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Supplementary Information

Quantitative image cytometry for analysing intracellular trafficking of G protein-coupled receptors on a chemically-trapping single cell array

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Experimental

1. Plasmid construction and retroviral gene transduction

We constructed a plasmid for expression of the Sortase A (SrtA)-recognition sequence (LPETGGGGG, $LPETG_5$) and HA tagged human G2A in mammalian cells. In detail, the $LPETG_5$ and HA was designed to be fused at the *N*-terminus of G2A, resulting in expression of G2A tagged at its extracellular *N*-terminus ($LPETG_5$-G2A). To increase sufficiently the expression level for fluorescent microscopic observations, the codons of the tagged human G2A gene were optimized by GeneOptimizer® from Thermo Fisher Scientific Co. (Tokyo, Japan), and the optimized DNA fragment with EcoRI and BamHI recognition sites at the 5' and 3' ends was synthesized by GeneArt® from Thermo Fisher Scientific Co. (Tokyo, Japan). The synthesized cDNA was digested with EcoRI and BamHI, and cloned into the upstream region of the internal ribosomal entry site (IRES) of the pMK-IRES-puro retrovirus vector. The expression vector was transfected into plat-E cells by Lipofectamine 3000 (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol. Plat-E cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 μg/ml puromycin and blastcydin (Gibco, Tokyo, Japan) for 2 d. The culture medium supernatant was filtered and placed on a Retronectin (Takara Bio Inc., Shiga, Japan)-coated 24-well plate. After incubation for 5 h, the supernatant was removed from the plate and a suspension of murine pro-B cells (Ba/F3 cells) in RPMI1640 medium supplemented with 10% FBS and 1 ng/ml IL-3 (from Gibco, Tokyo, Japan) was added to each well on the plate. After growing to 80% confluence, the culture medium was changed to the selection medium containing 3 μg/ml puromycin. After incubation for 2–3 d for selection, the puromycin-resistant cells were obtained and 95% of the cells were confirmed to express the tagged G2A on their surface by fluorescent immune-staining with FITC-conjugated anti-HA-IgG (Bethyl Laboratories, Tokyo, Japan) and confocal microscopy (LSM510 from Carl Zeiss Inc., Oberkochen, Germany).

2. Substrate preparation for a single-cell array

The substrates for the photo-induced single-cell array were prepared by modifying our previous method.[S1] A 35-mm glass-base dish with a diameter of 12 mm (Iwaki Glass, Chiba, Japan) was washed with an ultrasonic wave and coated with collagen (Cellmatrix type I-A, Kurabo Co., Osaka, Japan) according to the manufacturer's protocol. Briefly, the dish surface was dipped in a collagen solution overnight and rinsed three times with distilled water, followed by drying on a clean bench for 1 h. Next, the collagen-coated surface was modified with photo-cleavable PEG-lipid 1 for anchoring cells in a light-

guided manner. Compound 1 was synthesized as described in our previous report.[S2] A solution of 1 in Dulbecco's phosphate-buffered saline (PBS) (100 μM) was applied to the collagen-coated dish and incubated at 37 °C under 5% CO_2. After incubation for 1 h, the solution was removed from the dish, and the dish surface was rinsed with MilliQ water six times, followed with drying as described above. The PEG-lipid-modified surface was irradiated with UV light (λ = 365 nm) at 1.5 J/cm^2 through a photomask using a UV spot light source (100-W Xe lamp, Asahi Spectra Co., Japan) and a band pass filter (LX0365, Asahi Spectra Co., Japan). The photomask had a dot pattern of chromium (circular cluster with a diameter of 14 μm and an interval of 25 μm) and was in contact with the PEG-lipid surface during light irradiation.

3. SrtA-mediated labeling of G2A on the single-cell array

The single-cell array of the Ba/F3 cell expressing the tagged G2A (G2A-Ba/F3 cells) was constructed on the PEG-lipid-modified glass base dish. A cell suspension of G2A-Ba/F3 cells (800 μl, 5.7 × 10^6 cells/ml) was added to the light-irradiated PEG-lipid surface. After incubation for 30 min, free cells were carefully washed away with PBS without detaching the immobilized cells on the non-irradiated spots. Before labeling and observation, the immobilized G2A-Ba/F3 cells on the single cell array were incubated in the preincubation medium (RPMI1640 medium supplemented with 0.1% BSA and 10 μM Lysophosphatidylcholine (LPC)) for 1 h. LPC treatment was reported to suppress the internalization of G2A, leading to accumulation of G2A on the cell surface.[S3] Accordingly, we added LPC into the medium before and during SrtA-mediated labeling to increase the amount of labeled G2A and to retain the labeled G2A on the cell surface until labeling was finished.[S4] During the first step of labeling, cells were incubated in the cleavage medium (RPMI1640 medium supplemented with 0.1% BSA, 10 μM LPC, 30 μM SrtA and 1 mM triglycine) for 1 h and then, at the second step, were incubated in the labeling medium (RPMI1640 medium supplemented with 0.1% BSA, 10 μM LPC, 30 μM SrtA and 10 μM AF488-labeled DLPETGG peptide) for 1 h. After labeling, the single-cell array surface was rinsed with the preincubation medium three times to remove non-specifically adsorbed labeled peptide from the cell surface.

4. Single-cell analysis of G2A trafficking

The time-lapse image of intracellular G2A trafficking was obtained under acidic and slightly basic pH conditions by confocal microscopy. After labeling, the medium was replaced with an acidic one (RPMI1640 medium supplemented with 0.1% BSA, 5 mM HEM, adjusted to pH 6.6), and the dish was placed on the microscope stage with an

incubation system, where the single-cell array on the dish was incubated at 37 °C under 5% CO_2. After obtaining the images under the acidic condition, the pH of the medium was increased to pH 7.7 by adding 14 μl of a 10% $NaHCO_3$ solution to 1 ml of the acidic medium in the dish. Immediately after the pH change, the time-lapse observation was performed for 20 min at 1-min intervals. For re-acidification of the medium, the pH 7.7 medium was adjusted to the acidic one (pH 6.6). Just after re-acidification, the time-lapse observation was re-started and performed for 40 min at 5-min intervals. The obtained time-lapse images were automatically analyzed by a custom software.

5. Experiments for selective labeling and co-localizing with an endosomal marker.

We performed the SrtA-mediated labeling experiment of both G2As tagged at their extracellular *N*-terminus and the intracellular *C*-terminus. A plasmid for expression of G2A fused with V5 tag at its *N*-terminus and both SrtA-recognition sequence and HA tag at its *C*-terminus (G2A-$LPETG_5$) was constructed similarly as described in the method for the *N*-terminus tagged G2A ($LPETG_5$-G2A). To distinguish the cells expressing the two kinds of tagged G2A, the cytosols of Ba/F3 cells expressing G2A-$LPETG_5$ and $LPETG_5$-G2A were stained with the ViVidFluor Cell Blue CMAC (Wako Pure Chemical Industries, Osaka, Japan) and CytoRed (Dojindo Laboratories, Kumamoto Japan), respectively. After cytosol staining, cells are immobilized on the glass substrates coated with PEG-lipid-modified collagen. The immobilized cells were pre-incubated in RPMI medium supplemented with 0.1% BSA and 25 μg/ml Alexa Fluor 647 (AF647) transferrin conjugate (AF647-transferrin, from Thermo Fisher Scientific Co. ,Tokyo, Japan) for 20 min at 37 °C under 5% CO_2. AF647-transferrin was used as an endosomal marker. After rinsing, the cells were served for SrtA-mediated labeling as described above. Then, the fluorescence of the labeled G2A was monitored at pH6.6 and 7.7 by confocal microscopy as described above. Simultaneously, co-localization of the endosomal marker was also observed 35 min after shifting the extracellular pH to 7.7.

After SrtA-mediated labeling, the green fluorescent-labeled G2A was observed on the $LPETG_5$-G2A-expressing cell whose cytosol was stained in red (Fig. S1, **a** and **e**). On the G2A-$LPETG_5$–expressing cell (stained in light blue), no AF488 fluorescence was founded (Fig. S1, **a** and **d**). These results clearly indicate that the fluorescence labeling was selectively done at the extracellular SrtA-recognition sequence tagged on G2A.

In addition, after incubation at pH7.7, the labled G2A was internalized and co-localized with the endosomal marker (Fig. S1**k**). From this result, G2A was internalized into endosomes in Ba/F3 cells as reported in other cells.[S5]

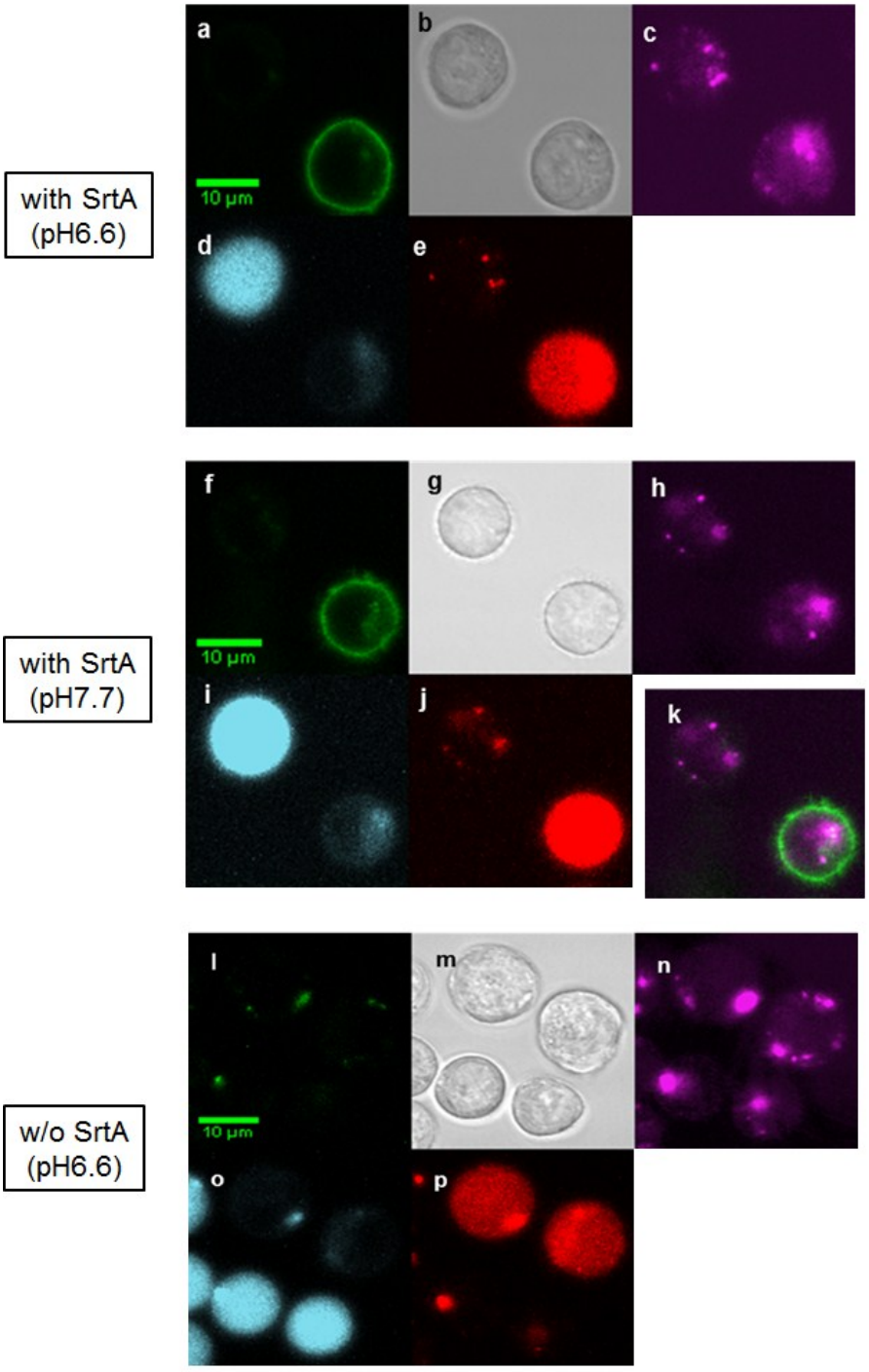

Fig. S1 Confocal microscopic images of the immobilized Ba/F3 cells expressing the G2A tagged with SrtA-recognition sequence at its *C*- and *N*-terminus (G2A-LPETG$_5$ and LPETG$_5$-G2A) after staining endosomes and SrtA-mediated labeling. (**a-e**) The cells were treated with SrtA and AF488-labeled DLEPTGG peptide and incubated at pH6.6, and then, (**f-k**) incubated for 35 min at pH7.7. (**l-p**) The cells were treated with only the labeled peptide and incubated at pH6.6 (negative control). (**a, f, l**) The

fluorescent images of AF488-labeled G2A (in green), (**b, g, m**) the differential interference contrast (DIC) images, (**c, h, n**) the fluorescent images of AF647-transferrin (endosomal marker, in pink), (**d, i, o**) those of ViVidFluor Cell Blue CMAC (the cytosol of G2A-LPETG$_5$–expressing cells in light blue) and (**e, j, p**) those of CytoRed (the cytosol of LPETG$_5$-G2A–expressing cells in light blue) were obtained with confocal microscopy. (**k**) The merged image of the fluorescent images of AF488-labeled G2A and AF647-transferrin was obtained. The scale bars are 10 μm.

6. Quantitative analysis of time-lapse single-cell images

We analyzed the time-lapse fluorescent images of the individual cells on the single-cell array to quantify each intracellular distribution change of fluorescently-labeled G2A under acidic and neutral pH conditions. A custom software was constructed to perform the image analysis rapidly and automatically by modifying our previous method[S5]. This software was consisting of the following procedures.

The single-cell images of the individual cell were obtained by cutting out from the original time-lapse fluorescent image series of the single-cell array. In this single-cell image, the cytosol was stained with red fluorescent dye, and the labeled G2A was observed in the green fluorescent image. These two-color fluorescent images were separated and used for image analysis as follow. The red one was used for preparing the mask of the cytosol region, and the green one was used for both preparation of the mask of the cell surface region and quantification of intracellular G2A distribution. First, the red cytosol image (Fig. S2**A**) was shrunken to avoid overlapping the cytosol and membrane regions (Fig. S2**C**), and binalized by otsu threshold method[S6] (Fig. S2**E**). Second, the green G2A image (Fig. S2**B**) was binalized by adaptive threshold method[S7] implemented in OpenCV 2.4.13(Fig. S2**D**). The binalized image of cytosol (**E**) was inverted to the cytosol mask (Fig. S2**F**), and it was used to define the intracellular compartment region. The image of G2A in intracellular compartment region (Fig. S2**H**) was obtained by subtracting the brightness of the image **D** with **F** (i.e. if the pixel value in image F(x,y) = 1, then forcefully change the pixel value in same position of image D(x,y) = 0. Else do nothing.). That in the cell surface region (Fig. S2**G**) was obtained by subtracting the brightness of the image **D** with **E**. If the nonspecific granular images (Fig. S2**J**) were detected in the cytosol region by human (operator), the G2A image in intracellular compartment region (**H**) was subtracted with the image **J**. Finally, the brightness at all the pixel was summarized on the G2A image in the cell surface (**G**) and the intracellular compartment regions (**H**), respectively.

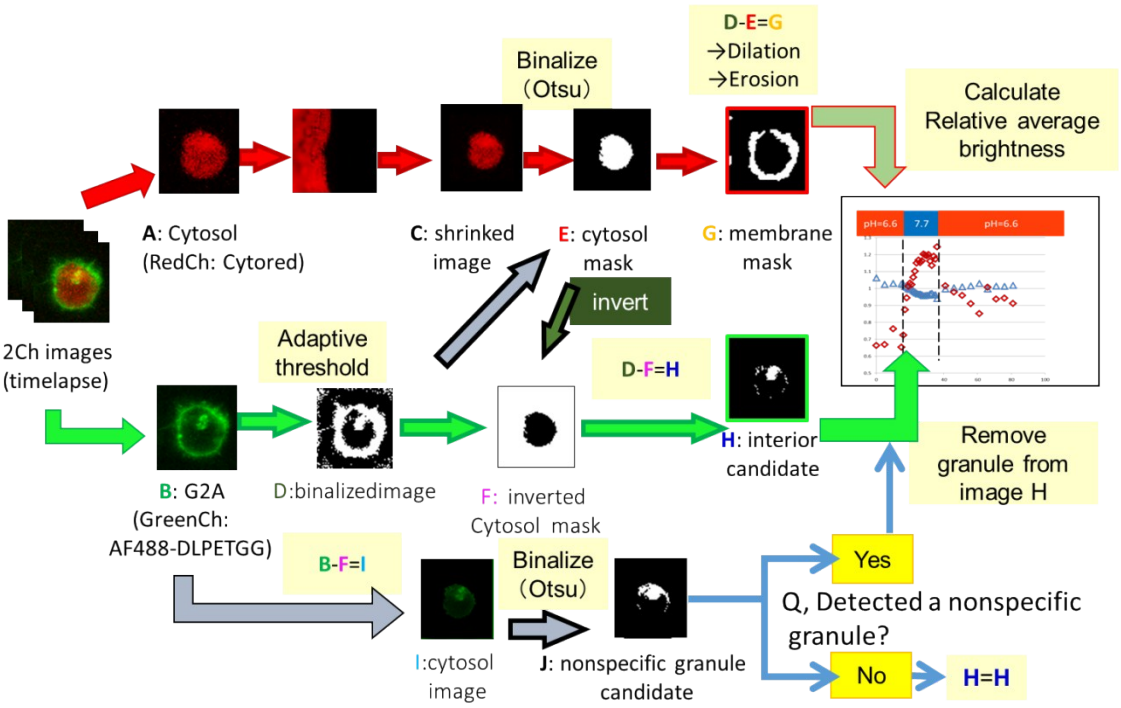

Fig. S2 Schematic diagrams of single-cell quantitative image analysis of intracellular G2A trafficking from the image of the single-cell array.

7. **The effect of G2A anchoring effect by LPC**

LPC was reported to have an effect to anchor G2A on the plasma membrane[S5]. To confirm G2A kinetics in Ba/F3 cell under treatment of LPC at neutral pHs, the time-lapse fluorescent images of fluorescently-labeled G2A was observed on the single-cell array in a medium (RPMI1640 medium supplemented with 0.1% BSA and 10 μM lysophospholipid (LPC)) (Fig. S3).

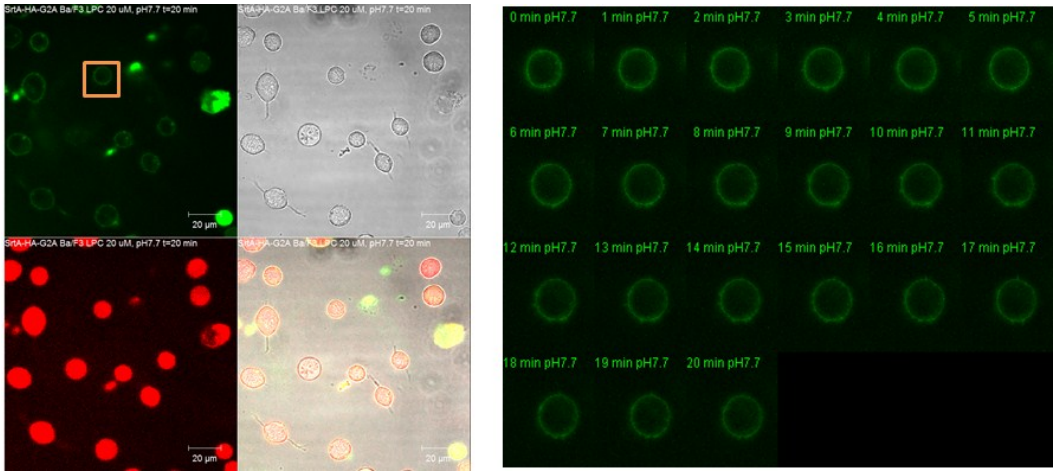

Fig. S3 pH dependent G2A localization change in the presence of LPC

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