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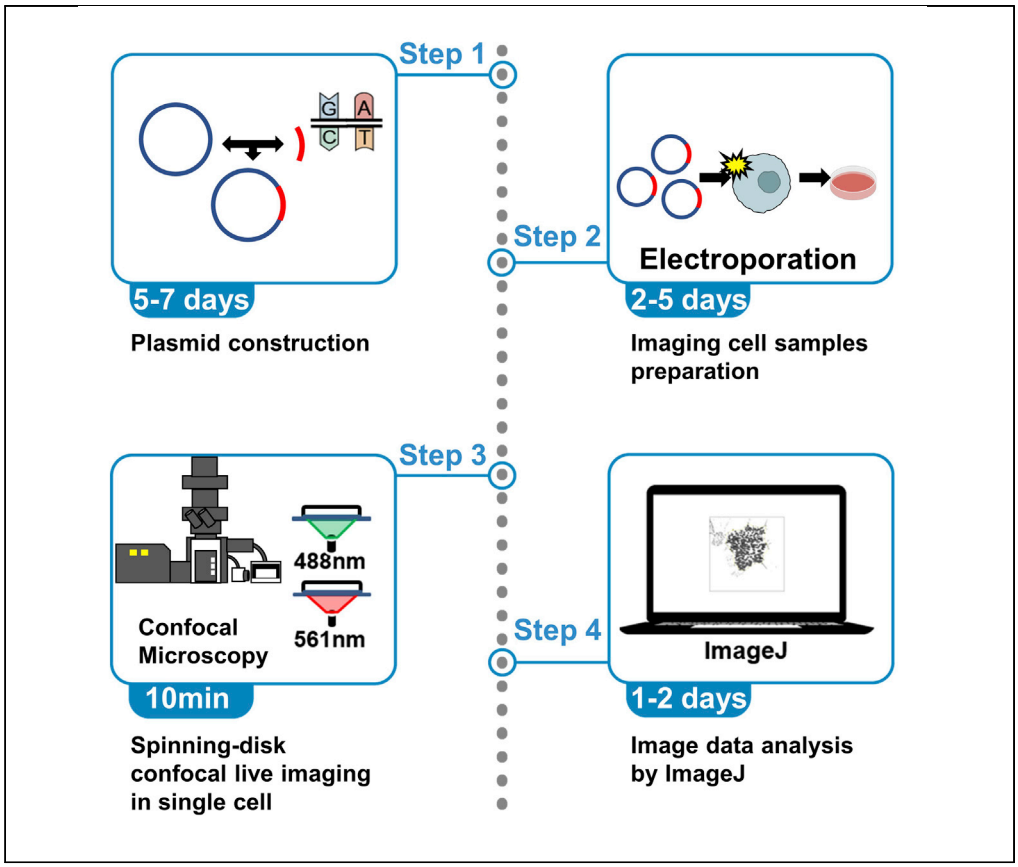
A live-imaging protocol for tracking receptor dynamics in single cells



Adjacent membrane receptors can show different cellular responses to ligand stimulation. Here, we describe a super-resolution microscopy imaging protocol for tracking the dynamics of two different membrane-bound receptors in single cells. We describe the transfection protocol by electroporation. We detail the imaging procedure for receptors in a single cell. We then outline the data analysis pipeline. We have applied this protocol to imaging of endocytosis of the LOX-1 and AT1 in CHO-K1 cells, but the protocol can be applied to a variety of membrane receptors in other cell lines.

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Highlights

Tracking of plasma membrane-bound receptors in single cells

Transfection of two different receptor genes by electroporation

Automated quantification using the ImageJ plugin

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A live-imaging protocol for tracking receptor dynamics in single cells

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SUMMARY

Adjacent membrane receptors can show different cellular responses to ligand stimulation. Here, we describe a super-resolution microscopy imaging protocol for tracking the dynamics of two different membrane-bound receptors in single cells. We describe the transfection protocol by electroporation. We detail the imaging procedure for receptors in a single cell. We then outline the data analysis pipeline. We have applied this protocol to imaging of endocytosis of the LOX-1 and AT1 in CHO-K1 cells, but the protocol can be applied to a variety of membrane receptors in other cell lines.

For complete details on the use and execution of this protocol, please refer to Takahashi et al. (2021).

BEFORE YOU BEGIN

Many GPCRs form homo- and heteromers at the cell surface. Thus, GPCR signaling is not only determined by conformational changes induced by agonist binding, but is also allosterically regulated by interactions with other receptors (Audet and Bouvier, 2012). Receptor interactions are not limited to GPCRs. We have found that a pattern recognition receptor (PRR), lectin-like oxidized low-density lipoprotein (oxLDL) receptor (LOX-1) and a GPCR, angiotensin II type 1 receptor (AT1) form a complex (Yamamoto et al., 2015) and that the cellular uptake of oxLDL was mediated by β-arrestin-dependent endocytosis of AT1 (Takahashi et al., 2021). The interaction of the receptors was supported by experiments including immunoprecipitation, in situ PLA assay (Yamamoto et al., 2015) and live-cell imaging of the two receptors (Takahashi et al., 2021). Here, we provide a protocol of the latest experiment that will allow for live-cell imaging of endocytosis of the two adjacent membrane receptors, LOX-1 and AT1 in a single cell (Takahashi et al., 2021). Complex formation is observed by the merge of labeled receptors, and endocytosis is observed by the instantaneous disappearance of receptors. This phenomenon was quantitatively analyzed by the alteration of number of the puncta which is a cluster of fluorescence-tagged receptor using images before and after stimulation with ligand. We showed that oxLDL, compared with vehicle, reduced number of red puncta representing LOX-1 upon stimulation. The phenomenon was not observed in cells transfected with AT1 which has mutations in the arrestin-binding sequence (Takahashi et al., 2021). The protocol described here modified the original technique that required manual counting of the puncta (Takahashi et al., 2021) by utilizing the ImageJ software, which enable the puncta to be counted automatically. The essential materials of this protocol are, SpinSR10 inverted spinning disk-type confocal super-resolution microscope (Olympus, Japan), ImageJ and its plugin, Red and Green Puncta Colocalization Macro. SpinSR10 allows for 120 nm resolution and image acquisition speeds as fast as 0.005 s per frame, as well as reduced phototoxicity, which is useful for live observation of separate receptors labeled

in two different colors. This macro was originally designed to analyze the co-localization of GFP-LC3 and RFP-LC3 puncta in cells transfected with the RFP-GFP-LC3 tandem reporter construct to assess intracellular autophagic fluxes (Pampliega et al., 2013).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Poly-L-Lysine	ScienCell, USA	Cat#0413
Resuspension buffer (buffer R)	Invitrogen	MPK1096
Electrolytic buffer (buffer E)	Invitrogen	MPK1096
Electroporation device	Digital Bio Technology	MP-100 Microporator
Neon pipette	Invitrogen	MPK1096
Neon 10 μL tips	Invitrogen	MPK1096
Neon tube	Invitrogen	MPK1096
Experimental models: Cell lines		
CHO-K1	ATCC	ATCC CCL-61
Recombinant DNA		
pmScarlet_C1	Addgene	Cat#85042
pcDNA3-EGFP	Addgene	Cat#85042
Other		
Super-resolution microscope	Olympus, Japan	SpinSR10
Lens	Olympus, Japan	100× NA-1.49
Camera	Hamamatsu Photonics KK, Japan	ORCA-Flash 4.0 V2
CellSens Dimension 1.11	Olympus, Japan	SpinSR10
3D deconvolution algorithm	Olympus, Japan	SpinSR10
Microscope incubation system	TokaiHit, Japan	STX Series Stage Top Incubator System
ImageJ1.53K Java 1.8	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Red and Green Puncta Colocalization Macro	(Pampliega et al., 2013)	https://imagejdocu.tudor.lu/plugin/analysis/colocalization_analysis_macro_for_red_and_green_puncta/start
Chemicals, peptides, and recombinant proteins		
Trypsin	Lonza, USA	Cat#CC-5012
Trypsin neutralized solution (TNS)	Lonza, USA	Cat#CC-5002
HEPES Buffered Saline Solution (HBSS)	Lonza, USA	Cat#CC-5022
D-PBS	FUJIFILM Wako, Japan	Lot NO.04529795
10 cm cell cultured dish	Corning	Cat#353801
35 mm glass coverslip bottom cell cultured dish	Iwaki, Japan	Cat#0161951
Cell culture medium (Ham's F-12 Nutrient Mix)	Gibco	Cat#2193045
Fetal Bovine Serum (FBS)	Gibco	Cat# A31605
Antibiotics Penicillin-Streptomycin	Gibco	Cat#2185242

MATERIALS AND EQUIPMENT

Imaging buffer		
Reagent	Final concentration	Amount
NaCl	125 mM	3.65 g
KCl	5 mM	186.38 mg
MgCl2	1.2 mM	57.13 mg
CaCl2	1.3 mM	72.15 mg
HEPES	25 mM	3 g
D-glucose	3 mM	270.24 mg
NaOH (5 N)	Adjust PH 7.4	n/a
ddH_2O	n/a	Fill to 500 mL
Total	n/a	500 mL

Store at 4°C. The maximum time for storage is 6 months.

STEP-BY-STEP METHOD DETAILS

Construction of plasmid vectors for the receptors of interest

Timing: 5–7 days

The receptors of interest are tagged with fluorophores in different colors for easy visualization. In this protocol, we used Human LOX-1 (GeneBank NM002543) and Human AT1R (GenBank NM_000685) as receptors of interest. LOX-1 tagged with V5-6×His at the C-terminus (V5-LOX-1) was subcloned into the EcoRI/EcoRV site of pmScarlet_C1 (Plasmid #85042, Addgene) (mScarlet-LOX-1), fluorophores located at N terminal. HA-FLAG-hAT1 was subcloned into the EcoRI/SalI site of pcDNA3-EGFP (Plasmid #85042, Addgene) (hAT1-EGFP), fluorophores located at C terminal. In this experiment, fluorophores were inserted into the intracellular regions of LOX-1 and AT1.

Note: Whether to place the fluorophore at the C- or N-terminal end of the receptor to be labeled must be based on an understanding of the characteristics of the receptor and its intracellular or extracellular localization.

Preparation of the samples for the imaging experiment

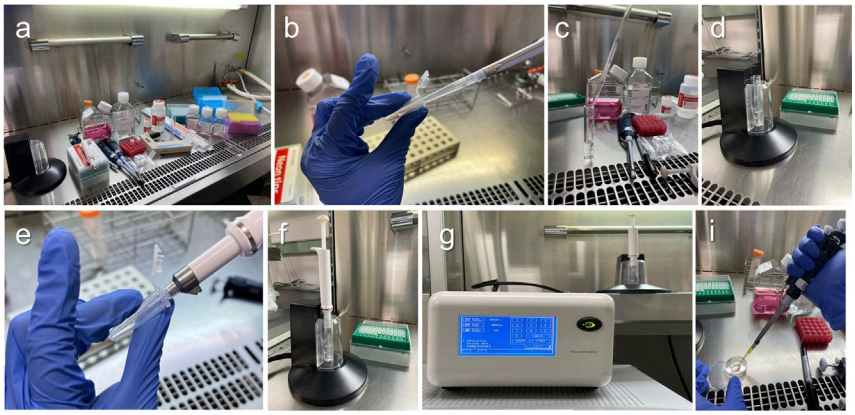
Timing: 2–5 days

This step includes cell culture, preparation and transfection by electroporation.

1. Defrost cells.

Firstly, defrost one CHO-K1 stock from liquid nitrogen under 37°C water bath 3 min, then equally split cells liquid into two 10 cm dishes containing 10 mL Cell culture medium (medium+10% FBS+1%antibiotic) and culture in a 5% CO2 incubator at 37°C 12 h. If cultured cells are obtainable, you can skip this step.

2. Cell passage.
   a. Each 35 mm glass coverslip bottom dish is pre-coated with 200 μL 10 mg/mL Poly-L-Lysine (1:1000) diluted in ultrapure distilled water at a 37°C incubator for an hour.
   b. Wash each 35 mm glass base dish with 200 μL 37°C PBS three times.
   c. Bring the cells in two 10 cm dishes from incubator, wash each 10 cm dish with 2 mL 37°C HBSS once, then add 2 mL 37°CTrypsin to detach cells in a 5% CO2 incubator at 37°C for 2 min.
   d. Add 2 mL 37°CTNS to neutralize Trypsin, then transfer the mixture from each dish to one 50 mL tube, and centrifuge at 210 × *g* for 5 min at 25°C.
   e. Aspirate supernatant, add 10 mL 37°C Cell culture medium (medium+10%FBS) to suspend cells, count cell number, transfer 5×10^4 cells to each 1.5 mL tube, and centrifuge at 210 × *g* for 5 min at 25°C.
3. Electroporation.
   a. Place Neon tube, pipette and tips, buffer R, buffer E and other required experimental tools in a biological safety cabinet (Figure 1A).
   b. Suspend cells (5×10^4 cells) with 10 μL buffer R per 1.5 mL tube.
   c. Add 400 μL Cell culture medium (medium+10%FBS) to another pre-prepared 1.5 mL tube (Figure 1B).
   d. Turn on the Neon device and enter the electroporation parameters. Our parameters in this experiment were, Pulse voltage 1,560 V, Pulse 5 ms, Pulse number 10, Cell density 5×10^6 cells/mL Transfection efficiency 90%, and tip type 10 μL.
   e. Fill the Neon tube with 3 mL buffer E (Figure 1C).
   f. Insert the Neon tube into the Neon Pipette Station until it "clicks" into place (Figure 1D).



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Electroporation parameters

Pulse voltage (v)	Pulse width (ms)	Pulse number	Cell density (cells/ml)	Transfection efficiency	Viability	Tip type
1,560	5	10	5×10^6	90%	93%	10 µl
1,650	10	3	5×10^6	88%	91%	10 µl
1,620	10	3	5×10^6	91%	92%	100 µl

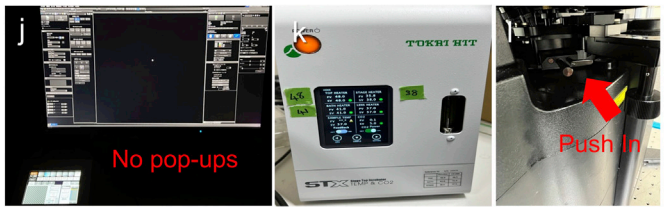

Figure 1. Pictures in the order shown in the electroporation protocol and SpinSR10 accessory equipment

(A–L) Electroporation was performed on CHO cell lines with the Neon™ Transfection System (A–G and I). Required experimental buffers and tools in the biological safety cabinet (A). Transfer 400 µL culture medium into pre-prepared 1.5 mL tube (B). Transfer 3 mL buffer E into Neon tube (C). Insert the Neon tube into Neon Pipette Station (D). Aspirate the cell-DNA mixture into 10 µL Neon tip (E). Place the Neon pipette into Neon Pipette Station (F). Neon device touchscreen displays "Complete" (G). Place 200 µL cell suspension into 35 mm glass coverslip bottom dish (I). Electroporation parameters of CHO-K1 cell line from Neon Transfection System Cell Line Data (H). Established connection between the microscope and the PC software CellSens Dimension 1.11 (J). Microscope accessory of heating machine (K). Microscope shade plate on push-in position (L).

g. Add 1.5 µg/35 mm glass coverslip bottom dish AT1-EGFP and 0.5 µg/35 mm glass coverslip bottom dish mScarlet-LOX-1 to the 1.5 mL tube from step b containing cells and mix gently.
h. Insert a 10 µL Neon tip into the Neon pipette, then press the push-button on the Neon pipette to the first stop and immerse the Neon tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon tip (Figure 1E).
i. Insert the Neon pipette with the sample vertically into the Neon tube placed in the Neon Pipette Station until it "clicks" into place, then press Start button on the Neon device touchscreen (Figure 1F).
j. Neon device touchscreen displays "Complete" to indicate that electroporation is complete (Figure 1G). Electroporation parameters of CHO-K1 cell line from Neon Transfection System Cell Line Data is shown in Figure 1H.
k. Remove the Neon pipette from the Neon Pipette Station and immediately transfer the sample mixture from the Neon tip into the prepared 1.5 mL tube from step c containing 400 µL Cell culture medium (medium+10%FBS).
l. Transfer 200 µL cell suspension from above 1.5 mL tube into each pre-coated 35 mm glass coverslip bottom dish, incubate in a 5% CO2 incubator at 37°C for 12 h (Figure 1I).

Note: Depending on cell types, you need to check the optimal electroporation parameters.

*Neon Transfection System Cell Line Data and transfection parameters can be found on the manufacturer's website.

https://www.thermofisher.com/jp/ja/home/life-science/cell-culture/transfection/neon-transfection-system/neon-transfection-system-cell-line-data.html.

⚠ CRITICAL: To ensure reproducibility and eliminate variation of the transfection conditions within or between experiments, use each Neon tip only at single time. Discard the used Neon tip into an appropriate biological hazardous waste container.

Neon Transfection System manual can be found on the manufacturer's website.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/neon_device_man.pdf.

Note: It is recommended that experiments be performed with cells in the same condition as much as possible. In our experiments, we defrost the cells after each experiment and use them as soon as their condition is restored, in order to avoid changes in cell properties due to differences in the number of passages and contamination during culture. Cells that have undergone repeated passages are not used.

Note: Cells should be grown at 80%–90% confluence when you passage the cells.

Note: The pre-coating of Poly-L-Lysine is used to enhance cell adhesion after electroporation.

Note: The Neon pipette is used only with Neon tips. Do not use any other tips for the Neon pipette.

Note: The medium used in electroporation process should not contain antibiotics in order to keep cell viability after electroporation.

Note: Preliminary experiments are required to determine the optimal transfection ratio of two plasmid vector, in this protocol, we used 1.5 μg/35 mm glass coverslip bottom dish AT1-EGFP and 0.5 μg/35 mm glass coverslip bottom dish mScarlet-LOX-1 to perform transfection.

Note: In this study, we are performing gene expression by electroporation. Other gene delivery methods, such as viruses, can be used for both in vitro and in vivo experiments (Fus-Kujawa et al., 2021).

Spinning-disk confocal live-cell imaging of receptors in the single cell

Timing: 10 min

In this part, we describe how to make dynamic recordings of the above transfected cells under super-resolution microscopy.

4. Wash the sample dish three times with pre-warmed 37°C 900 μL/35 mm glass coverslip bottom dish imaging buffer and keep 900 μL/35 mm glass coverslip bottom dish to the sample dish before microscopic observation is initiated.
5. To start operating the super-resolution microscope, ensure a secure connection between the microscope and the PC software cellSens Dimension 1.11 (Olympus, Japan) (Figure 1J).

6. Turn on the 488 nm (green) and 561 nm (red) laser channels at 20% power, and subsequently adjust the laser power according to the image brightness. Set the film recording time to a total of 5 min, 60 cycles, and 5 s interval. Turn on the auto focus.
7. Add a drop of lens oil to the 100× NA 1.49 objective, then place the sample dish on it, fix the dish with the matching clamp, and turn on the heater provided by the manufacturer (STX Series Stage Top Incubator System, Tokai Hit, Japan) to keep the sample at ambient temperature at 37°C (Figure 1K).
8. Under the EYE1DIC (Differential Interference Contrast) mode, adjust the focus to obtain clear images of the cells, then switch the channel to EYE2-G (green channel) or EYE3-R (red channel) to check the fluorescence expression and seek the best target single cell.
9. After selecting the cell area, click "Start" to initiate image recording and add 100 μL of stimulus (1/10 final concentration diluted by imaging buffer, imaging buffer and oxLDL are used as vehicle and stimulus respectively in this protocol) or vehicle to the sample dish just after a 1-min image has been captured.
10. Green and red channels, and the backgrounds of the two channels are subtracted using the 3D deconvolution algorithm of cellSens Dimension 1.11, and finally the two processed channels are combined to form the merged image (Figure 2 and Methods video S1).

Note: If there is a problem with the connection between the microscope and the PC, a pop-up appears. At that time, please follow the displayed pop-up.

Note: Choose and keep the laser settings and microscope configuration consistent between conditions to get high quality quantitative data.

Note: It is very important to find an optimal visualization of the cell in both the green and red channels. The best cell exhibits clear green and red puncta that are moving temporally. If you cannot find the best cell from the software screen, you can look for them under the microscope eyepiece. Prior to turn on microscope eyepiece mode, make sure the microscope shade plate needs to be on push-in position Figure 1L).

Note: Gently add the stimulus to the sample dish without any disturbance or movement of the selected cell area. It might need to cease the imaging cycle for drug addition when focus is altered.

Note: The focus may shift during stimulation. Please stimulate in a gentlemanly manner-slowly push the pipette to release reagent to periphery of dish. In this experiment, we use a microscope with autofocus function, which allows us to obtain stable live images. You can also use a perfusion device as an alternative method.

Note: If bleach correction is required on the captured image, it should be performed prior to analysis.

EXPECTED OUTCOMES

Green and red puncta images with a clear background can be obtained by the above procedure. In addition, the movement and disappearance of the puncta after ligand stimulation can be analyzed during the 5-min image loading process.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 1–2 days

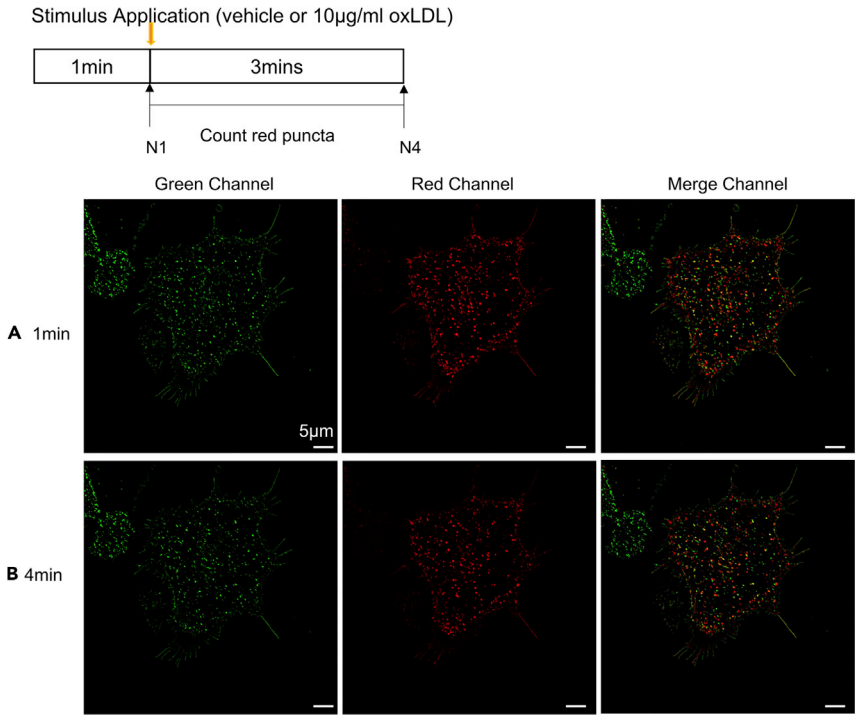

Figure 2. Two receptors internalization triggered by the stimulation

Imaging of CHO-K1 cells co-transfected with mScarlet-LOX-1 and control or AT1-EGFP. Cells were preincubated for 1 min and stimulated. In the current protocol, we use 10 μg/mL oxLDL for stimulation. The imaging of before (0 min) and 3 min after stimulation. The imaging was observed by a confocal super-resolution microscope.

Puncta counting was performed before application (N1; 1 min after the imaging acquisition) and at 4 min point (N4; 3 min after the stimulation), using separated images to observe red puncta (mScarlet-LOX-1) and green puncta (AT1R-EGFP). The cells were stimulated just after the 1 min point. Although the number of puncta was counted manually by a blind observer in a previous publication (Takahashi et al., 2021), we have currently updated the counting method using the ImageJ plugin to count puncta automatically. The percentage change of puncta is calculated by the following formula: (N1-N4)/N1 × 100%. The workflow of ImageJ is as follows:

1. Open red and green channel image files.
2. Image>Type>8-bit.
3. Image>Adjust>Threshold>click Dark background.
4. Polygon selections>define selected cell area in the green channel.
5. Analyze>Tool>ROI Manager>click add[t] to red channel.
6. Analyze> Analyze Particles>Size (20–200), Circularity (0.65–1.00)>click OK g. Obtain the counted puncta of green channel.
7. Repeat the previous step to obtain the count puncta for the red channel.
8. Plugins>Colocalization>Ratio 50%, check colocalized points 8-bit>click OK.
9. Repeat step c and d to merged images.
10. Open the ROI Manager window> click measure.
11. Open results window>Edit>Select All>Copy and paste these results into excel. Determine the number of puncta in the merge channel by counting the number of puncta in excel file with mean value above 0.
12. Count results are collected for N1 and N4 images (Figure 3).
13. Analysis of internalization of AT1 and LOX-1 to stimuli.

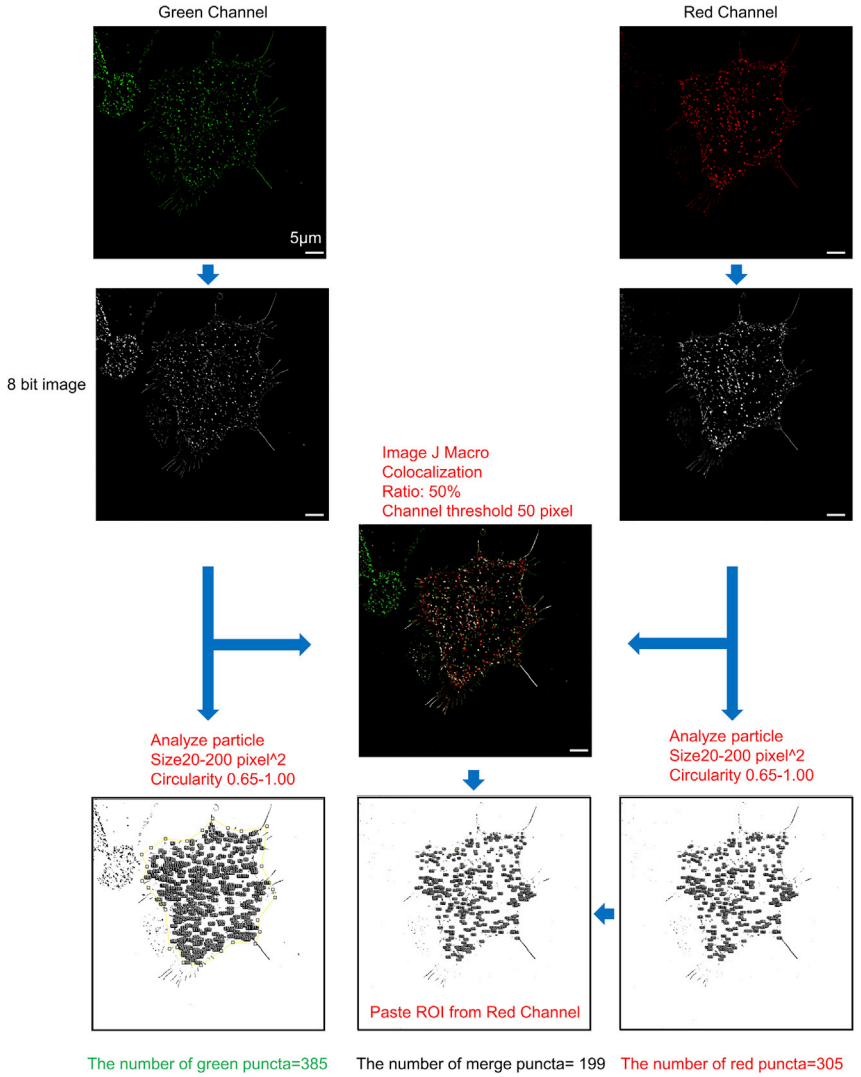

Figure 3. Puncta counting and colocalization analysis

A count of puncta was performed using separated images visualizing mScarlet-LOX-1 and AT1-EGFP just before (N1=1 min after the imaging acquisition) and 4 min point (N4; 3 min after the stimulation). The number of puncta at 1 and 4 min was determined by ImageJ. Change of the puncta was calculated as (N1-N4)/N1 × 100 (%).

(Result of the comparison between cells treated with vehicle and oxLDL in Figure 4).

LIMITATIONS

As the automatic count of puncta depends on the size of particles, very small puncta would be excluded from the analysis. The background particles with circularity and magnitude close to that of the real puncta might be included in the analysis. In this study, the rate of change was calculated by comparing images at two time points before and after stimulation and change over time was not calculated for all images. The ImageJ plugin can only analyze 8-bit images. This approach only can be applied to single cell but not to tissue or organ. This approach is also based on a cellular system of receptor overexpression and therefore difficult to play in native cells to study the relationship of endogenous receptors. In short, this approach does not meet the need to explore protein interactions of interest beyond engineered cell systems.

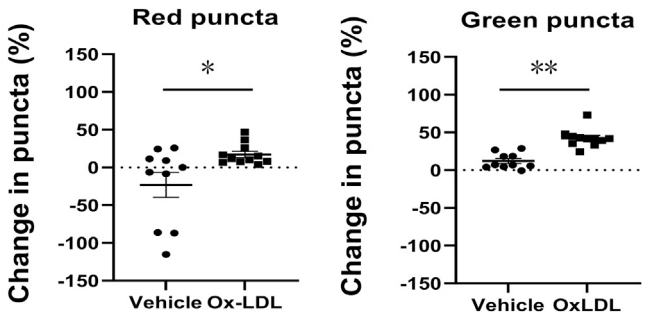

Figure 4. AT1 and LOX-1 internalization in response to oxLDL stimulation

Change in mScarlet-LOX-1 and AT1-EGFP puncta in CHO-K1 cells stimulated with vehicle (n = 10) or 10 μg/mL Ox-LDL (n= 11). Data are represented as mean ± SEM. The difference between the application of vehicle and oxLDL was determined by Student's t-test. *p < 0.05, **p< 0.01.

TROUBLESHOOTING

Problem 1

The electroporation device screen displays error message (related to step 2).

Potential solution

Make sure that the Neon pipette and cuvette are properly inserted into the Neon station separately. The metal tip of the Neon pipette should be tightly attached to the spherical plunger inside the pipette station. The side electrode of the Neon tube should be tightly attached to the spherical plunger inside the pipette station.

Problem 2

There is a connection failure message on electroporation device screen (related to step 2).

Potential solution

Check that the correct voltage value is entered. Make sure that the Neon tip is properly inserted into the Neon pipette and that there is no gap between the tip and the top cap of the pipette.

Problem 3

Cells have low viability after electroporation (related to step 2).

Potential solution

Avoid using excessively confluent cells or high-density cells. 80%–90% cell confluence is recommending. Ensure that the cell culture medium after electroporation is free of antibiotics.

Problem 4

The mobile activity of the cell puncta is lower than expected (related to steps 2 and 3).

Potential solution

This problem shares common solution with Problem 5. For complete details of this solution please refer to the Potential Solution of Problem 5. Regarding troubleshooting of SpinSR10 microscope operation, please refer to following manual.

Problem 5

The intensity of the receptor fluorescence is lower than expected (related to steps 2 and 3).

Potential solution

This problem may be caused by low transfection efficiency of electroporation, quality of plasmid DNA or low ambient temperature during cell observation. First, check whether the optimal electroporation

parameters are selected based on the cell type. Avoid creating any air bubbles in the Neon tip when aspirating the sample mixture. This is followed by preliminary experiments to optimize the concentration of DNA plasmids used in electroporation and to check the purity of the DNA plasmids. Finally, turn on the heater during the observation process and store the subsequent samples in a 37°C incubator.

Problem 6

Non-specific fluorescent signal and no required puncta (related to steps 2 and 3).

Potential solution

This problem could be raised by cell auto-fluorescence. First choose cells with good fluorescence expression of both green and red channels, which displays proper mobile activity of puncta and intensity of fluorescence. Then increase the laser power to fit in empirically. Always passage cells with compatible confluency levels.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Toshimasa Takahashi (toshimasa.takahashi@geriat.med.osaka-u.ac.jp).

Materials availability

All plasmids generated in this study are available from the lead contact without restriction.

Data and code availability

The data sets supporting the current study are available from the lead contact up on reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101347.

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AUTHOR CONTRIBUTIONS

T.T., H.Y., and K.Y. designed and performed the experiments and assisted in writing the manuscript. H.R. designed the experiments and assisted with writing the manuscript

DECLARATION OF INTERESTS

The authors declare no competing interests.

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