.Supporting Information (SI) for

Live Imaging of Cell Membrane-Localized MT1-MMP Activity on a Microfluidic Chip

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Experimental sections

1. Reagents and apparatus

SU-8 2050 Negative photoresist and developer were purchased from Microchem Corporation (Newton, MA, USA). PDMS prepolymers and initiators were purchased from Dow Corning (Midland, MI, USA). 5-TAMRA-βAlaSLAPLGLQRRK-5-FAM-C-NH2 was synthesized from Bankpeptide biological technology co., LTD (Hefei，China). Recombinant human MT1-MMP protein, rhFurin protein, transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor was purchased from R&D Systems (Minneapolis, MN, USA). Matrigel was bought from Corning, America. Marimastat was obtained from MedChem Express (Shanghai, China). CellTracker Red CMTPX dye was obtained from ThermoFisher Scientific (Shanghai, China). Hoechst was purchased from the Sigma (Shanghai, China). N-(4-Maleimidobutyryloxy) succinimide (NHS-Maleimido) and cell counting kit-8 (CCK-8) was bought from Dojindo Corporation (Tokyo, Japan). Fetal bovine serum (FBS) was from Hyclone

(Logan, UT), DMEM medium was from GIBCO (GrandIsland, NY). Native lysis buffer (R0030) was bought from Solarbio (Beijing, China). All chemical reagents were commercially available without further purification, unless noted otherwise. The three cell lines used in this work, HUVEC, HeLa and U87 cell were all obtained from the Cancer Institute & Hospital of the Chinese Academy of Medical Science (Beijing, China). Cell imaging was achieved by inversion laser confocal microscope Zeiss LSM780, and dynamic imaging were taken by DeltaVision. Fluorescence emission spectra were performed with spectrophotometer F-7000 (Hitachi, Japan). The absorbance was measured using the EnVision microplate reader (Perkin, England). Eppendorf Microfuge 5417C (Hamburg, Germany) was used to centrifuge and collect the protein in cells.

2. Design of microfluidic device for invasion assay

A home-designed three channel microfluidic device as described in our previous report1 was used to perform shear stress regulation and biochemical factors stimulation. The dimension (length × width) of each main channel is 15 × 1 mm, each connecting channel has dimension of 1 × 0.15 mm and the depth of all channels were 100 μm. The middle main channel and connective channels were filled with the matrigel to generate tissue-like structure, mimicking extracellular environments. The HUVECs were seeded in one side main channel with DMEM medium containing 1% FBS and flowed at a rate of 1 μL/min, 3 μL/min, 5 μL/min. In the other side main channel, the medium containing transforming growth factor beta 1 (TGF-β1, 10 nM/mL) and 1% FBS was filled and flowed. The detailed procedure for preparing the microfluidic device was as follows (Figure S1): Channels of microfluidic device were fabricated using PDMS (Sylgard 184, Dow Corning) by soft lithography and replica molding

techniques. First, a negative photoresist SU-8 2050 (Microchem) was spin coated onto a cleaned silicon wafer that precleaned by piranha solution. Then, the wafer was pre-baked in the oven (5 min at 65°C and then 4 min at 95°C). After cooling down to room temperature, the wafer covered by a shadow mask containing channels was exposed to UV light (7 mW) for 1 min. After developing, the silicon wafer was post-baked at 65°C for 5 min. PDMS (Sylgard 184, Dow corning) with the weight rate of 10:1 between monomer and curing agent was poured into the silicon mold and baked in the oven at 80°C for 2 h. Then the PDMS was peeled off from the wafer mold, and was treated with oxygen plasma (PDC-32G, Harrick Plasma, Ithaca, NY) for 90 s. After that, the PDMS with microchannels was irreversibly sealed with a glass slide. To avoid the cells being washed away, we performed a surface coating process to modify the microfluidic channel with polylysine (0.1 mg/mL) and then put the chip in an incubator for 4 h. After that, remove the polylysine solution and filled the channels with sterile deionized water, and then wash more than 3 times. Then, the coated chip was placed in a sterile dish at 80℃ in an oven for at least 24 h, but no longer than 72 h. After that, the chip could be used to culture cells.

3. Cell culture and small interfering RNA transfection

Human umbilical vein endothelial cells (HUVECs) and HeLa cells, which were used at passages 4-6, were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37℃ incubator. To silence MT1-MMP, HUVECs were transfected with 20 nM MT1-MMP-specific small interfering RNA (siRNA) (Human MMP14 Catalog: 4390824, Thermofisher, China) using lipofectamine 2000 reagent (Invitrogen, China).

And a scrambled siRNA sequence (Silencer^{TM} Negative Control No.1 siRNA Catalog: AM 4635) was used as a control. The transfection process was performed according to the reagent manual. To culture cells on microfluidic device, cells were first digested with trypsin, and then centrifuged and resuspended with a density of 10^7 cells/mL, followed by injecting the suspension into the microchannel from the inlet and cultured for 40 min in the incubator. After that, we covered the inlets and outlets with cell mediums to minimize evaporation. Thus, the cells were cultured for 12 h and subsequently were labeled with indicators under shear stress-regulated and biochemical factors-stimulated condition.

4. Evaluation of the indicator-response to MT1-MMP

To investigate the responses of indicator to MT1-MMP, first, we need to activate the recombinational human MT1-MMP (rhMT1-MMP). The rhMT1-MMP at a concentration of 200 μg/mL was activated with 0.86 μg/mL rhFurin in activation buffer (50 mM Tris, 1 mM $CaCl_2$, 0.5% (v/v) Brij-35, pH 9.0) and incubate at 37°C for 1.5 hours to obtain activity. Sequentially, the activated MT1-MMP was used in in the indicator-responsive experiments. The indicator (6 μM) was intensely activated by MT1-MMP (0.6 μg) at 37℃ and the fluorescence intensity was measured at excitation/ maximum emission wavelengths of 488 nm/525 nm. The indicator (6 μM) without the addition of MT1-MMP was used as a control. For inhibition experiments, we added inhibitor (marimastat) with a series of concentration (10 μM, 20 μM, 30 μM, 40 μM) to the MT1-MMP solution.

5. Protein quantification and western blot assay.

The cells were rinsed with PBS buffer for three times before homogenization in native lysis buffer (containing 150 mM NaCl, 50mM TrisHCl (pH8.0), 1% NP-40, 0.1 mM PMSF, 0.5%

sodium deoxycholate) at 4℃ for 15 min. After that, collected and centrifuged the lysates at 14000×g for 5 min. For the protein quantification, the supernatant of cell lysate was quantified by standard bicinchoninic acid (BCA) protein assay according to the standard protocol. Data shown were averaged from 3 experiments. 20 μL of the two samples was respectively added in 96-well plates and incubated with the working reagent of BCA kit (200 μL) for 15 min before measured by microplate reader at the absorbance of 562 nm. Finally, a constant amount of the total protein in HUVEC and Hela cells were separately mixed with 200 μL probe for 30 min at 37℃. The fluorescence intensity was measured by F-7000, where excitation was performed at 488 nm and emission was collected from 505 nm to 600 nm. For western blot assay, lysate from control and MT1-MMP-silenced cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After that, proteins were transferred to polyvinylidene difluoride membranes (PVDF), followed by being blocked overnight at 4°C with 5% defatted milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Next, membrane was further washed with TBST for three times at room temperature. And then the membrane was incubated with two kinds of primary antibodies directed against MT1-MMP and GAPDH at 4°C overnight. Further, the membrane was washed again in TBST, followed by a 1-hour incubation with horseradish peroxidase–conjugated second antibody, which was diluted in TBST containing 5% defatted milk. Finally, the membrane was visualized by ChemiDoc MP (Biorad, USA).

6. Chemical modification of cell membranes and cell imaging

Cells were cultured on confocal dishes (ϕ = 35 mm) until the confluency reached 80%. To chemically modify the cell membranes, cells were washed with PBS (2 mL) for three times and

treated with 50 μM NHS-maleimide (2 mL) for 15 min at 37℃. The 50 μM of NHS-maleimide (*Mw*=280.23 g/mol) was prepared by dissolving 2 mg of the reagent in 30 μL DMSO and then directly diluted with PBS for a final concentration of 50 μM. Then, cells were rinsed with PBS (2 mL) for three times to remove the free NHS-maleimide and further incubated with MT1-MMP-responsive peptide at a concentration of 6 μM (2 mL) at 37℃ for 15 min. The MT1-MMP-responsive peptide was prepared by directly dissolving with PBS buffer for a final concentration of 6 μM. Cells were then rinsed with PBS and refreshed with new cell medium. To make the cell images more clear, 2 mL of commercially available CellTracker Red CMTPX dye diluted with FBS-free cell medium to a concentration of 5 μM was used to stain cytoplasm at 37℃ for 40 min, and 2 mL of Hoechst 33342 (100×) was used to image the nuclear at 37℃ for 10 min. All microscopy measurements were operated on laser scanning confocal microscope (Zeiss780) inverted fluorescence microscope.

7. Cell viability assay

HUVECs, at passages 4-6, were cultured in a 96-wells plate with a density of 10^5 cells per well supplemented with 5% CO2 at 37°C for 24 h. Then the medium was discarded and replaced with fresh medium (100 μL) containing various concentrations of NHS-maleimide and substrate peptides for 15 min at 37°C, respectively, followed by a cytotoxicity investigation through the standard CCK-8 assay. 10 μL of CCK-8 working solution mixed with 100 μL culture medium was added into 96-well plate, a water-soluble tetrazolium salt WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-ni-trophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolim, monosodium salt] was reduced by dehydrogenase in living cells to give an orange colored outcome, WST-8 formazan, which was also soluble in the cell medium. The quantity of the formazan was directly

proportional to the amount of living cells after 1 h incubation, and results were detected by a microplate reader at 450 nm. Experiments were carried out in triplicate and repeated three times. To calculate the cell viability, we used the following method: *Cell viability (%)* = $\frac{Abs(drug) - Abs(blank)}{Abs(no\ drug) - Abs(blank)} \times 100\%$. Abs (drug) represents the absorbance of the well with cells, cell medium, CCK-8 reagent and drug solution; Abs (blank) represents the absorbance of the well with only cell medium and CCK-8 reagent; Abs (no drug) represents the absorbance of the well with cells, cell medium, CCK-8 reagent. To investigate the cell viability after an integrated modification, HUVECs, Hela cells at passages 4-6 and U87 human glioma cell lines at passages 3-5 were cultured in a 96-wells plate with a density of 10^5 cells per well supplemented with 5% CO_2 at 37°C for 24 h, followed by cell-surface modification according to the method "Chemical modification of cell membranes and cell imaging". After that, the cell viability was measured as mentioned above.

8. Cell responses to the regulation shear stress and stimulation of different proteins.

Cell responses to the regulation of a series of shear stress: Cell medium was infused into microchannel cultured with HUVECs at different flow rates. Fluid shear stress can be calculated using the Navier-Stokes formula: $\tau = 6\mu Q/wh^2$, where τ is fluid shear stress (dyn/cm^2), μ is liquid viscosity (cell medium at 37 °C is 7.8×10^{-4} N·S / m^2), Q is flow rate, w and h are channel width and height, respectively. The fluorescence signal on cell membrane was imaged by Zeiss780 and the average fluorescence intensity at different flow rate was calculated using software Nikon NIS Elements AR v4.3.

Cell responses to the stimulation of various biochemical factors: DMEM cell mediums containing 10 nM different biochemical factors (TGF-β1, VEGF, BSA, goat anti-mouse IgG)

were injected into the cell culture channels (HUVEC cells) at a series of flow rate (0 μL/min, 1 μL/min, 3 μL/min, 5 μL/min). The fluorescence signal on cell membrane was imaged by Zeiss780 and the average fluorescence intensity was calculated using software Nikon NIS Elements AR v4.3.

9. Statistical analysis.

All data were presented as means ± SD. All experiments were carried out in triplicate and repeated three times.

Supplementary Figures

To evaluate the enzyme activation of the designed MT1-MMP-responsive indicator, we measured the change of fluorescence intensity after the addition of standard MT1-MMP activated by furin protein. The fluorescence intensity of the indicator was apparently proportional to the volume of MT1-MMP with a fixed concentration of 0.2 μg/μl after incubation for 30 min at 37℃ (Figure S1). The time-lapse imaging further showed that the proportion sustained steady with the passage of time (Figure S2).

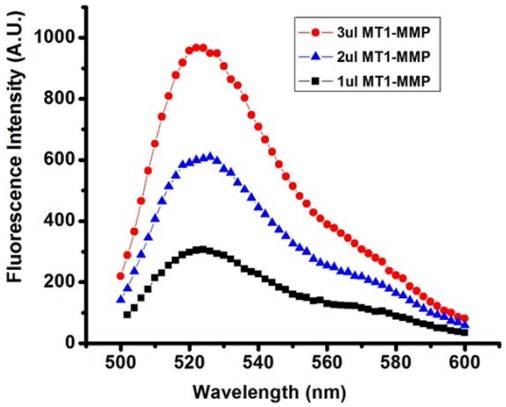

Figure S1. Representative spectra of the indicator upon cleavage for 30 min at 37℃ with different amount of MT1-MMP. Excitation was performed at 488 nm and emission was collected from 505 nm to 600 nm.

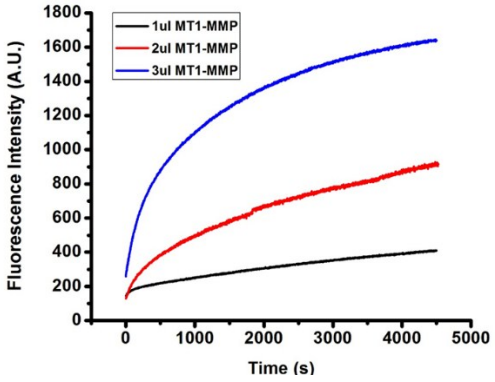

Figure S2. Time courses of the indicator fluorescence signal when treated with different volume of MT1-MMP. Excitation was performed at 488 nm and the maximum emission wavelength was 525 nm.

To validate the different expression levels of MT1-MMP, HUVEC and Hela cells were lysed to obtain the cell lysate and then incubated with our indicator. For a constant amount of the total protein in these two lysate, a standard bicinchonininc acid (BCA) analysis was performed (Figure S3). Consistently, much lower fluorescence intensity was observed in MT1-MMP-deficient HeLa cells, whereas relatively stronger fluorescence intensity was found in HUVEC cells lysates (Figure S4), confirming that our indicator can be effectively trace the cell-surface-localized MT1-MMP.

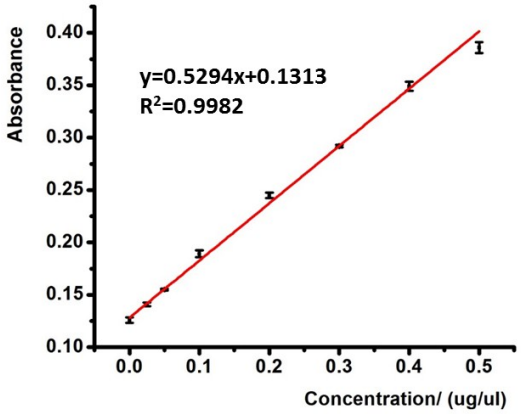

Figure S3. Standard curve for protein quantitative analysis by BCA method.

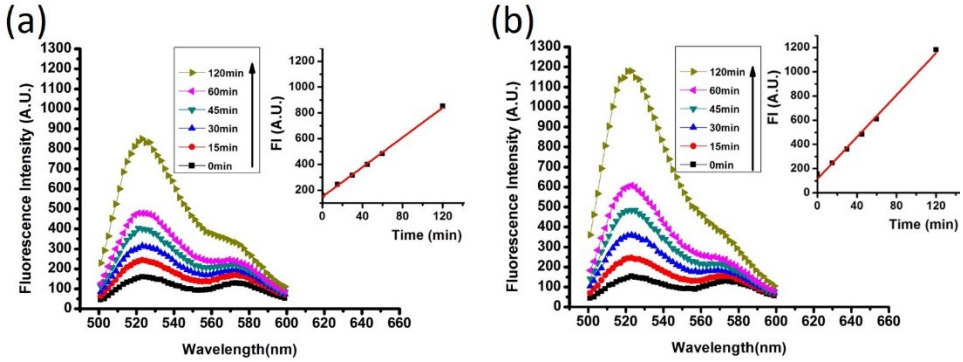

Figure S4. The fluorescence enhancement of the indicator (6 μM; in PBS buffer; pH 7.4) when incubated with the lysate of HeLa cells (a) and HUVECs (b) at different time intervals (0-120 min, Ex=488) at 37℃.

Inset shows the fluorescence intensity at the maximum emission wavelength changed with the incubation time.

The concentration of the indicator conjugated on cells membrane was determined by the cytotoxicity assays. Various concentrations of the linker molecule and peptide indicator were incubated with HUVECs for 15 min, where the cells had been seeded in a 96-wells plate with a density of 10^5 cells per well for 24 h. After that, the cell viability was measured with a standard CCK8 assay. The results showed that the peptide indicator had negligible cytotoxicity to cells, while the cells were tolerant to the linker molecule at the concentration $\leqslant$50 μM. Finally, we used 50 μM linker molecule and 6 μM peptide substrate in all experiments unless otherwise specified. Additionally, we investigated the viability of three kinds of cells after an integrated modification, no significant cytotoxicity was observed using the same reagent concentration as mentioned above. These results suggested that this indicator could be applied to a real-time imaging of MT1-MMP activity with biocompatibility. The cell viability was calculated according to the formula mentioned in methods.

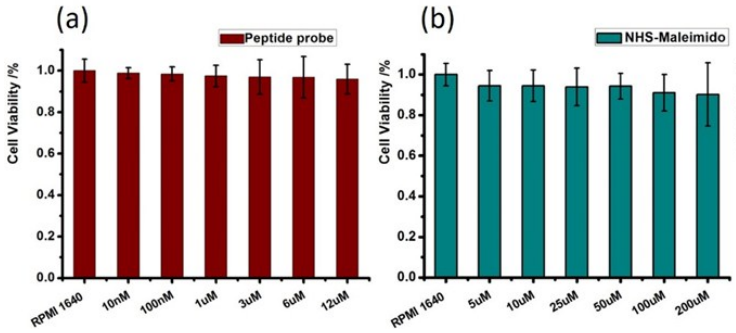

Figure S5. The optimization of the two-step-modified indicator concentration. (a) The concentration optimization of peptide indicator. (b) The concentration optimization of the linker molecule.

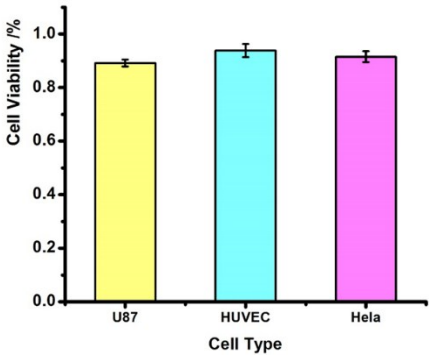

Figure S6. The cytotoxicity evaluation of the integrated modification on various kinds of cells (U87, HUVEC, Hela).

To evaluated the stability of our indicator, we cultured HUVECs as follows. As we can see in Figure. S7a, only some parts of cell surface appeared fluorescence in the first 12 h without TGF-β1 stimulating, suggesting a relatively low activity of MT1-MMP in this condition. Gradually, the bright fluorescent outlines of the cell membranes were clearly observed after the cell culture medium was replaced to contain TGF-β1 factor (Figure. S7b). As we can see, almost no

fluorescent signal was observed in the intracellular regions. Continuously, the fluorescence signal became increased with the passage of time (Figure. S7c and S7d), implying an enhanced activity of MT1-MMP under the stimulation of TGF-β1.

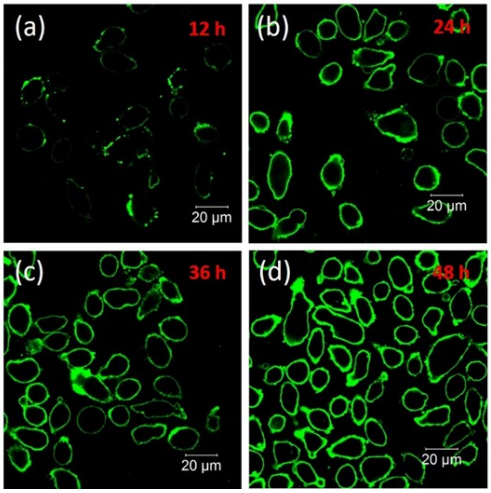

Figure S7. Typical time series fluorescence imaging of cell-surface-localized MT1-MMP on HUVECs to verify the stability of the indicator. The laser intermittently irradiated the sample every 12 h.

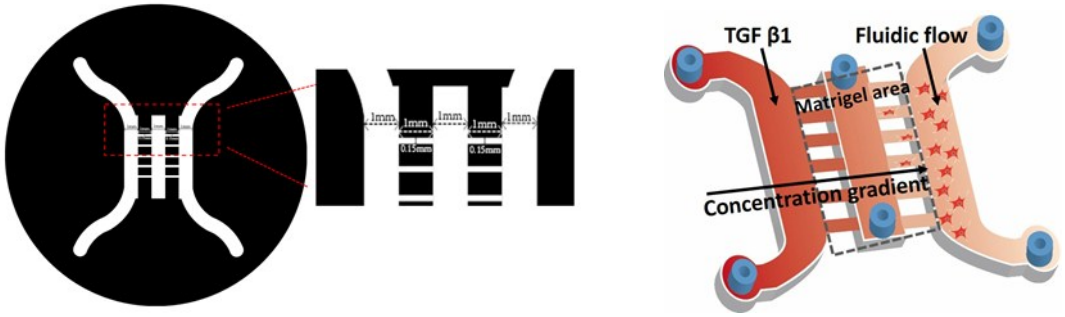

Figure S8. Schematic design of the microfluidic device. The dimensions of the mask (left) were shown. The microfluidic model (right) illustrated the duple controllable biochemical and mechanical stimuli. TGF-β1 was infused into left main channel at a flow rate of 2 μL/h and would diffuse into the right main channel. The middle main channel and connective narrow channels were filled with Matrigel to mimic the tissue-like niche. HUVECs were cultured in the right main channel and suffered from a fluid flow. The lateral solid arrow indicated a concentration gradient of TGF-β1.