

Macropinoscope: Real-time Simultaneous Tracking of pH and Cathepsin B Activity in Individual Macropinosomes

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ABSTRACT: A fluorescent sensor that allows simultaneous analysis of environmental factors in a limited cellular space is useful for understanding precise molecular interactions in live cells and their biological responses. Macropinocytosis is a ubiquitous endocytic pathway for massive uptake of extracellular fluids, resulting in formation of macropinosomes. Although macropinocytosis may impact intracellular delivery and cancer proliferation, information on the intracellular behaviors of macropinosomes is limited. Here, we aimed to develop a macropinoscope, a sensor that simultaneously detects pH and cathepsin B activity in individual macropinosomes. A macropinosome-specific marker, dextran (70 kDa), was employed as a platform, onto which fluorescein, Oregon Green, and tetramethylrhodamine were loaded for ratiometric pH sensing and imaging. A cathepsin-B-cleavable peptide sequence bearing sulfo-Cy5 and the quencher BHQ-3 was also mounted; cleavage of the sequence was detected as an increase in sulfo-Cy5 fluorescence. A steep decrease in pH was observed 5–10 min after macropinosome formation, which was accompanied by an immediate increase in cathepsin B activity. Our design concept will lead to the development of other macropinoscopes for the simultaneous detection of other parameters in individual macropinosomes.

Macropinocytosis is a form of endocytosis that allows cells to engulf large volumes of external materials and solutes.^{1–3} While most other forms of endocytosis (e.g., clathrin-mediated and caveolae-mediated endocytosis) are accompanied by pit formation on the plasma membrane to form a vesicular compartment with a diameter of 100–200 nm, macropinocytosis is accompanied by actin rearrangement, protrusion of the plasma membrane, and formation of membrane ruffles, followed by formation of a large vesicle with a diameter of 0.2–10 μm that is called a macropinosome.⁴ Macropinocytosis can be a means of large-scale cellular uptake. In particular, cancer cells take up serum albumin and cellular debris as nutrients for their growth.^{5–7} Macropinocytosis may also be utilized as a route for intracellular delivery of bionanomaterials and biopharmaceuticals.^{8–9} Therefore, a detailed understanding of macropinocytic uptake and the conditions inside macropinosomes is required to understand how to control cancer growth and metastasis and to develop effective intracellular delivery systems. Emerging evidence has shown that the pH inside endosomal compartments influences proliferation, migration, and sensitivity to chemoradiation therapy in a variety of cancers,¹⁰ highlighting the importance of analyzing pH inside macropinosomes.

Nakata et al. reported a pH endoscope that successfully monitored the local pH in the endosomal lumen using ratiometric fluorescence analysis of fluorescein (CF) and tetramethylrhodamine (CR) assembled on a DNA origami scaffold.¹¹ CF is a pH-sensitive dye with decreasing fluorescence intensity upon a decrease in pH ($\text{pK}_a = 6.5$), and CR is a pH-insensitive dye as a reference, allowing ratiometric pH analysis by calculating the ratio of the fluorescent intensities of CF/CR. This suggests the validity of the strategy for designing sophisticated biosensors

that integrate multiple fluorophores with varying optical characteristics through the controlled assembly of appropriate dyes on a single scaffold. In fact, a variety of ratiometric pH sensors have been developed and applied for the analysis of intracellular and endosomal pH.^{12–17} Although useful information has been obtained by ratiometric analysis using CF and CR, this system has a difficulty in precise pH analysis when the pH is < 5.5 because of the marginal change in fluorescence intensity of CF.¹⁸ It has been proposed that endosomal pH is reduced along with endosomal maturation, reaching a pH of about 6.5, 5.5, and 4.5, in early endosomes, late endosomes, and lysosomes, respectively.^{19–20} Therefore, to follow pH changes during macropinosome maturation, a wider pH detection range is needed for the pH endoscope.

Considering the need to detect a wider range of pH fluctuations, the additional fluorophore Oregon Green (OG), which has a lower pK_a of 4.8 when compared to CF ($\text{pK}_a = 6.5$), can be used for ratiometric imaging along with CF and CR.²¹ In fact, polyacrylamide-based nanoparticles bearing CF/OG/CR were reported to successfully allow pH measurement in the endosomal lumen of living cells²² and in the pharyngeal and intestinal lumen of *Caenorhabditis elegans*²³ in the range of 3.5–7.5. Although the applicability of time-resolved pH measurements in endosomes and lysosomes in living cells was demonstrated in the former case, the cellular uptake pathway for the sensor was not specified, and the sensors could not be employed to track pH changes in a single vacuole.²² Recently, Rennick et al. reported an elegant approach for analyzing subcellular pH, including endosomal and lysosomal pH, that uses pH-dependent fluorescence lifetime imaging microscopy, in which the engineered pH-sensitive protein mApple was fused with cellular

proteins at specific intracellular locations.²⁴ However, it may not be easy to evaluate subcellular pH when specific proteins for the target organelles and intracellular spaces are not known, as in the case of macropinosomes.

Lysosomal enzymes, such as cathepsins, play a central role in lysosomal proteolysis and are known to show optimal activity at acidic pH.^{25–26} Lysosomal proteolysis is related to cellular nutrient uptake, which is also closely linked to cancer growth. Lysosomal proteolysis has also been employed in the decomposition of invading viruses and bacteria. Abnormalities in the lysosomal degradation system are associated with both rare and common disorders, including neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Lewy body dementia (LBD).^{27–28} A precise understanding of the stages of action of lysosomal proteases, along with endosomal maturation as a function of endosomal pH, is highly important for understanding the modes of cellular degradation systems and their biological implications. Among the proteases in the cathepsin family, cathepsin B (CTSB) plays an important role in lysosomal proteolysis and is an attractive enzyme for analyzing the relationship between endosome maturation and the level of enzymatic activity.^{29–34}

The aim of this study was to develop a sensor that can precisely analyze pH by referring to lysosomal enzyme activity and that can be used to track macropinosome maturation in live cells. To specify the uptake route for macropinocytosis, 70 kDa dextran (Dex70) was used as the canonical marker.³⁵ Dex70 was modified with the pH-sensitive fluorophores (OG and CF) and the pH-insensitive fluorophore (CR) for ratiometric pH measurement in a wider pH range. A peptide segment cleavable by CTSB was also employed for the assessment of cathepsin B activity in macropinosomes as a function of pH via an increase in sulfo-Cy5 fluorescence intensity following cleavage (Fig. 1). The installation of a prefixed number of multiple sensing units on the same scaffold should allow real-time and simultaneous analysis of different environmental factors within a single vesicular compartment. Although approaches have been reported that simultaneously employ multiple fluorophores or fluorescent proteins to allow monitoring of multiple intracellular reactions or factors,³⁶ the significance and advantage of our approach is the capability of placing multiple sensing probes of fixed ratios in a space of interest, allowing for a more precise and reliable analysis of the intracellular microenvironment.

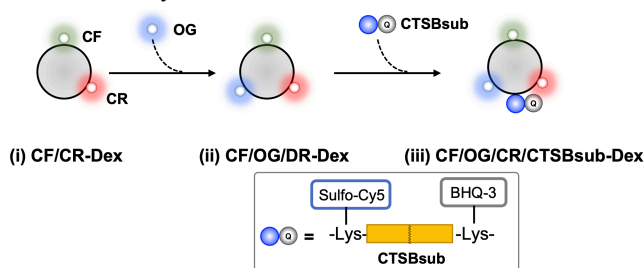


Figure 1. Concept for the design of a real-time macropinosome sensor for multiple environmental factors called a macropinoscope. (i) Modification of 70 kDa dextran (a macropinosome-specific marker) with 6-carboxyfluorescein (CF, pKa 6.5) and 6-carboxytetramethylrhodamine (CR, pH-insensitive) may yield a ratiometric macropinosome pH sensor CF/CR-Dex. (ii) The attachment of 5-carboxy-OregonGreen (OG, pKa 4.8) should allow detection at a lower pH range (CF/OG/DR-Dex sensor). (iii) A modular assembly with a cathepsin B (CTSB)-cleavable peptide sequence bearing sulfo-Cy5 and the quencher BHQ-3 (CTSBsub) should yield a real-

time sensor for multiple environmental factors (i.e., pH and CTSB activity) in individual macropinosomes in live cells.

EXPERIMENTAL SECTION

Labeling of Dextran with fluorophores for preparation of CF/OG/CR-Dex. Solution of 70 kDa aminodextran (Thermo Scientific) (50 nmol) in 110 μ L of 100 mM NaHCO₃ aq. (pH 8.0) and 6-carboxyfluorescein, succinimidyl ester (CF-NHS) (400 nmol), Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer (OG-NHS) (400 nmol), 6-carboxytetramethylrhodamine, succinimidyl ester (CR-NHS) (400 nmol), in 110 μ L of dimethylsulfoxide (DMSO) were mixed and stirred at room temperature for 6 h. Reaction mixture were diluted by 50 mM phosphate buffer (pH 7.0) (PB), were purified on size exclusion PD-10 columns using PB as the mobile phase, and were desalted with PD-10 column using water as the mobile phase. The eluate was lyophilized to yield CF/OG/CR-Dex. The degree of labeling (DOL: moles of dye per mole of dextran) was determined from UV-vis spectra (CF+OG=6.4 and CR=2.3). In these cases, CF and OG could not separate on UV-vis spectrum. The molar extinction coefficient of 75,619 M⁻¹ cm⁻¹ at 496 nm for CF+OG (the average of them), and 13,908 M⁻¹ cm⁻¹ at 496 nm and 59,387 M⁻¹ cm⁻¹ at 557 nm for CR (50 mM phosphate buffer at pH 9.0) were used for calculation of DOL. It should be noted the average of the recovery yield after the two times of size exclusion purification were estimated as 59%, which was determined as the average of three times of independent experiment.

Preparation of cathepsin B probe (CTSBsub). CTSBsub was synthesized via Fmoc-solid-phase peptide synthesis using a Rink amide resin as previously described. For modifying both fluorophore and quencher on CTSBsub, sulfo-Cyanine5 succinimidyl ester (sulfo-Cy5-NHS) and BHQ-3 succinimidyl ester (BHQ3-NHS) were used. The synthesized CTSBsub was purified by reversed-phase HPLC on a C18 column ULTRON VX-ODS (20 $\times$ 150 mm, eluent A: 0.05% TFA containing H₂O, eluent B: 0.05% TFA in 80% acetonitrile, with the gradient of eluent B increased from 40% to 75% in 35 min at a flow rate of 3.0 mL min⁻¹) and characterized by MALDI-TOF mass spectrometry (AXIMA-LNR, Shimadzu, CHCA matrix) as follows: CTSBsub, m/z 2306.0 (calcd. for [M+H]⁺ 2306.2).

Labeling of Dextran with fluorophores for preparation of CF/OG/CR/CTSBsub-Dex. Solution of 70 kDa aminodextran (Thermo Scientific) (50 nmol) in 50 μ L of 100 mM NaHCO₃ aq. (pH 8.0) and *N*-succinimidyl 4-[(5-aza-3,4,7,8-dibenzocyclooct-1-yne)-5-yl]-4-oxobutyrates (DBCO-NHS) (500 nmol) in DMSO 50 μ L was mixed and stirred at room temperature for 4 h. Then, CF-NHS (250 nmol), CR-NHS (250 nmol) and OG-NHS (250 nmol) in DMSO (100 μ L) were mixed and stirred at room temperature for 12 h. Reaction mixture were purified on size exclusion PD-10 columns using PB as the mobile phase, and desalted with PD-10 column using water as the mobile phase. The eluate was lyophilized to yield CF/OG/CR/DBCO-Dex. The DOL was determined from UV-vis spectra (CF+OG=5.7 and CR=1.6) as described above. CF/OG/CR/DBCO-Dex (10 nmol) in PB (130 μ L) and CTSBsub (10 nmol) in DMSO (70 μ L) were mixed and stirred at 37 $^{\circ}$ C for 48 h. Reaction mixture were diluted by DMSO, purified on size exclusion PD-10 columns using PB as the mobile phase, and desalted with PD-10 column using water as the mobile phase. The eluate was lyophilized to yield CF/OG/CR/CTSBsub-Dex. The DOL was determined from

UV-vis spectra (CTSB_{sub}=1.0). The molar extinction coefficient of 254,253 M⁻¹ cm⁻¹ at 656 nm for sulfo-Cy5, and 69,329 M⁻¹ cm⁻¹ at 656 nm for BHQ3 (90% DMSO aq.) were used for calculation of DOL.

Fluorescence spectra measurement and pH titration. Fluorescence spectra were measured by TECAN Infinite M Nano+ in the 96-plate (Greiner Microplate, 655906, 96-well, PS, F-bottom (chimney well) mCLEAR®, black, non-binding). CF+OG, CR, and sulfo-Cy5 were excited at 460 nm, 520 nm, and 620 nm, respectively. The buffer containing 25 mM acetate (pH 3.6 to 5.5), MES (pH 6.0 to 6.5) or HEPES (pH 7.0 to 8.0) with 140 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose were used for pH titration.

Enzymatic assay in test tube. All enzymatic assays were performed in 100 mM sodium acetate, 1 mM EDTA, 0.02% Tween20 (pH 5.5) at 37°C. The CTSB was activated in a buffer containing 100 mM sodium acetate, 1 mM EDTA and 1 mM DTT (pH 5.5) for 15 min under 37°C. CF/OG/CR/CTSB_{sub}-Dex was excited and monitored at 460 nm and 520 nm for CF+OG, 520 nm and 580 nm for CR, and 620 nm and 670 nm for sulfo-Cy5, respectively. In the case of inhibitor assay, the activated CTSB, which was treated with E64d, Z-FA-FMK, or CA-074 (20 μM each) for 5 min at 37 °C, was used.

Confocal laser scanning microscopy. HT1080 cells were seeded onto 35 mm glass-bottom dishes (Iwaki, Shizuoka, Japan) and were allowed to reach 50–60% confluence in 24 h. The cells were washed with PBS twice and then incubated with CF/OG/CR-Dex or CF/OG/CR/CTSB_{sub}-Dex (0.5 mg/mL) in FluoroBrite DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine (Gln). Then the cells were observed using an FV3000 CLSM system (Olympus, Tokyo, Japan) equipped with a 100×/1.30 numerical aperture oil-immersion phase contrast objective lens (UPLFLN, Olympus). For time-lapse imaging, an STX stage top incubator (TOKAI HIT, Shizuoka, Japan), humidified with 5% CO₂ at 37 °C, was attached to the FV3000 CLSM system. Time-lapse images were acquired every 30 or 60 sec. Image acquisition conditions are as follows; CF and OG channel: excitation 488 nm, emission 500–540 nm; CR channel: excitation 561 nm, emission 570–630 nm; sulfo-Cy5 channel, excitation 640 nm, emission 650–750 nm.

in situ pH calibration. HT1080 cells treated with CF/OG/CR-Dex or CF/OG/CR/CTSB_{sub}-Dex for 1 h were washed with PBS three times and then incubated with K⁺-rich calibration buffer at each pH (4.0 - 7.5) containing nigericin (10 μM) for 10 min on the stage top incubator at 37 °C attached to the FV3000 CLSM system.

Image analysis. All image analyses were done using Fiji/ImageJ (2.9.0/1.53v). For quantification of (F_{CF} + F_{OG})/F_{CR} in each macropinosome in each calibration buffer, the region of interest (ROI) of dextran-positive macropinosomes was determined first. Briefly, the image of CR channel was processed by smoothing, and the threshold was set to generate a binary image and ROI masks. Using ROI masks, raw images of the (CF + OG) channel and the CR channel were processed to calculate (F_{CF} + F_{OG})/F_{CR} in each ROI. For tracking macropinosomes in time-lapse imaging, ROI was determined in the raw images by manual tracking using a polygon tool to calculate (F_{CF} + F_{OG})/F_{CR} in each ROI. For generating ratio images and movies, the smoothed images of the (CF + OG) channel and the CR channel were processed using the ROI masks. Similarly, for quantification of F_{sulfo-Cy5}/F_{CR} in each macropinosome in the

cathepsin inhibitor study, ROI was determined using CR channel and F_{sulfo-Cy5}/F_{CR} was calculated using each raw image.

RESULTS AND DISCUSSION

Design and preparation of the CF/OG/CR-based wide-range pH sensor. Dextran 10 kDa (Dex10) has been reported to be taken up via not only macropinocytosis but also other endocytic pathways including clathrin-mediated endocytosis. However, Dex70 is exclusively taken up by macropinocytosis.³⁵ Therefore, Dex70 has been employed as a macropinosome-specific marker to trace the intracellular fates of endosomes formed by macropinocytosis (i.e. macropinosomes).³⁷ In this study, we therefore employed Dex70 as a platform to create a real-time sensor for multiple environmental factors to analyze the luminal conditions of macropinosomes. Prior to developing a sensor capable of analyzing both pH and CTSB activity, we first confirmed the validity of using OG (pK_a = 4.8) as an additional fluorophore to CF and CR to allow a wider range of detection at a pH < 5.5.

Dex70 functionalized with amino moieties was used as the platform. Dex70 has more than 20 amino functions per molecule. OG, CF, and CR were introduced by treatment with the corresponding *N*-hydroxysuccinimide (NHS) esters (5 eq for Dex70) to yield CF/OG/CR-Dex (Fig. S1). For reference, Dex70 bearing only CF and CR (CF/CR-Dex) and Dex70 bearing only OG and CR (OG/CR-Dex) were prepared in a similar manner. The number of dye molecules loaded on one Dex70 platform was controlled by tuning the conditions for dye-loading reactions, and was further estimated from the UV-visible absorption unique to each dye upon modification. Typically, 6.4 and 2.3 molecules of OG+CF and CR, respectively, were incorporated into one Dex70 molecule (see Experimental Procedures in Supporting Information). The UV and fluorescence spectra of CF/OG/CR-Dex, CF/CR-Dex, and OG/CR-Dex are shown in Fig. S2.

pH sensing using the CF/OG/CR-Dex sensor. Ratiometric titration of the CF/OG/CR-Dex sensor at various pH conditions indicated the feasibility of this sensor for the analysis of pH values ranging from 4 to 8. The fluorescence intensities of CF and OG at 520 nm (F_{CF} and F_{OG}, respectively) were recorded as their sum (F_{CF}+F_{OG}) upon excitation at 460 nm, and fluorescence intensities were recorded at 580 nm for CR (F_{CR}) upon excitation at 520 nm, providing the ratio (F_{CF}+F_{OG})/F_{CR}. The pH titration of the CF/OG/CR-Dex sensor yielded a sigmoidal curve of (F_{CF}+F_{OG})/F_{CR} with pK_a values of 4.3 and 6.1 (Fig. 2, CF/OG/CR-Dex).

On the other hand, the CF/CR-Dex and OG/CR-Dex sensors—missing either OG or CF—resulted in pH detection in narrower ranges: the CF/CR-Dex sensor showed a linear CF/CR ratio (F_{CF}/F_{CR}) between pH 5 and 7 (Fig. 2, CF/CR-Dex, pK_a = 5.8), and the OG/CR-Dex sensor showed a linear OG/CR ratio (F_{OG}/F_{CR}) between pH 4 and 5.5 (Fig. 2, OG/CR-Dex, pK_a = 4.3). The pH titration curve of the CF/OG/CR-Dex sensor revealed a summed feature of those of CF/CR and OG/CR, allowing reliable analysis over a wider pH range (pH 4–7) together with a larger ratiometric response, that is, (F_{CF}+F_{OR})/F_{CR}.

The above features of CF/OG/CR-Dex should meet the requirements for the analysis of the pH range expected for macropinosomal lumina. Dex70 is a specific marker of macropinocytosis. In addition, macropinosomes are generally 0.2–10 μm in diameter.^{4,35} These features should allow

CF/OG/CR-Dex to be used to trace the maturation process of a single macropinosome following its formation from the plasma membrane. The validity of tracing pH changes in single macropinosomes by CF/OG/CR-Dex was demonstrated using a human-fibrosarcoma-derived HT1080 cell line. The cell contains an activated N-Ras oncogene, which is constitutively active without the need for external stimuli such as growth factors.³⁸

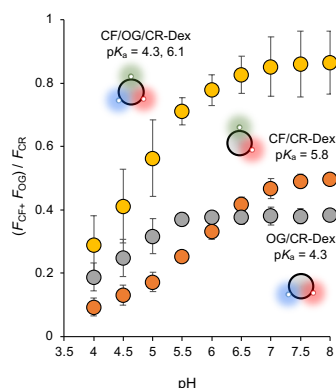


Figure 2. Fluorescence pH titration of fluorophore-modified dextran in test tubes. The ratios of emission intensity of CF/OG/CR-Dex, CF/CR-Dex, or OG/CR-Dex at 520 nm excited at 470 nm ($F_{CF} + F_{OG}$) to that at 585 nm excited at 530 nm (F_{CR}) were plotted against varying pH values ranging from 4 to 8. The emission intensity ratios $[(F_{CF} + F_{OG})/F_{CR}]$ of CF/OG/CR-Dex, CF/CR-Dex, or OG/CR-Dex were calculated by dividing the fluorescence intensity at 520 nm (excitation at 470 nm) by that at 585 nm (excitation at 530 nm). The pK_a value was estimated to be at ambient temperature (approximately 25 °C). Data are represented as the mean $\pm$ standard deviation (SD) ($n = 3$).

The relationship between $(F_{CF} + F_{OG})/F_{CR}$ and macropinosomal pH in cells was then analyzed in situ using the established procedures for endosomal pH adjustment using nigericin³⁷ (Fig. S3). The cells were incubated with CF/OG/CR-Dex for 1 h, and the medium was changed to a K^+ -rich buffer with varying pH (pH 4.5–7.5) (calibration buffer) containing nigericin. Nigericin is an ionophore that exchanges K^+ for H^+ and clamps the pH of the macropinosome to that of the cytosol, reflecting the pH of the calibration buffer (Fig. S3A). Using phase-contrast microscopy, macropinosomes were typically observed as large, round, white structures with a diameter of 1–10 μm , as reported previously³ (Fig. S3A, phase-contrast image). Colocalization of the (CF+OG) and CR signals with the large, round, white structures in the phase-contrast image suggested that CF/OG/CR-Dex detected changes in the pH of macropinosomes (Fig. S3A, white arrows as examples). Note that the signal intensities in the CF+OG channel became less intense at lower pH values, while there were no significant pH-dependent changes in the signals of the CR channel. Thus, ratios of $(F_{CF} + F_{OG})/F_{CR}$ were obtained at pH 4.5–7.5 (Fig. S3B, left). A linear relationship of $(F_{CF} + F_{OG})/F_{CR}$ as a function of pH was obtained in the range of pH 4.5–7.0 (Fig. S3B, right), suggesting the applicability of the CF/OG/CR-Dex sensor to analyze pH in macropinosomes.

Next, we analyzed the pH change in macropinosomes in live cells using the CF/OG/CR-Dex sensor. HT1080 cells were treated with CF/OG/CR-Dex for 5 min and time-lapse images were obtained. Live-cell time-lapse imaging of the cells allowed serial tracing of the pH alteration of macropinosomes bearing the CF/OG/CR-Dex sensor (Fig. 3A and B, see also

Supporting Information Video 1). Note that the pH calibration shown in Fig. S3 was performed for every measurement to obtain the $(F_{CF} + F_{OG})/F_{CR}$ value of each macropinosome.

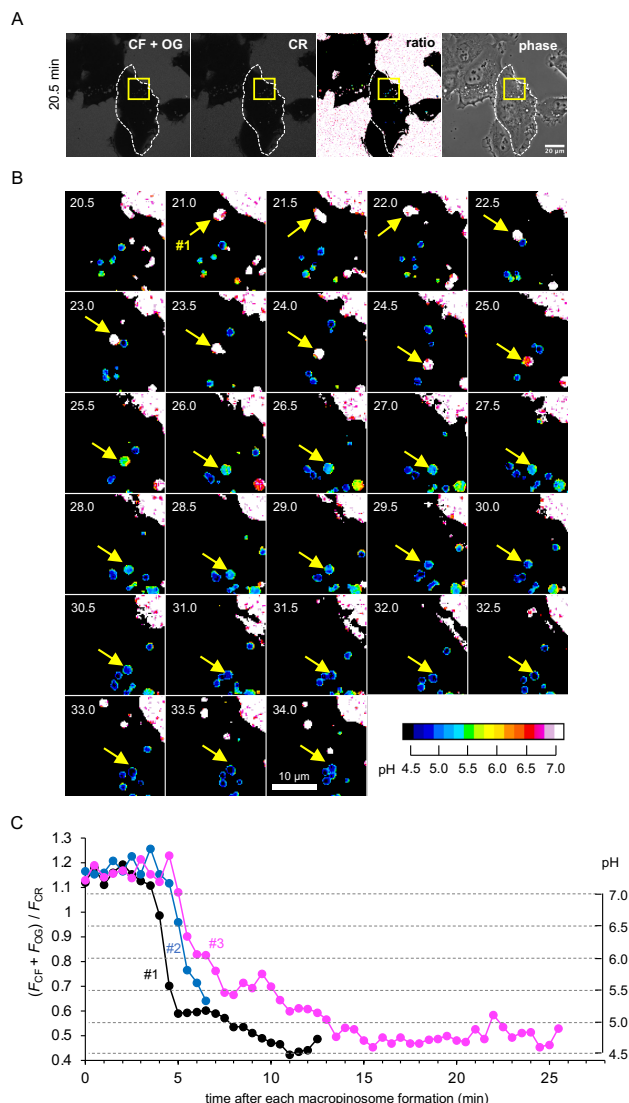


Figure 3. A typical example for the analysis of pH alteration in macropinosomes in live cells using the CF/OG/CR-Dex sensor. (A) Observation of the cells 20.5 min after incubation with CF/OG/CR-Dex. The ratio represents $(F_{CF} + F_{OG})/F_{CR}$. Scale bar, 20 μm . (B) The following change in the $(F_{CF} + F_{OG})/F_{CR}$ ratio in the area marked by a yellow box in (A) is shown. The $(F_{CF} + F_{OG})/F_{CR}$ values are represented in a 16-color scale, as shown by a color scale bar with pH values. The numbers in the corners of the tops of the images are the time (min) after incubation of CF/OG/CR-Dex with cells. The macropinosome analyzed (#1) has a round shape of 2–3 μm in diameter and is shown with a yellow arrow. Scale bar, 10 μm . See Fig. S2 to view other macropinosomes (#2 and #3). (C) Time-dependent decrease in $(F_{CF} + F_{OG})/F_{CR}$ for each macropinosome. Time zero represents the time of formation of each macropinosome, which is when it pinched off from the plasma membrane. The corresponding pH is given on the right. The decrease in pH in macropinosomes #1–3 in Figs. 3B and S4 is shown.

As described above, macropinosomes are significantly larger in size than vesicles produced via other endocytic pathways,^{4,40} and Dex70 is a specific marker of macropinosomes.³⁵ Thus, CF/OG/CR-Dex allows us to trace the modes of intracellular

trafficking of single macropinosomes in real time beginning immediately after their formation from the plasma membrane. Fig. 3C shows the results of pH alterations of three representative macropinosomes (#1 in Fig. 3B, and #2 and #3 in Fig. S4; see also Supporting Information Video 1). We observed that the pH in these macropinosomes remained approximately neutral in 4–6 minutes after macropinosome formation, followed by a steep decrease to a pH of about 5.5 in 2–3 minutes, and then gradually reached a pH of about 4.5–5 (Fig. 3C). No significant decrease in the pH to a value below 4.5 was observed. Thus, the feature of pH alteration in macropinosomes was illustrated at a single-vesicle level using the CF/OG/CR-Dex sensor.

In a previous report on CF/CR-based endoscope, Nakata et al.¹¹ employed a DNA nanostructure (DNA origami) as the scaffold, where, a cationic intracellular delivery peptide SN21-LK15⁴¹ was employed to facilitate encapsulation of the CF/CR-based endoscope to endosome to promote the endocytic uptake of the negatively charged scaffold bearing the sensor units, and a fine adjusting of calibration curves to eliminate the effect of SN21-LK15 was needed. However, the CF/OG/CR-Dex sensor was constructed on a canonical macropinosome marker (70 kDa dextran) and the sensor was readily taken up into macropinosomes per se without need of additives to promote the cellular uptake, which improved fidelity of the sensing system.

Design and preparation of a sensor to simultaneously detect macropinosomal pH and cathepsin B activity. A variety of proteases may exert their activity at different stages of endosomal maturation; sensors should be useful for analyzing enzymatic activity as a function of pH or the stage of endosomal maturation. CTSB is a lysosomal cysteine protease that is involved in proteolysis and cancer progression.⁴² The enzyme is known to have an optimum pH of approximately 5,⁴³ and its activity may increase along with a pH decrease during macropinosome maturation. Therefore, we developed a sensor that simultaneously detects macropinosomal pH and CTSB activity to investigate the feasibility of the fluorescent sensor for multiple environmental factors.

Using CF/OG/CR-Dex as a framework, a CTSB activity sensing unit (CTSBsub; **3** in Fig. S5A) was conjugated to produce CF/OG/CR/CTSBsub-Dex (**4** in Fig. S5A; see also Fig. S5B for the preparation of **3** and Fig. S6 for the structures and characteristics of the fluorophores employed). Referring to previous reports by ourselves and others,^{34,44} the sensing unit was designed to have a substrate sequence specifically cleaved by CTSB (Cha-Leu-Glu(OBzl)-Arg-Hex: Cha = 3-cyclohexyl-L-alanine; Hex = 4-aminohexanoic acid). Sulfo-Cy5 (Ex: 646 nm, Em: 662 nm) and the dark quencher BHQ-3 were placed on the N-terminal and C-terminal sides of the CTSB substrate sequence, respectively, via the side chains of Lys residues. 5-Azidovaleric acid was conjugated to Dex70 via a click reaction so that cleavage of the CTSB substrate sequence led to the liberation of the BHQ-3 quencher from the sensor, which in turn increased the fluorescence emission intensity of sulfo-Cy5 (i.e., off-to-on mode). Amino-functionalized Dex70 (**1**) was treated with NHS esters of CF, OG, and CR (5 eq each) together with DBCO-NHS (10 eq) to yield **2** (Fig. S5A(a)). The numbers of dyes incorporated in one Dex70 were determined to be 5.7 for CF+OG and 1.6 for CR (see Experimental Section). Conjugation of **3** with **2** was then conducted via a copper-free click reaction to yield CF/OG/CR/CTSBsub-Dex (**4**) (Fig. S5A(b)). The UV and fluorescence spectra of CF/OG/CR/CTSBsub-Dex

are given in Fig. S7, and the number of CTSBsub on one Dex70 was estimated to be 1.0.

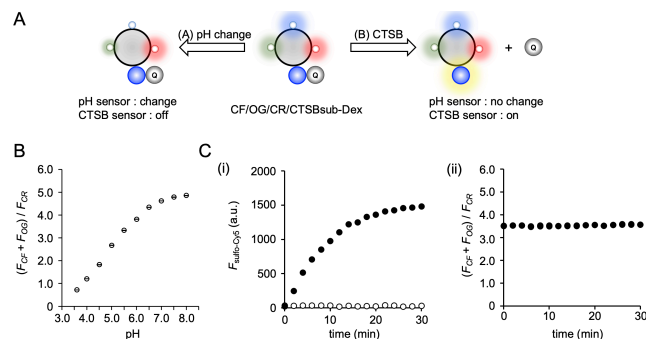


Figure 4. Analysis of pH and cathepsin B activity using the CF/OG/CR/CTSBsub-Dex sensor in test tubes. (A) Schematic illustration of the analysis of pH or cathepsin B activity using the CF/OG/CR/CTSBsub-Dex sensor. (B) A plot for the emission intensity ratio $[(F_{CF} + F_{OG})/F_{CR}]$ at various pH values ranging from 3.5 to 8.0. The pK_a values were estimated to be 6.0 ± 0.1 and 4.3 ± 0.1 at ambient temperature (approximately 25 °C). Data are represented as the mean $\pm$ standard deviation (SD) ($n = 3$); error bars denote SD. (C) Time-course observation of (i) $F_{sulfo-Cy5}$ and (ii) $(F_{CF} + F_{OG})/F_{CR}$ in the presence (filled circle) or absence (open circle) of cathepsin B (200 nM). Almost complete overlap of filled and open circles occurred in (ii).

The addition of CTSBsub had no significant effect on pH sensing with CF/OG/CR/CTSBsub-Dex (Fig. 4A, B). As shown in Fig. 4B, a titration profile of fluorescence ratios at 520 nm ($F_{CF} + F_{OG}$) over that at 580 nm (F_{CR}) with pK_a values of 4.3 and 6.3 was obtained that was very similar to the titration profile of CF/OG/CR-Dex ($pK_a = 4.3, 6.1$) in Fig. 2. This result thus demonstrates the validity of pH analysis using CF/OG/CR/CTSBsub-Dex in the pH range of 3.5–8.0.

Cleavage of the CTSBsub sequence by CTSB led to the liberation of sulfo-Cy5 from the BHQ-3 quencher, recovering sulfo-Cy5 fluorescence intensity. The feasibility of using CF/OG/CR/CTSBsub-Dex to monitor proteolysis by CTSB was confirmed by an increase in fluorescence at 670 nm ($F_{sulfo-Cy5}$, excitation at 620 nm) upon incubation with CTSB (Fig. 4C(i)). Since the CTSBsub sequence is highly susceptible to CTSB,^{34,44} a relatively low concentration of enzyme was employed as the substrate in this study, with the expectation of convenient monitoring of relatively slow cleavage kinetics. A significant time-dependent increase in F_{670} , suggesting cleavage of the CTSBsub sequence, was observed upon addition of CTSB (Fig. 4C(i), closed circle). In contrast, in the absence of CTSB, no significant increase in $F_{sulfo-Cy5}$ was observed even after incubation for 30 min (Fig. 4C(i), open circle). Cleavage of the CTSBsub sequence led to no marked alteration in the fluorescence ratio at 520 nm ($F_{CF} + F_{OG}$, excitation at 460 nm) when compared to that at 580 nm (F_{CR} , excitation at 520 nm), corresponding to $(F_{CF} + F_{OG})/F_{CR}$. These results clearly indicate orthogonality in the simultaneous analysis of pH and CTSB cleavage activity (Fig. 4C(ii)). The increase in $F_{sulfo-Cy5}$ levels increased with increasing CTSB concentration (Fig. S8(i)) without a significant effect on $(F_{CF} + F_{OG})/F_{CR}$ (Fig. S8(ii)). No significant increase in $F_{sulfo-Cy5}$ in the presence of representative CTSB inhibitors [E-64d (Aloxistatin),⁴⁵ Z-FA-FMK,⁴⁶ and CA-074⁴⁷] further confirmed that the increase in fluorescence intensity was due to the cleavage of CTSBsub by CTSB (Fig. S9). These results support the validity of using the sensor

CF/OG/CR/CTSBsub-Dex to simultaneously monitor pH and CTSB activity.

We next confirmed the ability of this sensor to detect CTSB activity in live cells (Fig. S10). Significant sulfo-Cy5 signal was observed in HT1080 cells after treatment with CF/OG/CR/CTSBsub-Dex for 30 min (Fig. S10A, control; yellow arrowheads). Colocalization of sulfo-Cy5 and CR signals, together with the large, round, white structures in the phase-contrast image, indicated the detection of CTSB activity in macropinosomes by CF/OG/CR/CTSBsub-Dex. A significant reduction in sulfo-Cy5 signal in the cells following treatment with the CTSB inhibitors E-64d or Z-FA-FMK also suggested the capability of CF/OG/CR/CTSBsub-Dex to detect CTSB activity in macropinosomes in live cells (Fig. S10A, E-64d and Z-FA-FMK). E-64d and Z-FA-FMK are membrane-permeable and are thus able to reach macropinosomes, but CA-074 is membrane-impermeable; therefore, the difference in membrane permeability of the inhibitors should yield inconsistent inhibition of CTSB activity in macropinosomes. Indeed, no significant inhibition of the cleavage of the CTSBsub sequence was observed after treatment with CA-074 in cells (Fig. S10A, CA-074), whereas CA-074 had significant inhibitory activity in the test tube assay (Fig. S9(iii)). The ratio analysis of sulfo-Cy5 and CR fluorescence in macropinosomes (Fig. S10B) was also in agreement with the results shown in Fig. S10A. In summary, CF/OG/CR/CTSBsub-Dex can be used to detect CTSB activity inside macropinosomes in live cells.

Simultaneous sensing of pH and cathepsin B activity in macropinosomes using the CF/OG/CR/CTSBsub-Dex sensor in live cells. The CF/OG/CR/CTSBsub-Dex sensor was applied for simultaneous sensing of pH and cathepsin B activity in live cells. HT1080 cells were treated with CF/OG/CR/CTSBsub-Dex, and time-lapse ratiometric analysis of $(F_{CF} + F_{OG})/F_{CR}$ was conducted in parallel with sulfo-Cy5 signal intensity analysis.

As shown in Fig. 5, real-time tracking of pH for a single macropinosome using CF/OG/CR/CTSBsub-Dex showed similar pH alteration tendencies to those shown in Fig. 3. The ratio in Fig. 5A represents the ratiometric images of $(F_{CF} + F_{OG})/F_{CR}$ for cells incubated with CF/OG/CR/CTSBsub-Dex. The provided color scale represents the range of pH values. The $(F_{CF} + F_{OG})/F_{CR}$ values reported in Fig. 5B suggest that the pH remained almost neutral for the first 5 to 10 min after the formation of each macropinosome, followed by a steep decrease to reach a pH of about 5.5–6.0 (see also Fig. S11A, where the graph is arranged so that time zero represents the time of the formation of macropinosomes; see also Fig. S12 for calibration curve of CF/OG/CR/CTSBsub-Dex between pH 4.5–7.0). CTSB activity, monitored by the fluorescence of sulfo-Cy5 (Fig. 5A and dotted lines in Fig. 5B), gradually increased, followed by a decrease in macropinosomal pH to approximately 6.0. The above results suggest that the pH of macropinosomes is similar to that of the extracellular fluid before their delivery to the perinuclear area. A steep decrease in pH was observed when macropinosomes reached the perinuclear area, which was notably correlated with an increase in macropinosomal CTSB activity.

CF/OG/CR/CTSBsub-Dex also allows the observation of the formation and intracellular behaviors of single macropinosomes in real time in live cells; traces of four representative macropinosomes (#1–4) are shown in Fig. S11B. As typically observed for macropinosomes #1 and #2, relatively straight movements

to the perinuclear area were observed in the first 10 min or so after their formation, suggesting movement of the macropinosomes along microfilaments. The timing of the steep pH decrease may correspond to the time when macropinosomes reached the perinuclear area, possibly leading to fusion with acidic vesicles (i.e., lysosomes), which also corresponded to the timing of the increase in CTSB activity.

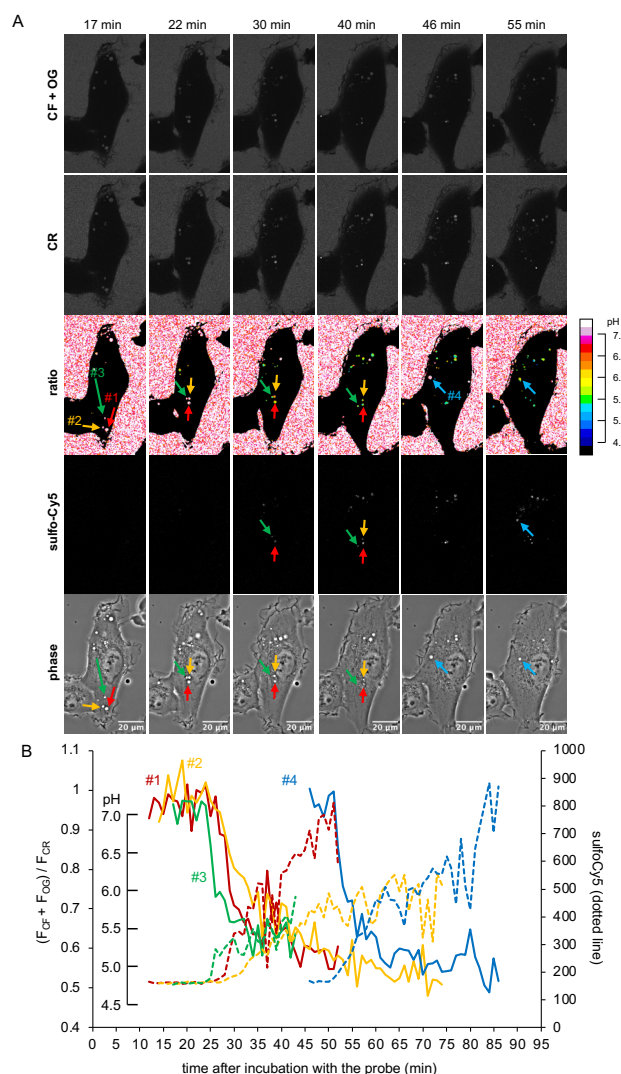


Figure 5. Time-lapse analysis of simultaneous sensing of pH and cathepsin B activity using CF/OG/CR/CTSBsub-Dex in live cells. (A) The representative time-lapse images of HT1080 cells incubated with CF/OG/CR/CTSBsub-Dex. The ratio means $(F_{CF} + F_{OG})/F_{CR}$. The 16-color scale represents pH values. A red arrow, a yellow arrow, a green arrow, and a blue arrow indicate macropinosomes #1, #2, #3, and #4, respectively. Scale bar, 20 μ m. See also Fig. S11 and Supporting Information Video 2. (B) Quantification of $(F_{CF} + F_{OG})/F_{CR}$ and fluorescence intensities of sulfo-Cy5 for each macropinosome in (A). Time zero represents the time when CF/OG/CR/CTSBsub-Dex was added to the cell culture. The corresponding pH is given on the left. Solid lines and dotted lines indicate $(F_{CF} + F_{OG})/F_{CR}$ and fluorescence intensities of sulfo-Cy5, respectively. Red, yellow, green, and blue indicate macropinosome #1, #2, #3, and #4, respectively. See also Fig. S11A where the graph is arranged such that time zero represents the time of the formation of each macropinosome.

Our study demonstrated that acidified macropinosomes had CTSB activity in HT1080 cells, indicating that lysosomes were fused with macropinosomes. CTSB is translated as a proCTSB protein and translocated in the lumen of ER.^{48,49} The proCTSB is then transported to lysosome through the trans-Golgi network (TGN) and late endosomes. Along with the pH decrease in those organelles, the proCTSB is converted to an active CTSB in lysosomes.⁴⁸ On the other hand, very few analyses of macropinosomes have confirmed if maturation of macropinosomes is accompanied by a decrease in pH and activation of CTSB. Using the sensor developed in this study, we shed light on the intracellular fate of macropinosomes for the first time, and exemplified that macropinosomal encapsulates should be finally transferred to acidic compartments bearing CTSB and presumably other digestive enzymes (i.e., lysosomes).

CONCLUSIONS

In this study, we developed a real-time fluorescent sensor that targets multiple environmental factors and allows simultaneous analyses of pH and CTSB activity in macropinosomes (i.e., a macropinoscope). A macropinosome-specific marker, Dex70, was used as a platform. Three fluorescent moieties (CF, OG, and CR) were loaded onto the platform as ratiometric sensing units of the luminal pH of macropinosomes. A CTSB-cleavable peptide sequence bearing sulfo-Cy5 and the quencher BHQ-3 (CTSB_{sub}) was also mounted on the platform; the cleavage of CTSB_{sub} by CTSB was detected as an increase in sulfo-Cy5 fluorescence. Using this sensor, modes of macropinosome maturation were traced in terms of pH and CTSB activity together with information on the cellular localization of macropinosomes.

We have shown in this study that two sensing units (i.e., pH and CTSB activity) loaded on a same platform worked without interfering each other. The conceptual significance of this sensor design approach is the feasibility of placing prefixed numbers or ratios of different types of sensor probes (e.g., pH, endo/lysosomal proteins/enzymes, and lipids) on a single platform that provide multiple types of information simultaneously in a specific cellular space in real time. Coincubation of materials of interest with such macropinosome sensors may also yield the information of the cellular localization and luminal conditions of macropinosomes encapsulating the materials. The same concept may be applicable to developing sensors not only within macropinosomes conditions, but for other local cellular conditions using appropriate platforms for the sensor units.

To our knowledge, this study is the first example that simultaneously detected multiple parameters in a single macropinosome in real time and thus monitored the intracellular behaviors of macropinosomes. We also expect the sensor to facilitate more detailed studies on the modes of macropinosomes in the future using different conditions and cell types, which should help elucidate the contribution of macropinocytosis to cancer proliferation and metastasis, as well as aid in the development of effective intracellular delivery strategies for functional molecules and biotherapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting experimental procedures (Materials, Cell culture, and statistical analysis); Figure S1-S12; description of Supporting

Information Video 1 and 2 (PDF); Supporting Information Video 1 and 2 (mp4).

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Author Contributions

H.H. and E.N. designed the experiments. E.N. is responsible for the preparation of the sensors and their characterization in test tubes. H.H. is responsible for the characterization and evaluation of sensors using live cells. Z.Z. and Y.S. were involved in sensor preparation and characterization. M.M. contributed to the evaluation of the data using live cells. M.T. and S.F. directed the project, analyzed the data and wrote the paper with H.H. and E.N. [#]These authors contributed equally.

Notes

The authors declare no competing financial interest.

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