Designing Protease Sensors for Real-time Imaging of Trypsin Activation in Pancreatic Cancer Cells

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SUPPORTING INFORMATION

Expression and purification. To express EGFP-based trypsin sensors, a single colony was inoculated into 20 ml of LB media with 30 μg/ml kanamycin at 37 ˚C. The cell culture was then agitated at 200 rpm overnight and transferred to 1 L of LB media with 30 μg/ml fresh kanamycin. The cell culture was further induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the optical density (OD) at 600 nm reached 0.6, and allowed to grow at 30 ˚C for another 16 to 20 h. The cells were harvested by centrifugation at 7,000×g for 20 min. The cell pellets were then resuspended in 10 ml of lysis buffer (20 mM Tris, 10 mM NaCl, 0.1% Triton X-100, pH 8.8) and sonicated to disrupt the cell membrane. The solution was centrifuged at 20,000×g for 20 min. The resulting supernatant was filtered and injected into an Aktaprime FPLC equipped with a nickel-chelating column loaded with 0.1 M nickel sulfate solution. After washing with buffer A (50 mM phosphate, 250 mM NaCl, pH 7.4), the bound protein was eluted with a gradient of imidazole from 0 to 0.5 M in phosphate buffer. The eluted protein was dialyzed in 10 mM Tris buffer with 1 mM DTT at pH 7.4 to remove imidazole. The concentration of the collected fractions was determined based on the absorbance at 280 nm measured using a UV-1601 spectrophotometer (Shimadzu Scientific Instruments Inc.), and then calculated using an extinction coefficient constant of 21,890 $M^{-1}cm^{-1}$ for EGFP. The purity of collected fractions was monitored using SDS-PAGE.

Spectral properties of trypsin sensors. The UV-visible spectra of EGFP-based trypsin sensors were monitored using a UV-1601 spectrophotometer scanning from 600 to 200 nm. The fluorescence spectra were measured in the emission region of 410 to 600 nm with an excitation wavelength at 398 nm using a fluorescence spectrophotometer (Photon Technology International Inc., Canada). Samples were buffered in 10 mM Tris, 1 mM DTT, at pH 7.4.

Dynamic range calculation for optical change. In order to evaluate designed trypsin sensors, the dynamic range calculation for optical signal change in absorbance or fluorescence upon trypsin digestion is determined by equation 3:

$$\text{or} \quad \begin{matrix} D = (A_{490a} / A_{398a}) / (A_{490b} / A_{398b}) \\ D = (F_{490a} / F_{398a}) / (F_{490b} / F_{398b}) \end{matrix} \qquad \text{Eq. 3}$$

where A_{490a} and A_{398a} are absorbance intensity values at 490 nm and 398 nm following trypsin cleavage; A_{490b} and A_{398b} are absorbance intensity values at 490 nm and 398 nm before trypsin cleavage; F_{490a} and F_{398a} are fluorescence intensity values at 490 nm and 398 nm excitation following trypsin cleavage; and A_{490b} and A_{398b} are fluorescence intensity values at 490 nm and 398 nm excitation before trypsin cleavage, respectively.

Inhibition of EGFP-T1 cleavage by leupeptin. In order to examine the inhibition effects of leupeptin (Sigma, St. Louis), a trypsin inhibitor, leupeptin at various concentrations (0, 12.5, 25, 50 and 100 nM) was mixed with EGFP-T1 and subsequently digested by trypsin with a final concentration of 5 nM. Kinetic studies of EGFP-T1 at various concentrations were performed at each inhibitor concentration level and the absorbance changes at 490 nm were monitored using a time-course model. Initial rates and trypsin sensor concentrations were fitted to obtain a plot to confirm inhibition type and calculate K_i value with Enzfitter (Bio-soft, Cambridge).

Cleavage specificity of EGFP-T1. In order to examine cleavage specificity of EGFP-T1 exposed to different proteases, thrombin (GE healthcare, USA), cathepsin B, tryptase, tissue plasminogen activator, kallikerin or chymotrypsin (Sigma, St. Louis) was used to digest trypsin sensor, EGFP-T1. Thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikrein or chymotrypsin was added to 15 μM EGFP-T1, buffered in their optimal reaction conditions (20 mM Tris, 20 mM $CaCl_2$, pH 7.5 for trypsin or chymotrypsin; 20 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.0 for thrombin; 50 mM Tris, 120 mM NaCl, 20 μg/ml Heparin for tryptase; 10 mM Tris, 75 mM NaCl, 2 mM EDTA and 2 mM Cysteine for cathepsin B; 20 mM Tris, 5 mM NaCl, 0.02% NaN_3, pH 8.0 for tissue plasminogen activator; and 20

mM Tris, 100 mM NaCl, 0.1% BSA, pH 7.5 for kallikrein), to a final concentration of 20 nM, respectively. The cleavage of EGFP-T1 following trypsin, thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikrein or chymotrypsin overnight digestion was verified using SDS-PAGE. To confirm optimal reaction buffer conditions for EGFP-T1 cathepsin B digestion, the fluorescence signal change of Z-FR-MAC for cathepsin B was detected by fluorescence spectroscopy following cathepsin B digestion.

Construction of EGFP-T1-mito and EGFP-T1-ER. In order to track the protease activation in various subcellular compartments, the ER retention sequence, KDEL, was connected to the C-terminus, and the ER targeting sequence of calreticulin (CRsig), MLLSVPLLLGLLGLAAAD, was fused to the N-terminus of the EGFP-based protease sensors via PCR technique. The Kozak consensus sequence was placed at the N-terminus of the calreticulin sequence for optimal initiation of protein expression in mammalian cells. The commercial marker Mitotracker Red (Invitrogen, Molecular Probe) and DsRed-ER-marker (BD Bioscience, Clontech) were used as positive markers for mitochondria or ER to confirm co-localization of our designed protease sensors with signal peptide sequences.

Immunofluorescence. In order to identify the subcellular location of trypsinogen activation and EGFP-T1 expression, immunofluorescence was exploited to confirm the co-localization of trypsinogen activation following induction with 10 nM caerulein and EGFP-T1 expression in MIA PaCa-2 cells transfected with EGFP-T1. MIA PaCa-2 cells transfected with EGFP-T1 were stimulated with 10 nM caerulein for 1 h. Following the removal of cell culture media, the cells were washed one time using PBS buffer (140 mM NaCl, 2.7 mM KCl, 20 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and then incubated with 500 μl of 3.7% paraformaldehyde in the cell chamber to fix cells for 30 min at room temperature. The fixed cells were then incubated with 0.1% Triton X-100 for 15 min at room temperature and washed with TBS buffer three times at 15 min intervals. 500 μl of TBS buffer with 5% BSA was added in the cell chamber to block the cells for 3 h. Primary antibodies, rabbit polyclonal to α-trypsin and goat polyclonal to GFP conjugated with FITC (Abcam Inc.), were added into cell chamber with TBS buffer

containing 2.5% BSA in a 1:200 dilution to incubate with fixed cells for 3 h at room temperature. Following incubation with primary antibodies, the cells were washed with TBS buffer three times at 15 min intervals. Secondary antibody, rabbit IgG antibody conjugated with Texas Red (Abcam Inc.) was used to against rabbit polyclonal anti-trypsin for incubating 1 h at room temperature. Following incubation, extra secondary antibody was removed through washing with TBS buffer. One drop of mounting reagent was added on the glass coverslip and subsequently covered for cell imaging.

Expression of EGFP-T1-Mito and EGFP-T1-ER. Our developed trypsin sensor can be specifically targeted to the endoplasmic reticulum (ER) and mitochondria by adding the signal peptide sequence, ER retention sequence, KDEL, and ER targeting sequence of calreticulin, MLLSVPLLLGLLGLAAAD (*1, 2*), and by fusing to mitochondria signal peptide, PLLRGRCPARRH, from cytochrome C oxidase subunit VIII (COX 8) (*3*), respectively. The mitochondrial-targeted sensor (EGFP-T1-Mito) and ER-targeted sensor (EGFP-T1-ER) nicely overlap with the commercial marker Mitotracker Red (Invitrogen, Molecular Probe) and DsRed-ER-marker (BD Bioscience, Clontech), respectively (Figure S4A and S4B). These exciting results indicate that our trypsin sensor provides the potential to map dynamic activation/inhibition processes in specific subcellular environments.

Examination of pH change in living cells following caerulein induction. In order to determine whether the inducer for trypsinogen activation has an effect on pH change in living cells, one of the pH probes, 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Invitrogen, Molecular Probe, USA) was used to examine the pH change following 10 nM Caerulein stimulation. MIA PaCa-2 cells were grown on glass coverslips (0.5 - 1.0 × 10^6 cells/dish) in 35 mm culture dishes in Dulbecco's Modified Eagles Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 44 mM $NaHCO_3$, pH 7.2, and supplemented with 10% FCS (v/v), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep) at 37 °C with 5% CO_2 in a humidified incubation chamber. After the cells were seeded and grown overnight, the cells were incubated with BCECF-AM pH probe to a final concentration of 10 μM in modified Ringer solution at 37 °C for 45 min. Then the fresh modified Ringer solution was added to the cell dishes and incubated for another 20 min at 37 °C to ensure complete

hydrolysis of the probe and release of the fluorescent group in living cells. Cells were imaged on a Leica Inverted Microscope (Leica Microsystem, Germany) with a 40x oil objective lens and an EM CCD camera (Hamamatsu Corporation, Japan). Ratiometric emission imaging was acquired though a monochromator set (excitation wavelength: 490 ± 10 nm and 440 ± 10 nm; emission wavelength: 535 ± 10 nm) from a light source in a time course mode under the control of SimplePCI software (Hamamatsu Corporation, Japan). During image acquisition, 10 nM caerulein for trypsinogen activation were added to the cell plate to an appropriate final concentration for inducing trypsinogen activation. The ratiometric change between fluorescence emission for excitation at 490 and 440 nm was calculated at different time intervals to express pH change due to effects of protease inducers in living cells.

Statistical analysis. The data for normalization fluorescence intensity and fluorescence ratio change of living cells transfected with EGFP-T1 and EGFP-wt are represented as the mean ± SD for at least 6 living cells. A student's t-test analysis was performed to determine statistical significance comparing EGFP-T1 and EGFP-wt. Differences were considered statistically significant at $P < 0.05$.

SUPPORTING FIGURES

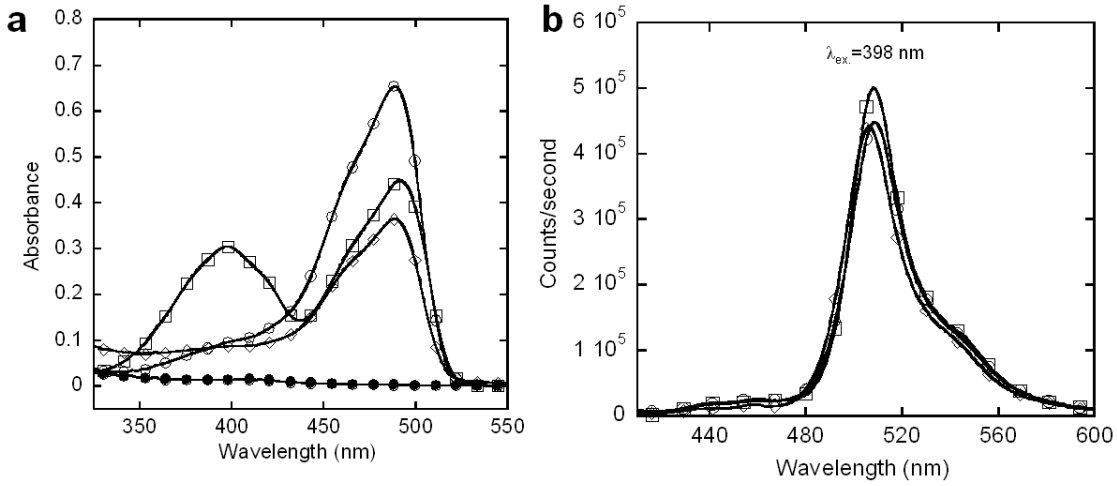

Figure S1. Optical properties of trypsin sensors. Visible absorbance (**a**) and fluorescence (**b**) spectra of EGFP-wt (○), EGFP-T1 (□), EGFP-T2 (◊) and EGFP-T3 (●) were measured in 10 mM Tris, 1 mM DTT, pH 7.4.

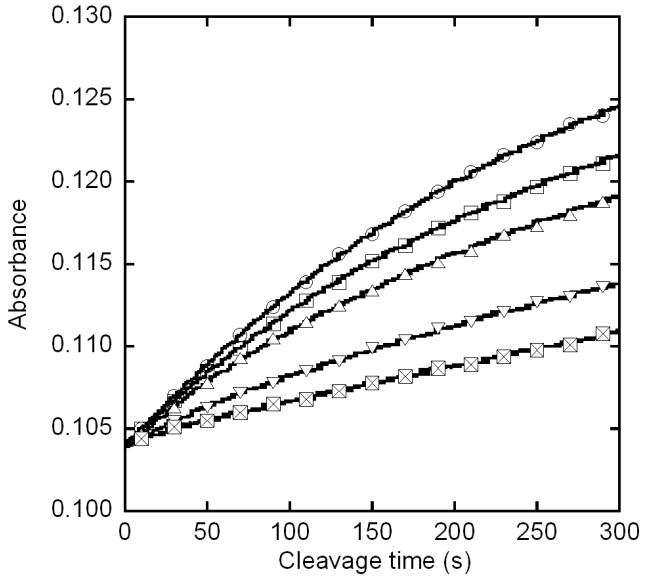

Figure S2. Inhibition effects on the cleavage of EGFP-T1. EGFP-T1 samples containing trypsin inhibitor, leupeptin of 0 nM (○), 12.5 nM (□), 25 nM (Δ), 50 nM (∇), 100 nM (⊠) were digested using 5 nM trypsin in 10 mM Tris, 20 mM $CaCl_2$, pH 7.4. Absorbance was monitored at 490 nm using a time course model.

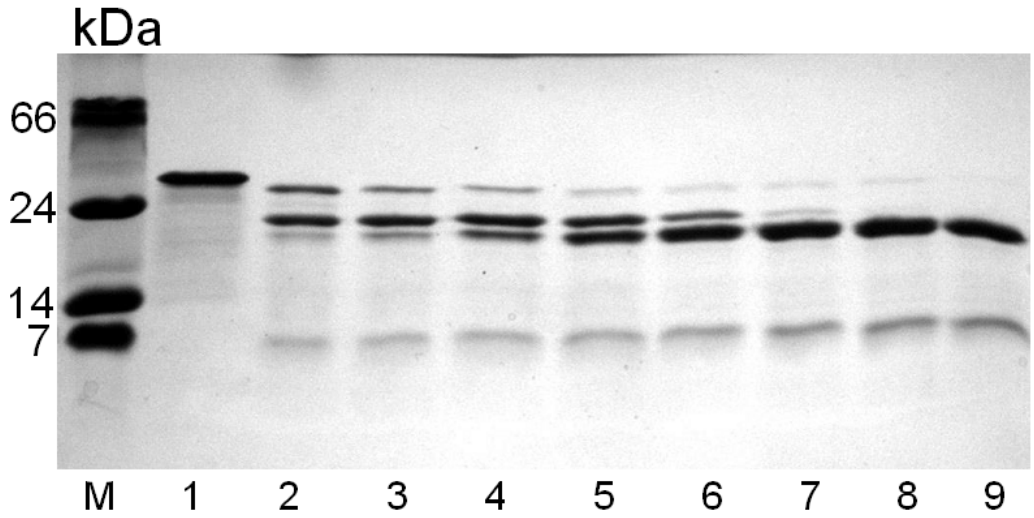

Figure S3. Trypsin digestion of EGFP-T1 in trypsin digestion buffer (10 mM Tris, 20 mM $CaCl_2$) under various pH conditions following 20 nM trypsin cleavage overnight at room temperature. M is protein marker. Lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9 are EGFP-T1 in 10 mM Tris, 20 mM $CaCl_2$, at pH 7.4 as the control, EGFP-T1 tryspin digestion in 10 mM Tris, 20 mM CaCl2, at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, 8.0, and 9.0, respectively.

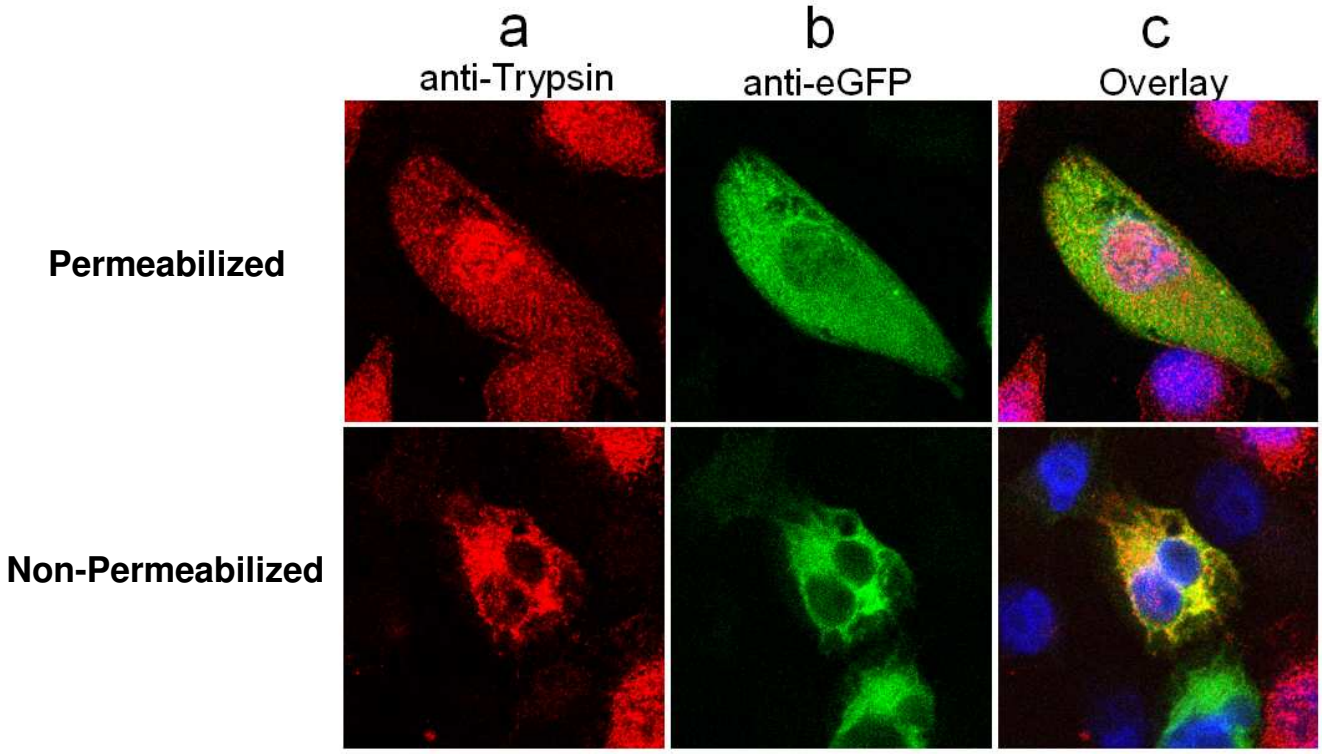

Figure S4. Co-localization of trypsin and EGFP-T1 expression was verified by immunofluorescence. Fluorescence signal of trypsin (**a**) against anti-trypsin antibody and EGFP-T1 (**b**) against anti-GFP antibody exhibited good overlay (**c**) in the cytoplasm of living MIA PaCa-2 cells following 10 nM caerulein stimulation, which suggests EGFP-T1 cleavage due to trypsin in the same subcelluar compartment, the cytosol of cells.

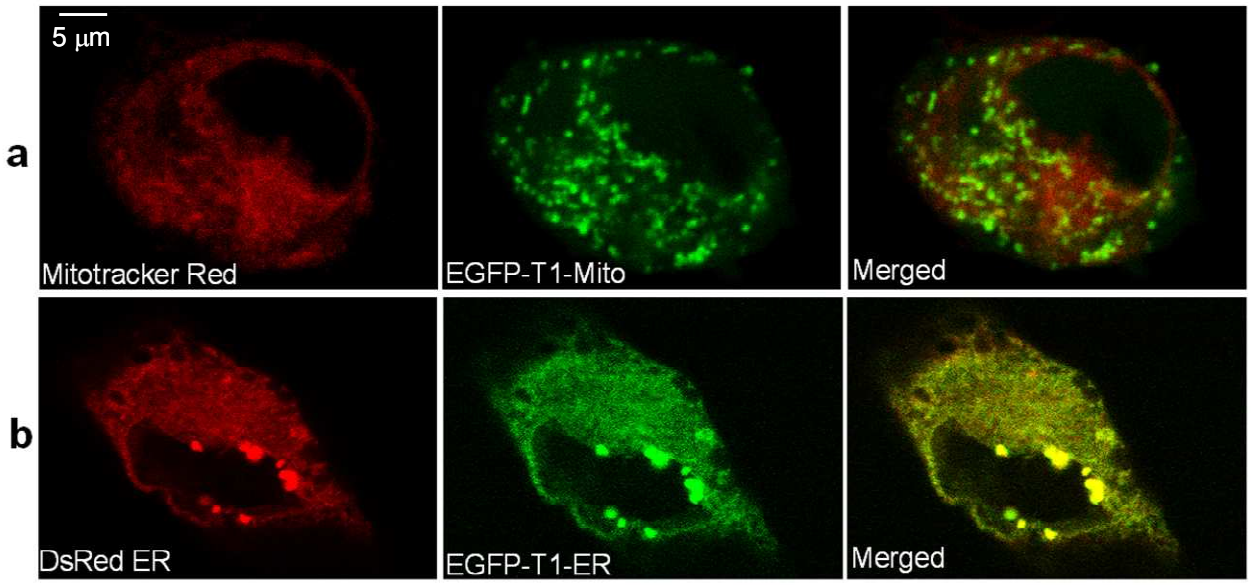

Figure S5. The sub-cellular localization of EGFP-T1-Mito and EGFP-T1-ER was confirmed using mitotracker Red and DsRed-ER. Our trypsin sensors, EGFP-T1-Mito (**a**) and EGFP-T1-ER (**b**) exhibited good overlap with the commercial mitotracker Red and DsRed-ER, respectively.

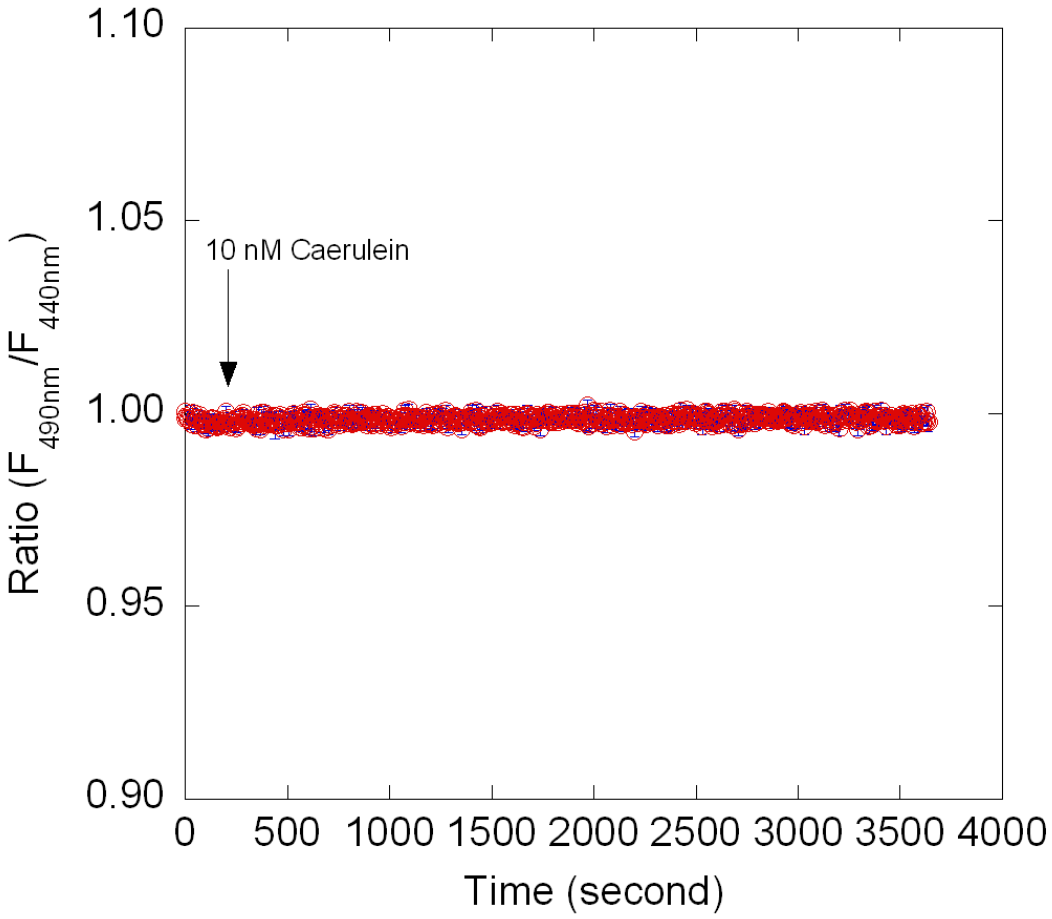

Figure S6. pH change analysis in living cells following Caerulein induction. pH change analysis was conducted using BCECF-AM pH probe in MIA PaCa-2 cells following 10 nM Caerulein induction. No obvious changes in ratiometric fluorescence signal (F_{490nm}/F_{440nm}) of the living cells incubated with BCECF-AM pH probe were observed following Caerulein induction, suggesting that the trypsinogen activation inducer, 10 nM Caerulein, didn't result in significant pH change during trypsinogen activation.

References

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