Supporting information

A facile combinatorial approach to construct a ratiometric fluorescent sensor: application for the real-time sensing of cellular pH changes

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Figure S1 Design of a DNA origami scaffold (rec-Ori). a) An illustration of the DNA scaffold (rec-Ori) indicates the ssDNA scaffold (M13mp18 ssDNA) in black line and staple strands in gray lines. The staple strands for both shorter sides (24 staple strands in total) in the original design ^{S1} were removed to prevent the intermolecular interaction between folded rec-Ori. b) An illustration of rec-CF15 indicates the calculated distance between each loading position of CF as a typical example. The staple strands appended with comp-tag1 at the 5'-position of it were shown in blue lines. Because CF shows a self-quenching property due to förster resonance energy transfer (FRET) with 44 Å of förster radius, ^{S2} the position to load each CF was at least 11 nm away from the neighboring CF loading position. c) Typical AFM image of rec-CF15.



Figure S2 Absorption and fluorescence emission spectra of CF-ODN1 and CR-ODN2. (a and c) Absorption spectra of (a) CF-ODN1 (5 μ M) and (c) CR-ODN2 (5 μ M). (b and d) Fluorescence emission spectra of (b) CF-ODN1 (1 μ M) with excitation at 470 nm and (d) CR-ODN2 (1 μ M) with excitation at 530 nm. These spectra were measured in a buffer (pH 7.0) containing 40 mM MES at ambient temperature.



Figure S3 Characterization of CF assembled rec-Ori (rec-0 and rec-CF15) by AFM imaging and gel electrophoreses. a) Design of rec-0 (left) and rec-CF15 (right). b) AFM images of rec-0 (left) and rec-CF15 (right). c) Images for gel electrophoretic analyses of rec-0 and rec-CF15 visualized by the emission of CF (left) and by ethidium bromide (EtBr) staining (right). Lane M: 1 kbp DNA ladder as marker; Lane 1: M13mp18 with staple strands without annealing; Lane 2: rec-0 after purification; Lane 3: rec-CF15 before purification; Lane 4: rec-CF15 after purification. The results indicated that excess amounts of staple strands including CF-ODN1 were removed after the purification procedure.



Figure S4 A linear relationship for the emission intensity and the number of CR on a single DNA origami scaffold (rec-Ori). (a) Illustrations for a series of rec-Ori loaded with various numbers of CR molecules (0: rec-0; 1: rec-CR1; 3: rec-CR3; 5: rec-CR5; 10: rec-CR10; 15: rec-CR15). (b) Fluorescent spectra of a series of CR loaded rec-Ori (5 nM) in a buffer (pH 7.6) containing 40 mM Tris–HCl, 20 mM acetic acid, 12.5 mM MgCl₂ at ambient temperature with excitation at 530 nm. (c) A plot of fluorescence intensities for a series of CR loaded rec-Ori at 582 nm against the number of CR assembled on a single rec-Ori.



Figure S5 Characterization of rec-CF5/CR5 by gel electrophoreses and AFM imaging. a) Design of rec-CF5/CR5. b-c) Image for the gel electrophoretic analysis of rec-CF5/CR5. (b) Visualized by the emission of CF (green) and CR (red) (c) Visualized by ethidium bromide (EtBr) staining. Lane M: 1 kbp DNA ladder as marker; Lane 1: M13mp18; Lane 2: Rec-CF5/CR5 before purification; Lane 3: Rec-CF5/CR5 after purification. d) AFM image of rec-CF5/CR5.



Figure S6 Fluorescence spectral changes of CF derivatives depending on pH. a) CF-AE (2-aminoethanol) (8 nM). b) CF modified with a single strand DNA (CF-ODN1) (10 nM). c) CF-ODN1 hybridized with comp-ODN1(CF-dODN1) (10 nM). The detail of the buffer used for each pH condition were shown in Experimental procedures.



Figure S7 Stability of rec-CF5/CR5 at various pH (pH 3-10) monitored by AFM imaging and gel electrophoretic analyses. a-b) Image for the gel electrophoretic analysis of rec-CF5/CR5. a) Visualized by the emission of CF (green) and CR (red). b) Visualized by ethidium bromide (EtBr) staining. Lane M: 1 kbp DNA ladder as marker (asterisk was dye (bromophenol blue) containing DNA ladder); Lane C: control (m13mp18); Lane 3-10: rec-CF5/CR5 after treatment at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or 10.0, respectively. c) AFM image of rec-CF5/CR5 after treatment at pH 3.0, 6.0 or 9.0. The detail of the buffer used for each pH condition were shown in Experimental procedures.



Figure S8 Fluorescent pH titration of CF and CR coassembled on rec-Ori (rec-CF5/CR5) (1.6 nM) with SN21-LK15. (a) Illustration for rec-CF5/CR5 with SN21-LK15. (b and c) Fluorescence emission spectral changes of (b) CF (excited at 470 nm) and (c) CR (excited at 530 nm) at various pH ranging from 3 to 10. (d) A plot for the ratio of emission intensity at 518 nm excited at 470 nm, corresponding to FC, to that at 582 nm excited at 530 nm, corresponding to CR, for rec-CF5/CR5 at various pH ranging from 3 to 10. The emission intensity ratios (518/582) was calculated by dividing the fluorescence intensity at 518 nm with excitation at 470 nm by fluorescence intensity at 582 nm with excitation at 530 nm. The p K_a value was estimated to be 5.9 ± 0.1 at ambient temperature. The detail of the buffer used for each pH condition were shown in Experimental procedures.



Figure S9 Characterization of rec-CR5 by gel electrophoreses and AFM imaging. a) Design of rec-CR5. b-c) Image for the gel electrophoretic analysis of rec-CR5 before and after purification. (b) Visualized by the emission of CR (red). (c) Visualized by EtBr staining. Lane M: 1 kbp DNA ladder as marker; Lane 1: M13mp18; Lane 2: Rec-CR5 before purification; Lane 3: Rec-CR5 after purification. c) AFM image of rec-CR5.



Figure S10 Fluorescence spectral changes of CR derivatives depending on pH. a) CR-AE (2-aminoethanol) (8 nM). b) CR modified with a single strand DNA (CR-ODN2) (8 nM). c) CR-ODN2 hybridized with comp-ODN2 (10 nM)(CR-dODN2). d) rec-CR5 (8 nM). The detail of the buffer used for each pH condition were shown in experimental procedures.

The emission of CR of rec-CR5, same as rec-CF5/CR5, was not changed in the pH range of 5 to10 though that was slightly decreased in the pH range of 3 to 5(Figure S10(d) and Figure 3(c)). As shown in Figure S10 (a) and (b), CR-AE and CR-ODN2 did not showed such the decrement in the pH range of 3 to 5, whereas CR-dODN2 showed slight decrement in the pH range of 3 to 5. These results indicated the decrement of emission of CR of CR-dODN2 and rec-CR5 in the pH range of 3 to 5 might be the cause of the destabilization of duplex formation.



Scale bar: 20 µm

Figure S11 Comparison of the difference of internalization efficiency of CR assembled rec-Ori (rec-CR5) in the presence and absence of macropinocytosis inducible peptide (SN21-LK15). (a) Illustration for the difference of rec-CR5 and rec-CR5/SN21-LK15. (b) Confocal microscopic images of Hela cells treated with rec-CR5 alone, rec-CR5 with LF2000 (lipofectamine 2000), rec-CR5 with SN21-LK15 under different charge ratio (1, 3, and 5).

These results strongly indicated fluorophore assembled DNA origami scaffold such as rec-5CR requires macropinocytosis-inducible peptide for enhancing the cellular uptake. The charge ratio of 1, 3, and 5 showed largely assembled fluorescent punctate, the strong fluorescence emission and well distributed punctate, and certain toxicity to the cell, respectively. Thus, we chose the charge ratio 3 for further investigation. The LF2000 treated cells also revealed endocytic uptake of rec-CR5. The uptake efficiency was not significantly different that observed for the SN21-LK15 complex at the same charge ratio (charge ratio 3).



Figure S12 Identification of the internalized rec-CR5 treated with SN21-LK15 (rec-CR5/SN21-LK15) costained with LysoDR. Confocal microscopic images of Hela cells treated with rec-CR5 (magenta) with SN21-LK15 and LysoDR (green). Expanded and time lapse images (from 40 min to 100 min) bounded by a square in these images were shown in Figure 4d.



Figure S13 (a) Titration of the fluorescence intensity ratio (518/582) against the solution pH. The titration plot were estimated by using confocal microscopy (see experimental procedures). (b) The determined pH value of the macropinosome against each time spot.

 pK_a values of rec-CF5/CR5 treated with SN21-LK15 under confocal microscopic observation were also determined by following the equation (1).^{S3}

$$R = R_{min} + \frac{(R_{max} - R_{min})}{1 + 10^{pK_a - pH}}$$
(1)

Where *R* is the ratio of fluorescence emission intensity at 518 nm over 582 nm (excited at 488 nm), R_{min} is the minimum ratio value, R_{max} is the maximum ratio value. The curves were fitted by OriginPro (2018-Academic) by using equation (1). The determined p K_a value (6.2±0.1) was consistent with the value determined in Figure S5 (5.9±0.1). To determine the pH in the macropinosome of each time spot, the ratio value was substituted to the equation (1) with the p K_a (6.2), R_{max} (0.549), and R_{min} (0.082) value to estimate pH value of each time spot. The determined pH value of each time spot were shown in Figure S13b.

Note S1 The comparison of CF property on rec-Ori with different assembling method

Three different methods 1 to 3 were adapted to locate fluorophore on rec-Ori and the fluorescence emission profile of each resulting CF-modified rec-Ori were compared. Methods (1) and (2) utilize hybridization of CF-ODN1 (CF was modified at the 5'-end of Ori-tag1) to its complementary sequence on rec-Ori.

Method 1: The complementally sequence for Ori-tag1 of CF-ODN1 (comp-ODN1) was appended to the 5'-end of five types of staple strands. Five molecules of CF-ODN1 were hybridized on rec-Ori with the fluorophore just facing to the rec-Ori surface to afford rec-CF5(5'-hy) (Figure S14a).

Method 2: The comp-ODN1 sequence was appended to the 3'-end of five types of staple strands to hybridize five molecules of CF-ODN1 on rec-Ori with the fluorophore locating 20-bp away from the rec-Ori surface to afford rec-CF5(3'-hy) (Figure S14b).

Method 3: CF was directly modified at each 5'-end of the five types of staple strands, thus five CF molecules were located on the surface of rec-Ori upon folding DNA origami rec-CF5(insert) (Figure S14c).

In all the three cases, five molecules of CR-ODN2 were co-assembled in a similar manner described for method 1 to provide an internal standard for the fluorescence emission of CF.

Fluorescent titration of CF and CR coassembled on rec-Ori were performed at various pH (Figures S15). Fluorescence emissions of CF on three types of rec-CF5 at pH 9.0, where CF dominantly exists in the deprotonated form, ^{S2} were compared to investigate the location dependency on the emission property of CF. Ratio of the fluorescence intensity of CF at 518 nm and CR at 582 nm (Ratio 518/582) was taken to normalize the emission intensity of CF at 518 nm as shown in Figure S15. Estimated p K_a obtained by using the ratio (Ratio 518/582) for rec-CF5(5'-hy)/CR5, rec-CF5(3'-hy)/CR5 and rec-CF5(insert)/CR5 were 7.1 ± 0.1, 6.7 ± 0.1 and 6.7 ± 0.1, respectively. Because rec-CF5(5'-hy)/CR5 provided the same p K_a value with CF-dsDNA (7.1 ± 0.1) as shown in Figure S6, method 1 was applied to assemble CF on the DNA scaffold.

a) rec-CF5(5'-hybri)/CR5



Figure S14. Three methods to assemble CF on the DNA scaffold rec-Ori. a) The comp-ODN1 sequence was appended to the 5'-end of five types of staple strands. Five molecules of CF-ODN1 were hybridized to rec-Ori with CF facing to the surface of rec-Ori. b) The comp-ODN1 sequence was appended to the 3'-end of five types of staple strands. Five molecules of CF-ODN1 were hybridized to rec-Ori with CF locating 20-bp away from the rec-Ori surface. c) CF was directly modified at the 5'-end of each staple strand. Five types of CF modified staple strands were located on the surface of rec-Ori upon folding DNA origami.

In all the cases, five molecules of CR-ODN2 were assembled in the similar manner as described for (a) to utilize as an internal standard for the fluorescence emission of CF.



Figure S15. Comparison of the fluorescence emission property of CF assembled on rec-Ori with different methods. (a-c) (left) Illustration for the location of CF in each assembly (2 nM) as described in Figure S14. (middle) Fluorescent pH titration of CF (excited at 470 nm) and CR (excited at 530 nm) coassembled on rec-Ori at various pH ranging from 3 to 10. (right) A plot for the emission intensity ratios (Ratio 518/582) at various pH ranging from 3 to 10. Ratio 518/582 was calculated by dividing the fluorescence intensity at 518 nm with excitation at 470 nm by fluorescence intensity at 582 nm with excitation at 530 nm. The pK_a value of rec-CF5(5'-hy)/CR5, rec-CF5(3'-hy)/CR5 and rec-CF5(insert)/CR5 were estimated to be 7.1 \pm 0.1, 6.9 \pm 0.1 at ambient temperature, respectively. The detail of the buffer used for each pH condition were shown in Experimental procedures.

Supplementary References

- S1. P. W. K. Rothemund, Nature, 2006, 440, 297–302.
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