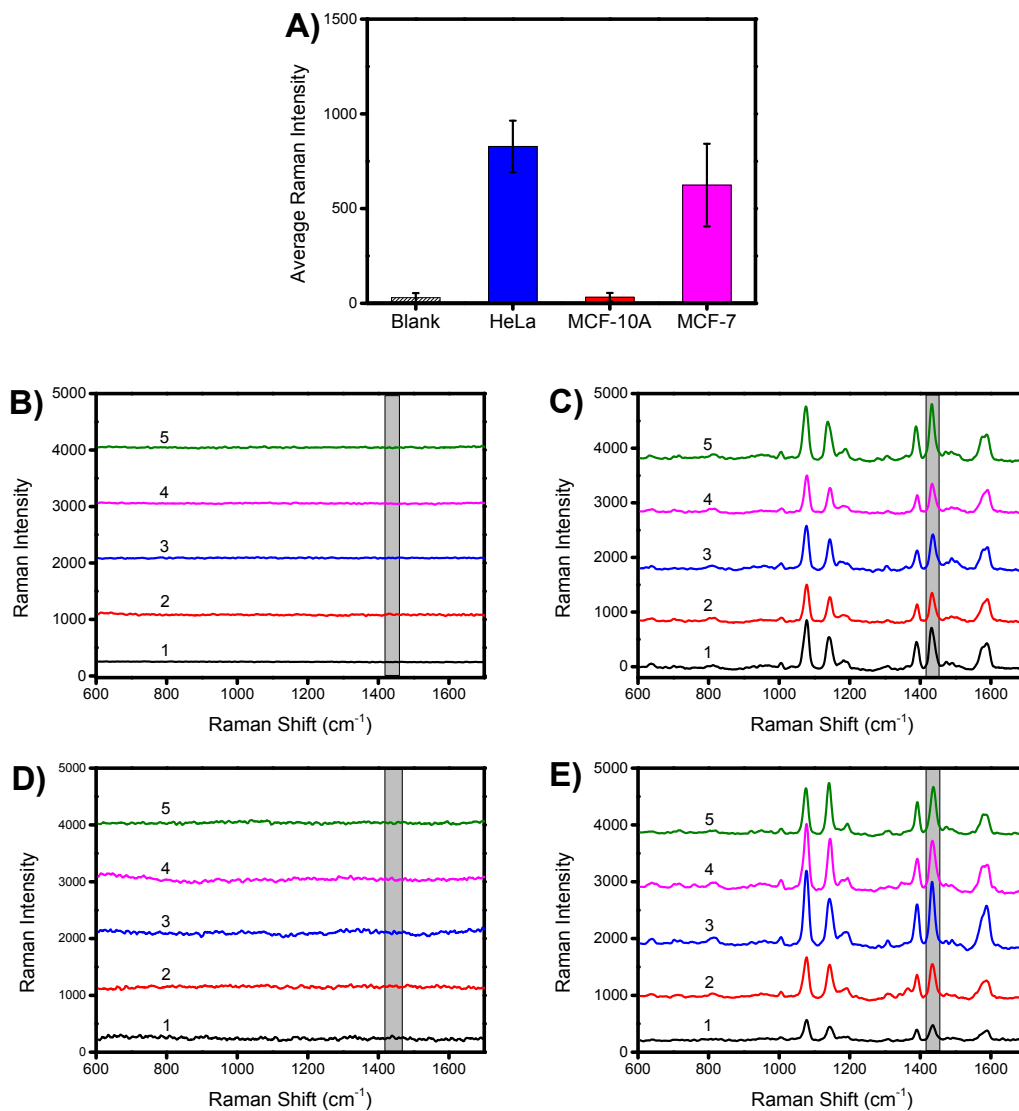
**Fig. S2** Dependence of the Raman intensity for miR-21 extracted by the extraction microprobe from miR-21 solutions of different concentrations on the extraction time. Error bars represent the standard deviations.



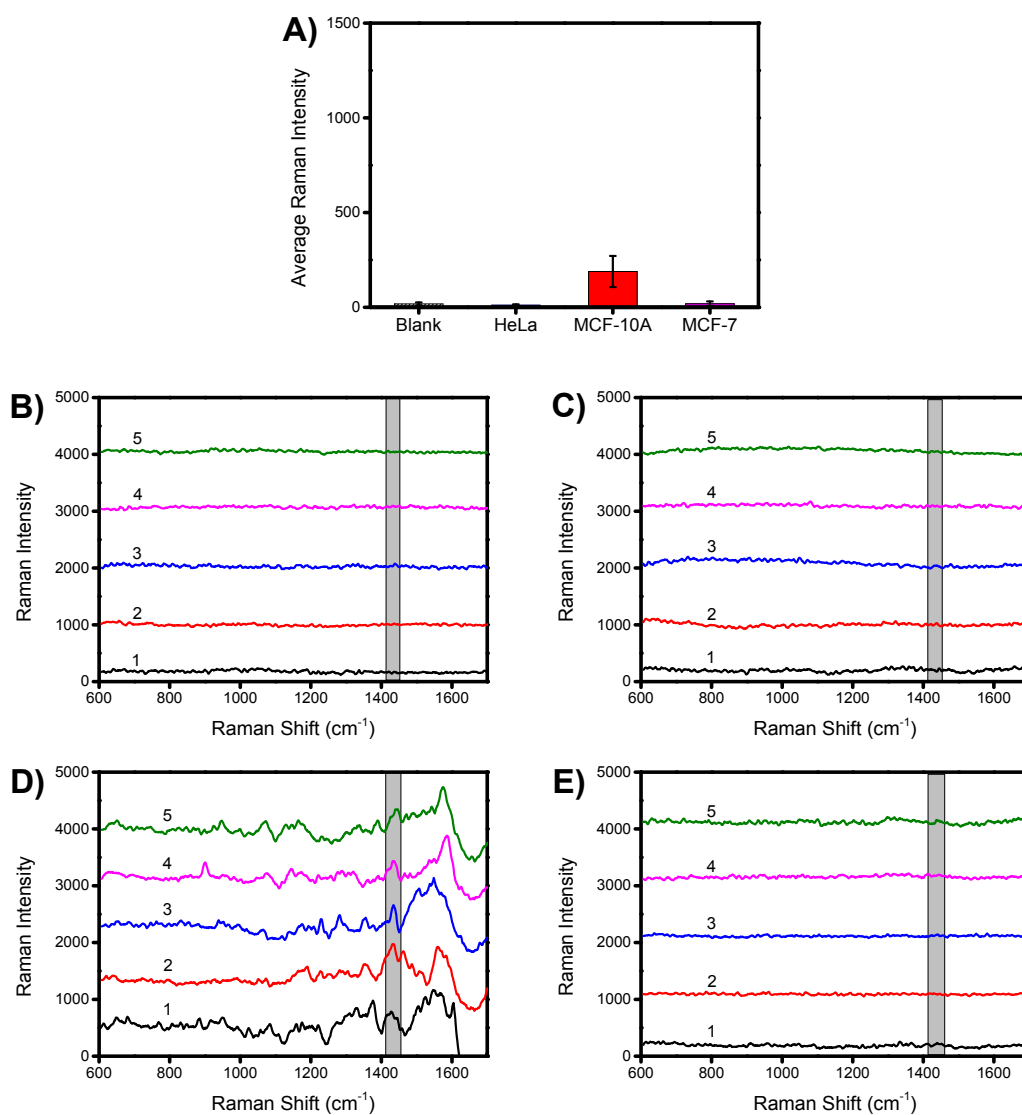
**Fig. S3** Signal variation of eight different microprobes for PASA of miR-21 in standard solutions.



**Fig. S4** Signal intensity for PASA of free-floating cytoplasmic miR-21 in single living cells of different cell lines. A) Average Raman intensity of free-floating cytoplasmic miR-21 in different cell lines. B-E) Representative Raman spectra of free-floating cytoplasmic miR-21 expression in individual cells of different cell lines. B) Blank sample: phosphate buffer (10 mM, pH 7.4). C) HeLa cell line. D) MCF-10A cell line. E) MCF-7 cell line.

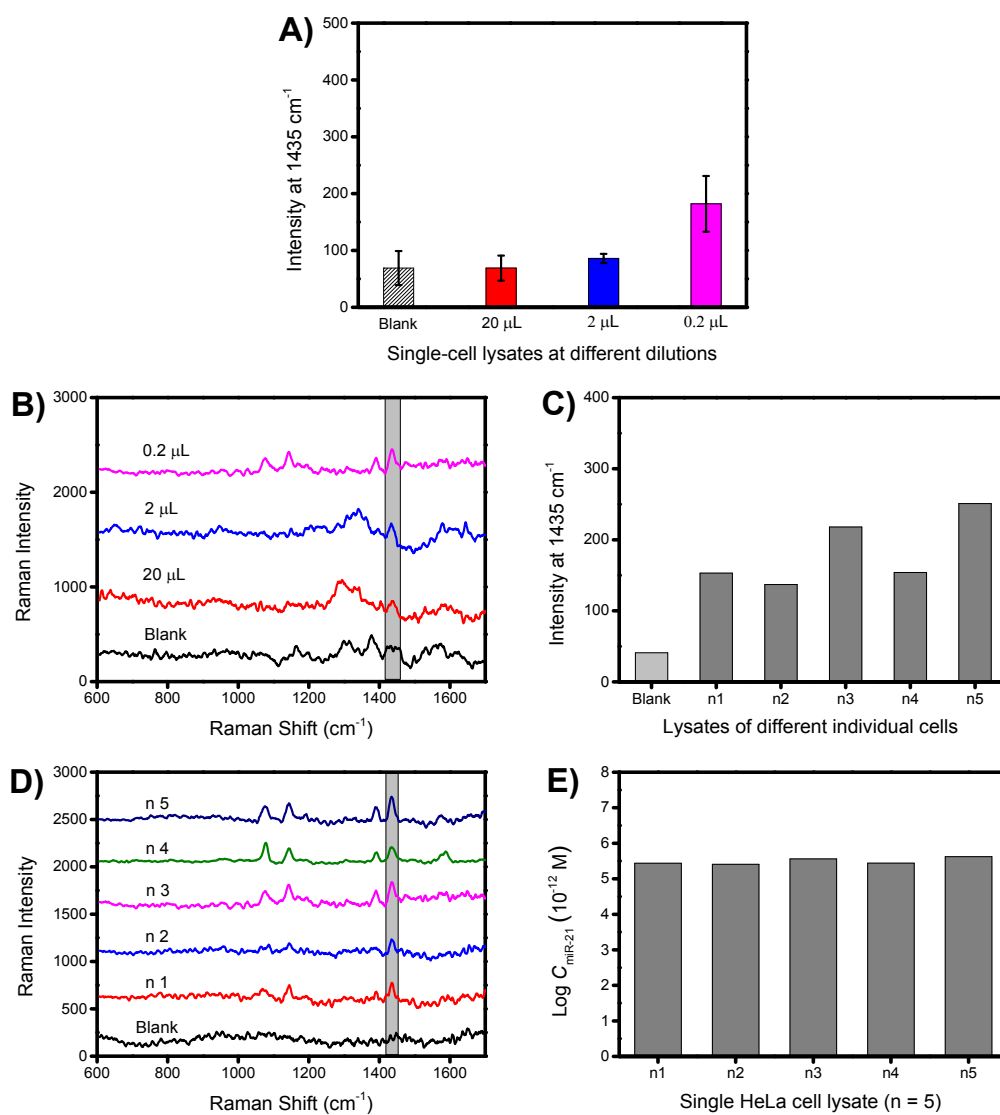


**Fig. S5** Signal intensity for PASA of free-floating cytoplasmic miR-155 in single living cells of different cell lines. A) Average Raman intensity of free-floating cytoplasmic miR-155 in different cell lines. B-E) Representative Raman spectra of free miR-155 in individuals of different cell lines: B) Blank sample: phosphate buffer (10 mM, pH 7.4). C) HeLa cell line. D) MCF-10A cell line. E) MCF-7 cell line.



**Fig. S6** Signal intensity for PASA of of free-floating cytoplasmic miR-203 in single living cells of different cell lines. A) Average Raman intensity of free-floating cytoplasmic miR-203 expression in different cell lines. B-E) Representative Raman spectra of free-floating cytoplasmic miR-203 in individuals of different cell lines: B) Blank sample: phosphate buffer (10 mM, pH 7.4). C) HeLa cell line. D) MCF-10A cell line. E) MCF-7 cell line.





**Fig. S7** A) Raman intensity for PASA of miR-21 in single-cell lysates at different dilutions. B) Representative Raman spectra for the PASA experiments in A. C) Raman intensity for PASA of miR-21 in lysates of individual HeLa cells dissolved in 0.2 μL of cell lysis buffer (the total dilution fold was about  $2 \times 10^7$ ). D) Raman spectra for the PASA measurements in C. E) Estimated concentration of miR-21 in the lysate of single cells according to obtained Raman intensities shown in C.

**Table S1.** The extraction sequences and labeling sequences used in this study.

<b>Target miRNA</b>	<b>Function</b>	<b>Sequence (5' to 3')</b>
miR-21	Extraction	UGAUAAGCUA-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>
	Labeling	SH-(CH <sub>2</sub> ) <sub>6</sub> -UCAACAUCAGU
miR-203	Extraction	AACAUUUCAC-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>
	Labeling	SH-(CH <sub>2</sub> ) <sub>6</sub> -CUAGUGGUCCU
miR-155	Extraction	UUAGCAUUA-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>
	Labeling	SH-(CH <sub>2</sub> ) <sub>6</sub> -CCCCUAUCACG
miR-29a	Extraction	GAUGGUGCUA-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>
	Labeling	SH-(CH <sub>2</sub> ) <sub>6</sub> -AACCGAUUUC
miR-29b	Extraction	AAUGGUGCUA-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub>
	Labeling	SH-(CH <sub>2</sub> ) <sub>6</sub> -AACACUGAUU

**Table S2.** MiRNAs tested in this study.

<b>Name</b>	<b>Sequence (5' to 3')</b>
miR-21	UAGCUUAUCA G ACUGAUGUUGA
miR-21-A	UAGCUUAUCA G ACUGAG <u>GU</u> UGA
miR-21-B	UAGCUUA <u>UG</u> A G ACUGAUGUUGA
miR-21-C	UAGCUUA <u>UG</u> A G ACUGAG <u>GU</u> UGA
miR-21-D	UAG <u>GU</u> UA <u>UG</u> A G ACUGAG <u>GU</u> <u>G</u> GA
miR-203	GUGAAAUGUU U AGGACCACUAG

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miR-155	UUAAUGCUAA UCGUGAUAGGGG
miR-29a	UAGCACCAUC U GAAUUCGGUU
miR-29b	UAGCACCAUU UGA AAUC AGUGUU

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