Supporting Information for

A novel intracellular signal amplification strategy for the quantification of ATP in single cells by microchip electrophoresis with laser induced fluorescence detection

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Reagents and materials

Trishydroxymethylaminomethane (Tris), sodium hydroxide, sodium dodecyl sulfate (SDS), borax and hydrochloric acid were purchased from Sinoponics Group Chemical Reagent Co., Ltd. (Shanghai, China). Exonuclease III and 10×NEB buffer 1 (0.1 M Bis-Tris-Propane-HCl ,0.1 M $MgCl_2$,10 mM DTT，pH7.0) were purchased from New England Biolabs Ltd. (Beijing, China). Targeted nucleic acid probe (TP) which was labeled with fluorescent group (sequence is: 5'-CCTCCTACCTGGGGGAGTA-TTGCGGAGGAA-GGTA-(FAM)-3'), non-targeting nucleic acid probe (NP) which was labeled with fluorescent group (sequence is: 5'-CCTCCTACCTGAGCCAGTA-TTTAGGAGGAAGGTA-(FAM)-3'), ATP, TTP, CTP, GTP, UTP, dATP, 5×TBE buffer and diethyl pyrocarbonate treated water (DEPC water) were purchased from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Lipofectamine®3000 kit (Lipofectamine®3000 and P3000^{TM} reagents are included), DMEM hyclone, RPMI1640 hyclone and Opti-MEM hyclone were purchased from Thermo Fisher Scientific Inc. Fetal bovine serum was purchased from Shanghai ExCell Biology Inc. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), trypsin-EDTA digestive juice (0.25%) and penicillin-streptomycin solution were purchased from Beijing Solarbio Science & Technology Co.,Ltd. TP stock solution (20 μM) was prepared with Tris-HCl buffer solution (20 mM Tris-HCl, 0.1 M NaCl, 20 mM KCl, 2.0 mM $MgCl_2$, pH=7.4), and stored at -20 °C in darkness. It was heated in a 90 °C water bath for 10 min before use, then cool naturally to room temperature. Electrophoresis buffer was 25 mM borate solution (pH 9.0) containing 25 mM SDS. All solutions were filtered through a 0.45 μm membrane filter. HepG2, Hela and HL-7702 cell lines were obtained from the cell/stem cell bank of the Chinese Academy of Sciences.

Apparatus and microfluidic chip

MCE-LIF system was designed and constructed by our laboratory (see following Figure 1). It consisted of an intelligent 8-channel high voltage power supply (0~5000 V), a microfluidic glass chip, a 473 nm semiconductor laser and a data acquisition system (chromatography workstation). The microchip was purchased from Dalian Tuo

Microchip Technology Co., Ltd. The chip design is shown in following Figure 2. All reservoirs are 3 mm in diameter and 1.5 mm in depth. All channel sections are approximately rectangular (70 μm width × 25 μm depth).

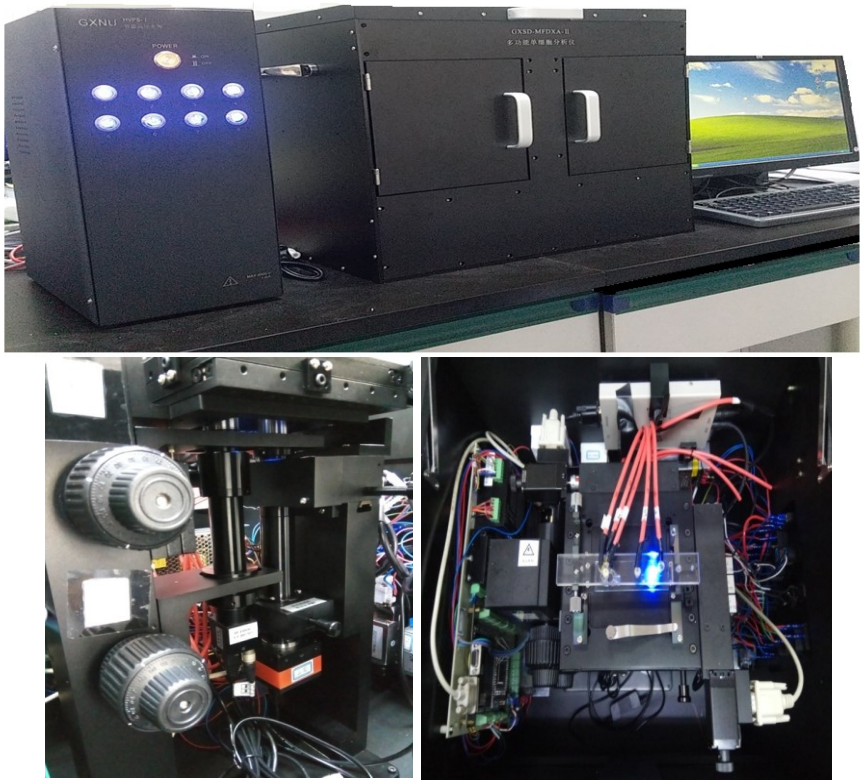

Figure 1. Microchip electrophoresis-laser induced fluorescence detection system designed and constructed for single cell analysis in our laboratory.

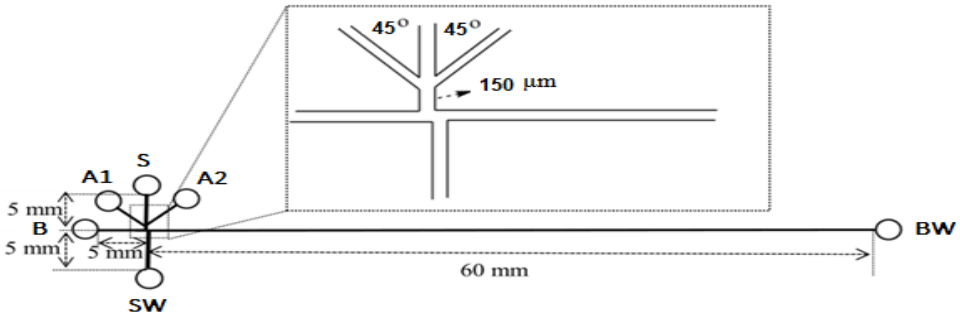

Figure 2. Layout and dimensions of the glass/glass microchip used in this work. S: sample reservoir; SW: sample waste reservoir; B: buffer reservoir; BW: buffer waste reservoir; A1: buffer reservoir; A2: buffer reservoir.

Cells culture

HepG2 cells and HL-7702 cells were grown in DMEM medium containing 10% fetal bovine serum, 100 IU/mL penicillin-streptomycin, respectively, and incubated at 37 °C in a 5% CO_2/95% air humidified incubator. HeLa cells were grown in RPMI 1640 medium.

Preparation of cell lysates

An appropriate amount of cells was transferred from a cell culture flask and re-suspended in PBS so that the cells density was 1×10^6 cells/mL. Then, 3 mL of the cell suspension was centrifuged at 1000 rpm for 5 min. After removing PBS solution, cells were re-suspended in 200 μL Tris-HCl solution. The cells were crushed at 0 °C for 20 minutes with an ultrasonic crusher, and centrifuged at 4 °C in an ultrafiltration tube with a trapped molecular weight of 3000. The filtrate was collected as a cell lysate.

Activation of microchip channels

Before use, vacuum pump was used to wash the microchip channels with methanol, ultrapure water, 1 M NaOH solution and ultrapure water sequentially for 30 min, and then the chip was soaked overnight in ultrapure water. Before each sample injection, the microfluidic channel was rinsed sequentially with ultrapure water for 5 min, 1M NaOH solution for 10 min, ultrapure water for 5 min and electrophoresis buffer for 5 min. Finally, all reservoirs were filled with the electrophoretic buffer solution. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoretic buffer solution for MCE-LIF assay.

Analysis of cells lysate

ATP solutions with different concentrations, or cells lysate sample solution were added into a series of centrifuge tubes, then 5 μL10×NEB buffer 1，20 U exonuclease III，10 μL 0.5 μM TP (targeted probe) solution and an appropriate amount of DEPC water were added to give a volume of 50 μL. The mixed solution was shaken well and incubated in 37 °C water bath for 120 min, then incubated in a 70 °C water bath for 20

min to terminate the reaction. An appropriate amount of reaction solution was detected by MCE-LIF. Prior to electrophoresis, electrophoretic buffer solution in reservoir S was replaced by 10 μL reaction solution, then different voltages were applied to respective reservoirs to complete sample injection, separation and detection. During the sample injection period, the sample reservoir S was applied 400 V, SW reservoir 0 V, B reservoir 200 V, BW reservoir 250 V. After 15 S, and potentials were switched to the separation and detection period immediately. During this period, S and SW reservoirs were applied 2000 V, B reservoir 2600 V, and BW reservoir 0 V.

Intracellular signal amplification

A 10 μL volume of Lipofectamine®3000 was mixed with 140 μL Opti-MEM to obtain solution A. 10 μL Lipofectamine®3000 was mixed with 5 μL P3000™, 5.2 μL exonuclease III, 6.5 μL TP solution (20 μM) and 123.3 μL Opti-MEM to obtain solution B. Then solution A was mixed with solution B to obtain solution C, in which the concentrations of TP and exonuclease III were 434 nM and 1.73 U/μL.

The cells were cultured in 6-well plates for 2 days. When the cell density reached about 80%, the culture medium was removed and cells were washed with PBS solution for 3 times. Then, 1 mL Opti-MEM medium was added into each well. After 1 h, 300 μL solution C was added, and the cells were incubated for 2 h. All cells in the 6-well plate were collected with a 15 mL centrifuge tube. The medium solution was removed by centrifugation for 5 min at 1000 r/min. Then, 500 μL Tris-HCl solution was added to resuspended the cells (cell density about 6×10^6/mL) and transferred to a 1.5 mL centrifuge tube. The above cells suspension was incubated at 70 °C for 20 min to deactivate the exonuclease III and terminate the amplification reaction. The cells were ultrasonically broken at 80 W power for 20 min with an ultrasonic instrument. The broken cell homogenates were centrifuged at a speed of 12000 r/min at 4 °C for 20 min. Appropriate amount of supernatant was diluted 10 times with Tris-HCl solution for MCE-LIF detection. At the same time, the blank control experiments were performed with solution C without exonuclease III and with NP replacing the TP, respectively.

Single cell analysis

HepG2, HL-7702 and HeLa cells were cultured in 6-well plates for 2 days respectively. When the cell density reached about 80%, the culture medium was removed and cells were washed with PBS solution for 3 times. Then, 1 mL Opti-MEM medium was added into each well. After 1 h, 300 μL solution C was added, and the cells were incubated for 2 h. All cells in the 6-well plate were collected with a 15 mL centrifuge tube. The medium solution was removed by centrifugation for 5 min at 1000 r/min. The cells were washed with PBS solution. Then, a certain amount of Tris-HCl solution was added to resuspend the cells (cell density measured by blood cell counting board was about 2×10^5 /mL), and transferred to a 1.5 mL centrifuge tube. The above cells suspension was incubated at 70 °C for 20 min to deactivate the exonuclease III and terminate the amplification reaction.

A 10-μL volume of cell suspension was added to the sample reservoir S. The single-cell analysis was divided into three periods. In the first period, the voltage applied at reservoir S was 200 V, reservoir SW at 0 V, and the electrodes in other reservoirs were suspended. When cell entering channel intersection, voltage was switched immediately to the second period, voltage of 500 V was applied in the reservoirs A1 and A2, and 0 V was applied in reservoir SW, electrodes of other reservoirs were suspended, running 1 s make a cell movement to the intersection of separation channel. When a cell entered the intersection of separation channels, the voltage is switched to period 3. At this stage, the voltage was applied to reservoir B at 2600 V, reservoir SW at 2000 V, and reservoir BW at 0 V, while the electrodes of other reservoirs were suspended. Dissolving of cell membrane, separation and detection of ATP were completed in the third period.

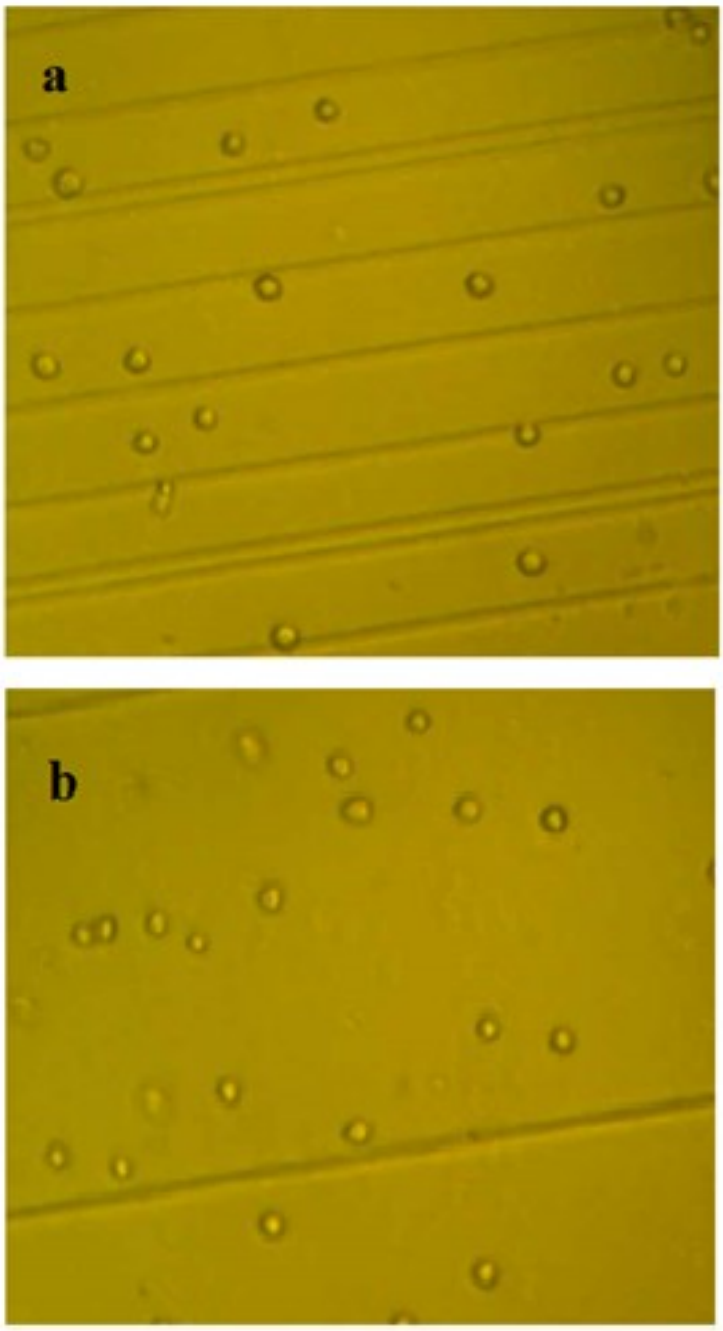

Figure S1. Microscopic images of Hela cells before (a) and after (b) incubation at 70 °C for 20 min.

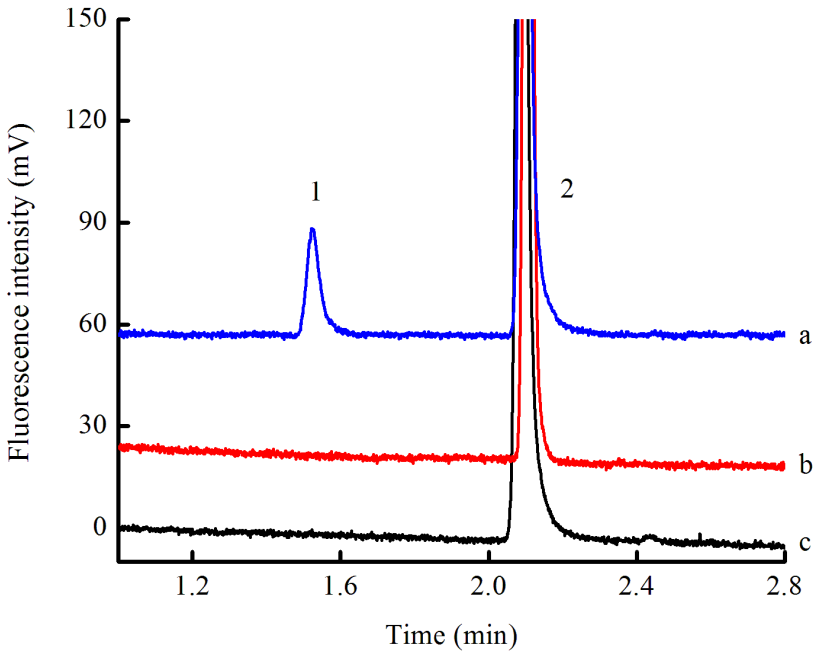

Figure S2. Electrophoretograms of Hela cell lysates after cell lysates were incubated with solutions containing different components: (a) a solution containing TP and exonuclease III; (b) a solution containing TP; and (c) a solution containing non-targeting probe (NP) and exonuclease III.

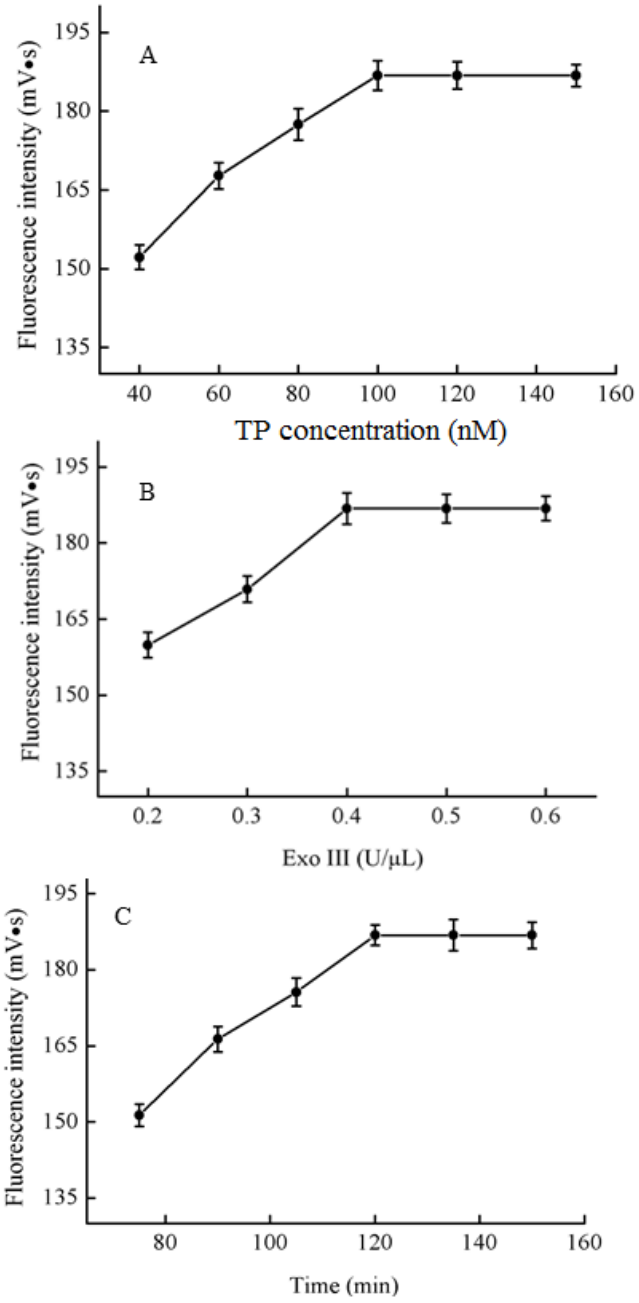

Figure S3. Optimization of reaction conditions for intracellular signal amplification. (a) TP concentration; (b) exonuclease III concentration; (c) reaction time.

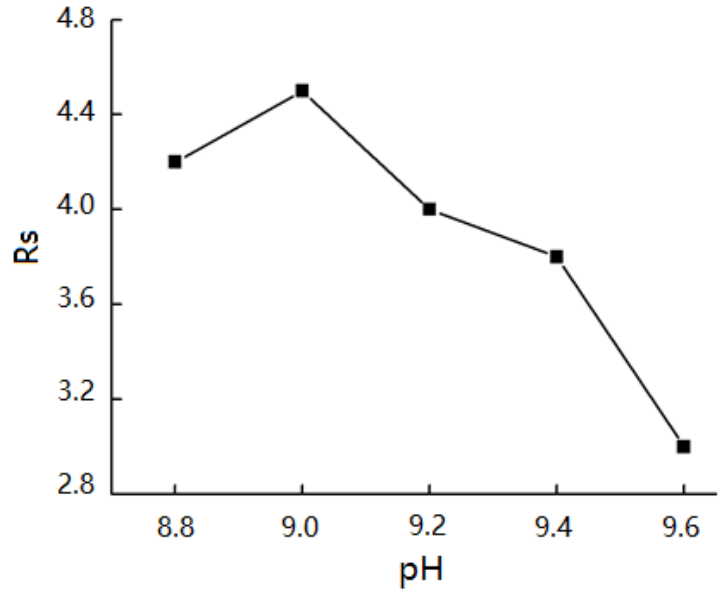

Figure S4. Optimization of electrophoresis buffer solution pH. Electrophoresis buffer was 25 mM borate solution containing 25 mM SDS.

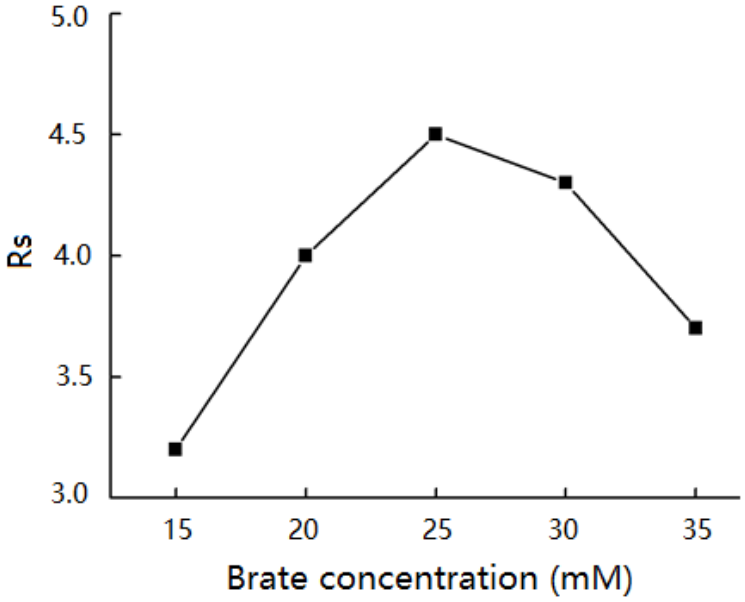

Figure S5. Optimization of borax concentration. Electrophoresis buffer was the borate solution (pH 9.0) containing 25 mM SDS.

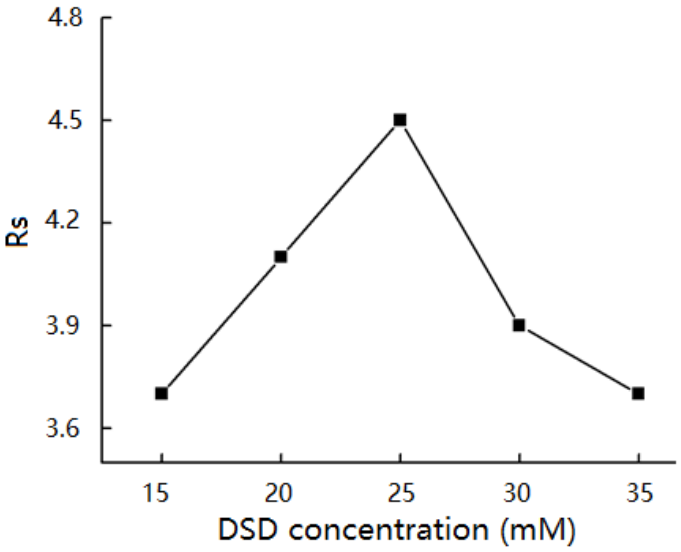

Figure S6. Optimization of the SDS concentration. Electrophoresis buffer was 25 mM borate solution (pH 9.0) containing SDS.

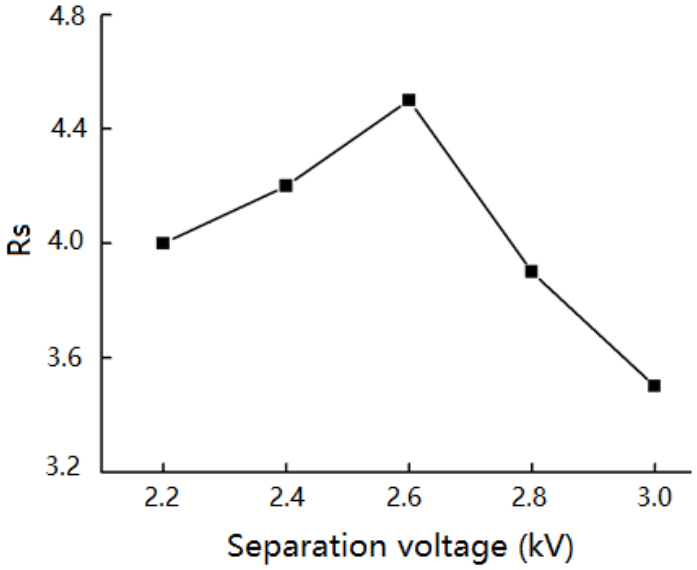

Figure S7. Optimization of the separation voltage. Electrophoresis buffer was 25 mM borate solution (pH 9.0) containing 25 mM SDS.

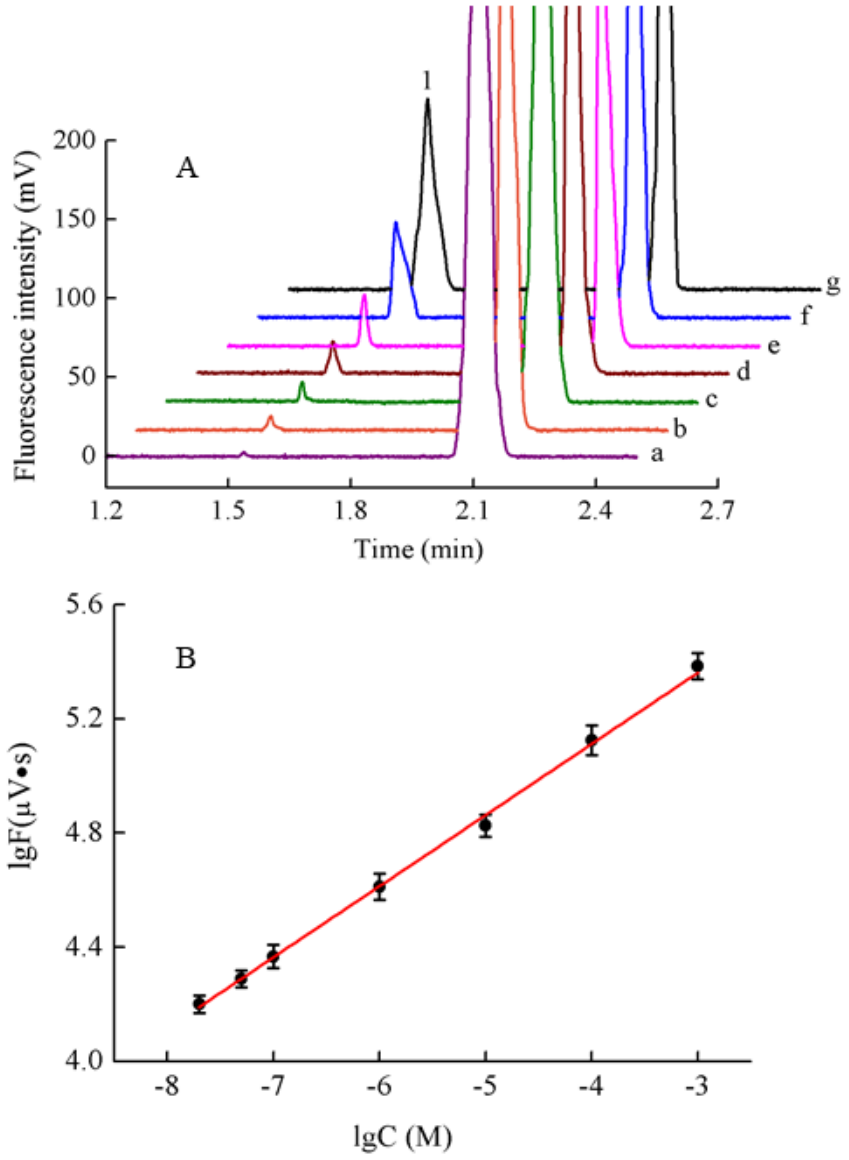

Figure S8. (A) Electrophoretograms of different concentrations of ATP standard solution. The concentration of ATP corresponding to a-g curve is 20 nM, 50 nM, 100 nM, 1μM, 10 μM, 100 μM and 1.0 mM, respectively. (B) The working curve for ATP quantification.

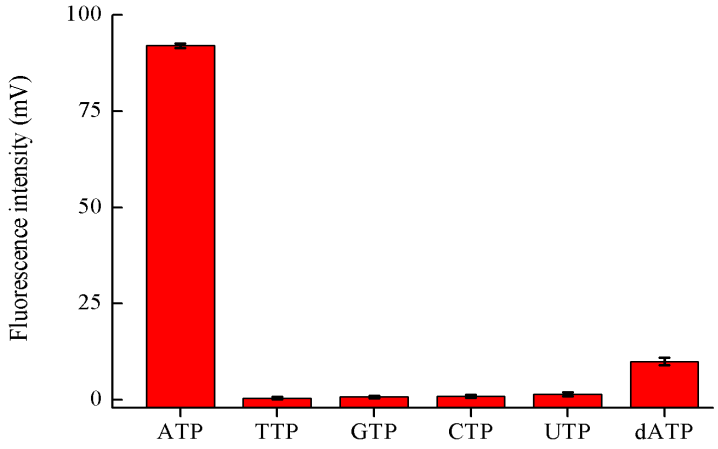

Figure S9. The specificity of present method for ATP detection. The concentration of ATP was 500 μM, and the concentration of other triphosphate deoxyribonucleotide was 1 mM.

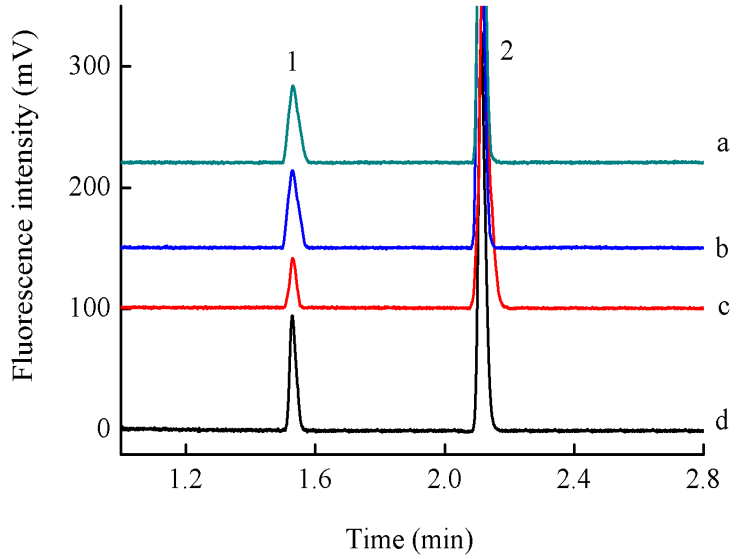

Figure S10. Electropherograms obtained from the analysis of cell lysate samples of Hela (a), HepG2 (b) and 7702 (c), as well as an ATP standard solution (500 μM) sample (d).

Table S1. The detection results of ATP contents in cells lysate

Samples (Cell)	Dilute cells lysate* (μM)	Cells lysate (mM)	Individual cell** (fmol/Cell)	RSDs (n=5, %)
Hela	251	1.51	101	3.6
HepG2	243	1.46	97.3	2.8
HL-7702	170	1.02	68.0	4.2

* Cells lysate samples were diluted 6 times with normal saline.

** Average ATP content in a single cell is calculated from the formula:

$$\text{Content} = CV \div 3.0 \times 10^6$$

where C (M) is the lysate ATP concentration and V (L) is the lysate volume.

Table S2. The detection results of ATP contents in cells lysate (colorimetric method)

Sample (cell)	Content (mM)	RSDs (n=5, %)
Hela	1.56	2.5
HepG2	1.47	3.1
HL-7702	1.05	2.6

Table S3. Results of recovery experiment with standard addition of ATP content in cell lysates

Samples (Cell)	Dilute cells lysate (μM)	Added (μM)	Total measured (μM)	Recovery (%)	RSDs (n=5，%)
Hela	251	200	443	96.0	2.1
HepG2	243	200	454	105.5	3.8
HL-7702	170	200	373	101.5	2.6