Direct imaging furin-controlled intracellular condensation with two-photon laser microscope

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Method Article

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Abstract

We developed a new “smart” Eu-based probe \(**2**) which is susceptive to furin, a protease overexpressed in cancer cells. Upon furin cleavage, **2** condenses to form oligomers and the latter self-assemble into Europium nanparticles \(Eu-NPs) on site. Two-photon laser microscopy \(TPLM) imaging of MDA-MB-468 cells incubated with **2** showed strong fluorescence signals from Golgi networks, suggesting **2** was under the action of furin and trapped at/near the locations of furin \(i.e., Golgi networks). TPLM imaging of MDA-MB-468 cells incubated with the scrambled control of **2** \(i.e., **2-Scr**) at same condition only exhibits uniform, weak fluorescence signals. These results suggest that **2** could be a useful probe for TPLM imaging of furin activity in cancer cells. We describe herein a detailed protocol of cell preparation and TPLM imaging with **2**.

Introduction

The trans-Golgi protease furin is a protein convertase playing crucial roles in homeostasis, and in diseases ranging from anthrax and Ebola fever to Alzheimer’s disease and cancer [1]. Increase of furin in tumors correlates with the increase of membrane type 1-matrix metalloproteinase \(MT1-MMP), which activates extracellular pro-MMP2 to induce rapid tumor growth and metastasis [2]. Therefore, noninvasive imaging of furin activity offers a valuable tool to probe tumor growth and migration in real time and directly assess the anti-cancer efficacy of drugs _in vivo_ [3]. It has been reported that the majority of human breast cancer cells overexpress furin [4].Traditional immunofluorescence staining of MDA-MB-468 cells indicates that furin is predominantly located in the trans-Golgi networks of this type of breast cancer cells [5]. While there are very few methods that have been reported to image furin activity directly, Rao and coworkers developed two methods of intracellular condensation and intramolecular macrocyclization for imaging furin activity in living cells using fluorescence probes [6-7]. Two-photon laser microscopy \(TPLM) is a fluorescence imaging technique that allows imaging of living tissues up to a very high depth. It uses red-shifted excitation light to excite fluorescent dyes. For each excitation, two photons of the infrared light are absorbed simultaneously. TPLM can be a superior technique due to its deep tissue penetration, efficient light detection and reduced phototoxicity [8]. Furin preferentially cleaves Arg-X-Lys/Arg-Arg↓X motifs, where Arg is arginine, Lys is lysine, X can be any amino acid residue and ↓indicates the cleavage site [9]. Combining these two advantages above, recently we developed Acetyl-Arg-Val-Arg-Arg-Cys\(StBu)-Lys\(Eu-DOTA)-CBT \(**2**) for imaging furin-controlled condensation in MDA-MB-468 cells \(Fig. 1) [10]. Its scrambled control, **2-Scr**, was studied in parallel. In brief, **2** contains a RVRR peptide sequence for furin cleavage and cell membrane translocation, disulfided Cys for supplying the 1,2-aminothiol group for condensation with the cyano group on the benzothiazole motif, Lys conjugated with Eu-DOTA for TPLM. With the probes designed, we successfully imaged the furin-controlled intracellular condensation of **2**, as well as the location and activity of furin \(Fig. 2). We describe herein a detailed protocol of cell preparation and TPLM imaging with **2**.

Reagents

1. Dulbecco’s modified eagle medium \(GIBCO) 2. Fetal bovine serum \(GIBCO) 3. PBS \(Sangon) 4. Paraformaldehyde \(Sinopharm Chemical Reagent Co.) 5. Glycerol \(Sangon) 6. Nail enamel \(from local store)

Equipment

Equipment 1. CO_2 incubator \(Thermo) 2. Pipettor \(Eppendorf) 3. Glass slide \(Sailing boat) 4. Two photon microscope \(Zeiss LSM 710) 5. A femtosecond mode locked Ti: sapphire laser \(Coherent Inc.; pulse width, <140 fs; repetition rate, 80 MHz) 6. Zeiss TPMT detector 7. Objective: Zeiss W plan-Apochromat 20×1.0 DIC 8. Software: Zen 2010 9. Beam splitters: MBS-InVis: MBS 690+ Equipment Setup 1. Irradiate the coverslips with ultraviolet rays for 30 min under a UV lamp \(500 W), sonicate in water for 15 min, wash with DI water for 3 times, immerse in ethanol. Before use, take out the coverslips from ethanol and dry them on an alcohol lamp in biological safety cabinet. 2. Prepare the solutions of **2** or **2-Scr** at 10 mM by dissolving the powders in PBS \(pH 7.4) and then filtrate with 0.2 μm filter membrane.

Procedure

1. MDA-MB-468 human breast adenocarcinoma epithelial cells were cultured in Dulbecco’s modified eagle medium \(GIBCO) supplemented with 10% fetal bovine serum \(FBS, GIBCO) in incubator supplied with 5% carbon dioxide humidified air at 37 °C. 2. Seed the healthy cells on the coverslips in each well of a 24-well plate at about 60% density, then incubate overnight. 3. Suck off the culture medium, add 500 μL DMEM containing 100 μM **2** or **2-Scr** \(mix 5 μL of PBS stock solution of **2** or **2-Scr** at 10 mM with 495 μL DMEM) into different each well respectively, then incubate for 8 h. 4. Suck off the medium, wash the cells with PBS for three times at room temperature. 5. Add 500 μL of 4% paraformaldehyde in PBS into each well to fix the cells at room temperature for 30 min, wash the cells with PBS a further three times. 6. Drop 1 μL of 50% glycerol PBS solution on a glass slide and then pick up the coverslip in 24-well plate, mount the coverslip on the glycerol drop with the cells facing to the glass slide, fix the edges of coverslip with glass slide using nail enamel.

Timing

Timing Step 2: 10 h Step 3: 8.5 h Step 4: 20 min Step 5: 50 min Step 6: 30 min per sample Step 7: 60 min per sample

Troubleshooting

Anticipated Results

The two europium probes developed in this protocol could be used as one pair for TPLM imaging furin activities in cancer cells. Figure 1 shows the chemical structures of the two probes used in this protocol, in which **2** has a RRVR peptide substrate for furin cleavage. Following this protocol to prepare cell samples, **2** condenses to form Europium nanparticles \(Eu-NPs) intracellularly resulting in strong fluorescence emission from the locations of furin \(i.e., Golgi networks), as exampled in figure 2a. In contrast, since **2-Scr** is not susceptive to furin, TPLM imaging of cancer cells incubated with **2-Scr** will exhibit uniform, weak fluorescence signal, as illustrated in Figure 2b. Figure 1: Chemical structures of **2** and **2-scr**. Figure 2: TPLM images \(λ_{ex} = 725 nm, λ_{em} = 565-636 nm) of MDA-MB-468 cells incubated with **2** \(a) or **2-Scr** \(b) at 100 μM for 8 h and then rinsed and fixed prior to imaging. Scale bar: 20 μm.

References

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Figures

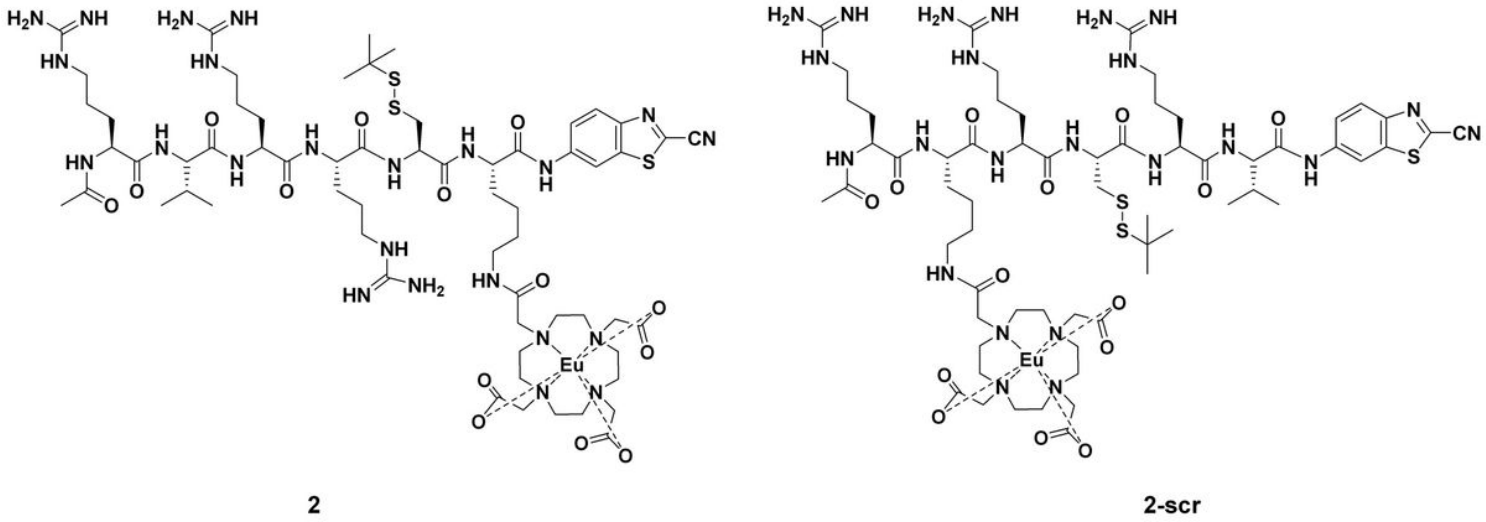

Figure 1

Chemical structures of the two probes used in this protocol Chemical structures of *2* and *2-scr*.

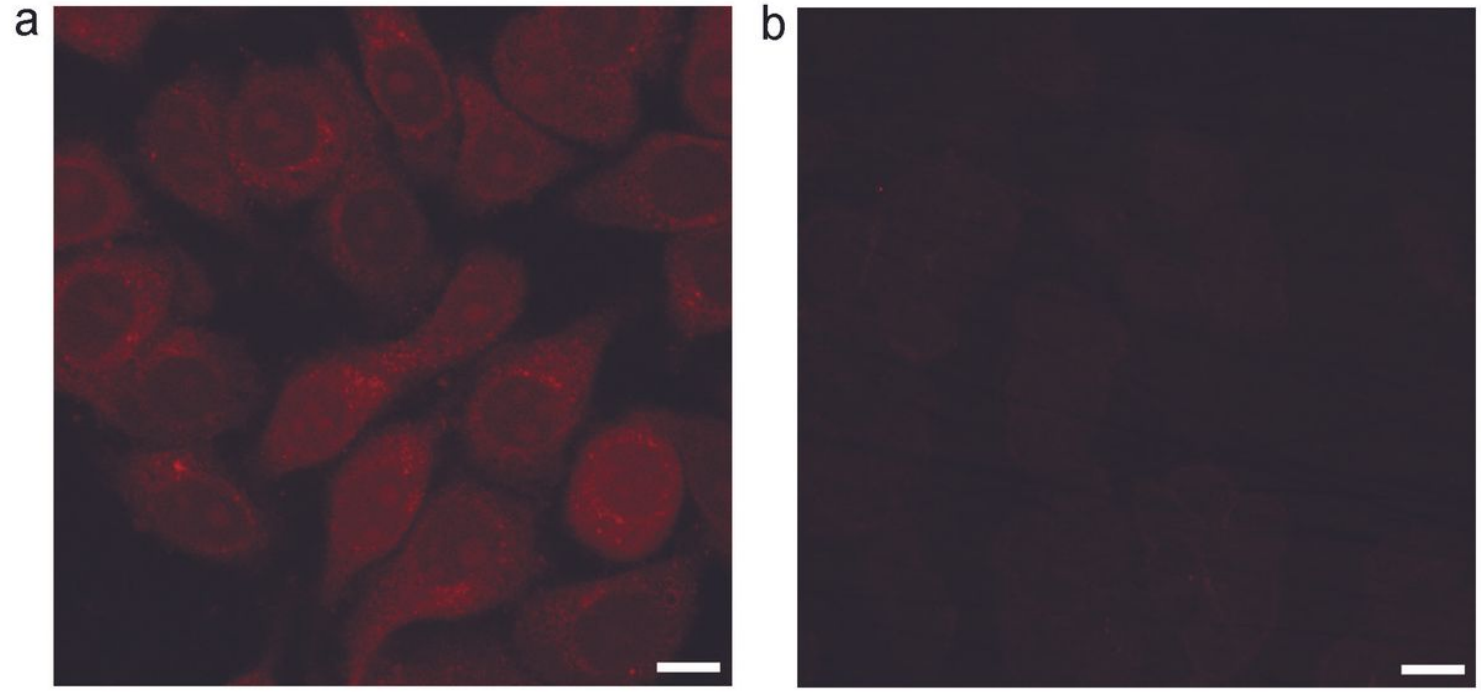

Figure 2

TPLM images of MDA-MB-468 cells incubated with *2* or *2-Scr* TPLM images (λ~ex~ = 725 nm, λ~em~ = 565-636 nm) of MDA-MB-468 cells incubated with *2* (a) or *2-scr* (b) at 100 μM for 8 h and then rinsed and fixed prior to imaging. Scale bar: 20 μm.

Step	Problem	Possible reason	Solution
6	Bubbles in the view field	Contact bubbles formed during dropping the mounting medium or covering the coverslip	Leave small portion of mounting medium in the tip, cover the coverslip starting with a contact angle
7	High background	Culture medium is not totally washed out	Totally wash out the culture medium at step 4
7	Low fluorescence intensity of cell image	Laser power is low, Master Gain value of TPMT is low	Increase the laser power, increase the gain value

Figure 3

Table 1 Trouble shooting Table 1 for Trouble Shooting