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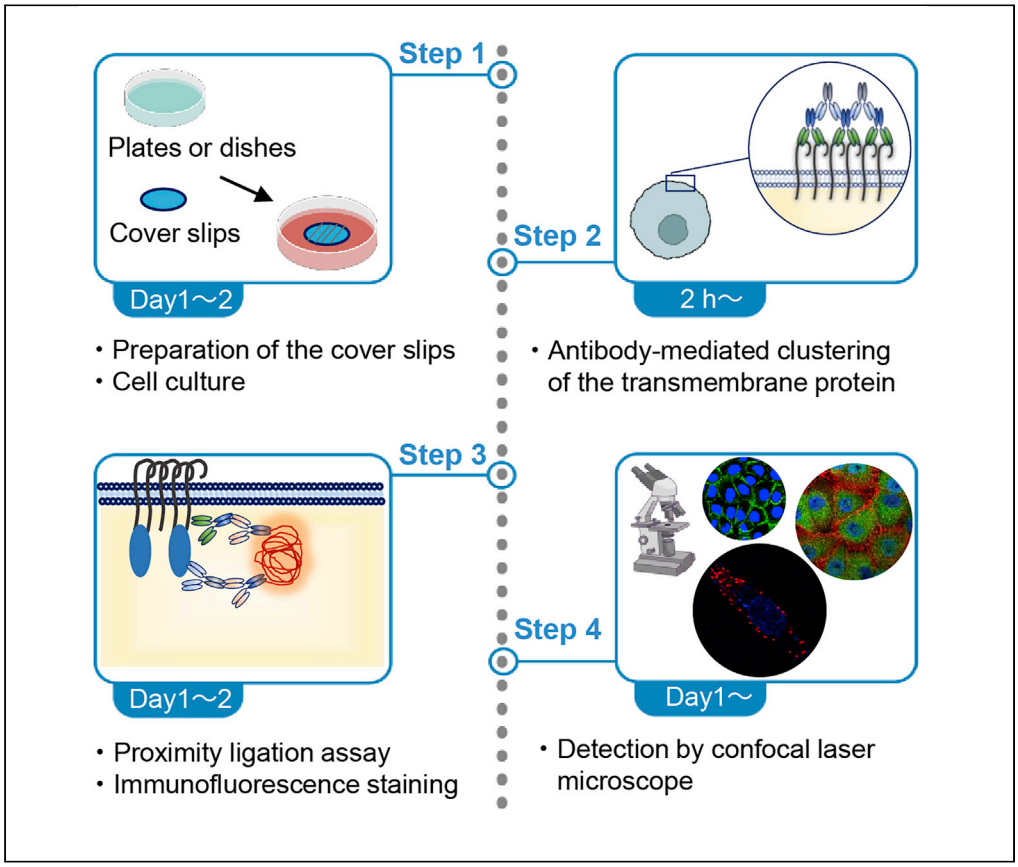
Protocol for visualizing conditional interaction between transmembrane and cytoplasmic proteins



This protocol visualizes dynamic interaction between a transmembrane protein and an intracellular protein induced by clusterization/oligomerization of the transmembrane protein. Association-dissociation of the intracellular region of the transmembrane protein with cytoplasmic protein(s) is detected by proximity ligation assay. Since a transmembrane protein often resists extraction, biochemical analysis of its dynamic interaction with cytoplasmic effectors is cumbersome. This protocol quantitatively visualizes protein-protein interaction occurring in the membrane periphery, providing a powerful tool to elucidate signal transduction across the membrane.

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Highlights

Antibody-mediated cross-linking induces functional clustering of membrane protein

PLA can visualize extracellular-signal-dependent intracellular protein interaction

This protocol is applicable for the analysis of dynamic protein network formation

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Protocol for visualizing conditional interaction between transmembrane and cytoplasmic proteins

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SUMMARY

This protocol visualizes dynamic interaction between a transmembrane protein and an intracellular protein induced by clusterization/oligomerization of the transmembrane protein. Association-dissociation of the intracellular region of the transmembrane protein with cytoplasmic protein(s) is detected by proximity ligation assay. Since a transmembrane protein often resists extraction, biochemical analysis of its dynamic interaction with cytoplasmic effectors is cumbersome. This protocol quantitatively visualizes protein-protein interaction occurring in the membrane periphery, providing a powerful tool to elucidate signal transduction across the membrane.

For complete details on the use and execution of this protocol, please refer to Ooki et al. (2019).

BEFORE YOU BEGIN

We describe a protocol for visualization of a protein-protein interaction that is induced by clustering of transmembrane proteins. Antibody-mediated cross-linking can be used for inducing clustering of transmembrane proteins instead of natural ligands (Ibuka et al., 2015; Toki et al., 2016; Chakravarty et al., 2017; Damiano et al., 2020). Interaction between transmembrane and intracellular proteins is easily visualized by a proximity ligation assay (PLA) without cell lysis and can be observed using a microscope. This protocol will be applicable as a method to investigate a novel interaction between a plasma membrane protein and an intracellular signaling molecule(s), the function of which is regulated by extracellular ligand-membrane protein interaction. The overview of purpose and principal in this protocol are shown in Figure 1.

This protocol was used in a recent study (Ooki et al., 2019) to determine whether extracellular high-molecular-weight hyaluronan (HMW-HA)-induced CD44 clustering is required for activation of the Hippo signal by inducing intracellular CD44-PAR1b interaction in mammary epithelial cells. The results showed that specific cross-linking of CD44 using an antibody mimics HMW-HA-mediated clustering of CD44 to activate the Hippo signal. Although we used CD44 as the transmembrane protein in that study, this method is applicable to other membrane-spanning proteins such as integrin family proteins, cell adhesion molecules, cytokine/growth factor receptors and G protein-coupled receptors (GPCRs) for investigating their intracellular effector/regulator proteins.

For clustering of a transmembrane protein by antibody-mediated cross-linking, selection of an antibody is the most critical point. You must use an antibody recognizing the extracellular domain of your targeted transmembrane protein. In addition, you should consider specificity of the antibody, appropriate concentration of the antibody, appropriate incubation time, and appropriate

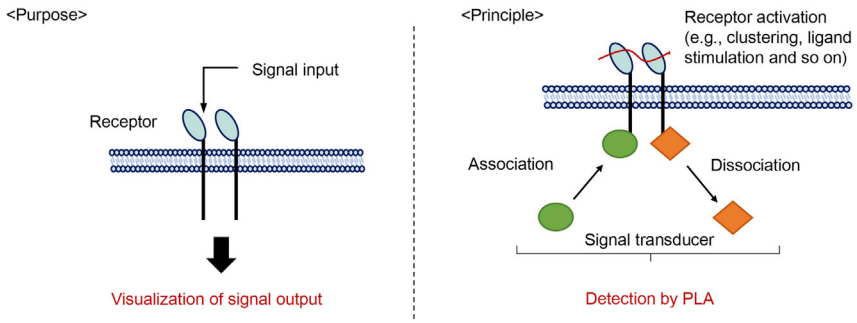

Figure 1. Overview of purpose and principal in this protocol

Left panel (purpose of this protocol):
Visualization of a signal output (e.g., protein-protein interaction, intracellular protein localization) that is induced by signal input (e.g., clustering of the transmembrane proteins, ligand stimulation and so on).
Right panel (principal of this protocol):
Association-dissociation of the intracellular region of the transmembrane protein with a cytoplasmic protein by the activation of receptors (e.g., clustering, ligand stimulation and so on) is detected by proximity ligation assay (PLA).

temperature. When you cross-link your membrane protein, you must use antibodies (both for primary and secondary antibodies) made by species that are different from those used for the PLA reaction.

For the PLA, as well as antibody-mediated cross-linking, you should consider specificity of the antibodies, appropriate concentration of the antibodies, appropriate incubation time and appropriate temperature (To decrease non-specific PLA signals, confirmation of the staining condition of your antibodies should be performed by immunofluorescence staining before performing the PLA.). You can use several species of antibodies as primary antibodies (mouse, rabbit, goat and human) and choose the PLA probes as needed (https://www.sigmaaldrich.com/japan/lifescience/proteomics/protein-detection/duolink/InSitu_PLA.html).

Preparation of collagen-coated cover slips

Timing: 1–2 days

1. Wash the cover slips (MICRO COVER GLASS, MATSUNAMI, Cat #C012001) with 18.2 Ω MilliQ sterilized H_2O containing tween 20 (at a final concentration of 0.1%) by stirring in a beaker for 3–4 h.
2. To remove the detergent (tween 20), wash the cover slips with ddH_2O by stirring overnight (12–16 h), (ddH_2O should be changed at least three times).

   Note: Tween 20 is used to remove small particles of dust and the oils and fats that are attached to the cover slips. Cleaning the cover slips will help cell adhesion and enhance coating.

3. Transfer the cover slips into another beaker and then wash the cover slips with 100% ethanol by stirring for 30–60 min.

   Note: All the manipulations should be done in clean bench to keep sterility (steps 4–9).

4. To sterilize the cover slips, thoroughly tap off the ethanol and then bake the cover slips using a gas burner on a clean bench (usually within one second). Troubleshooting problem 1

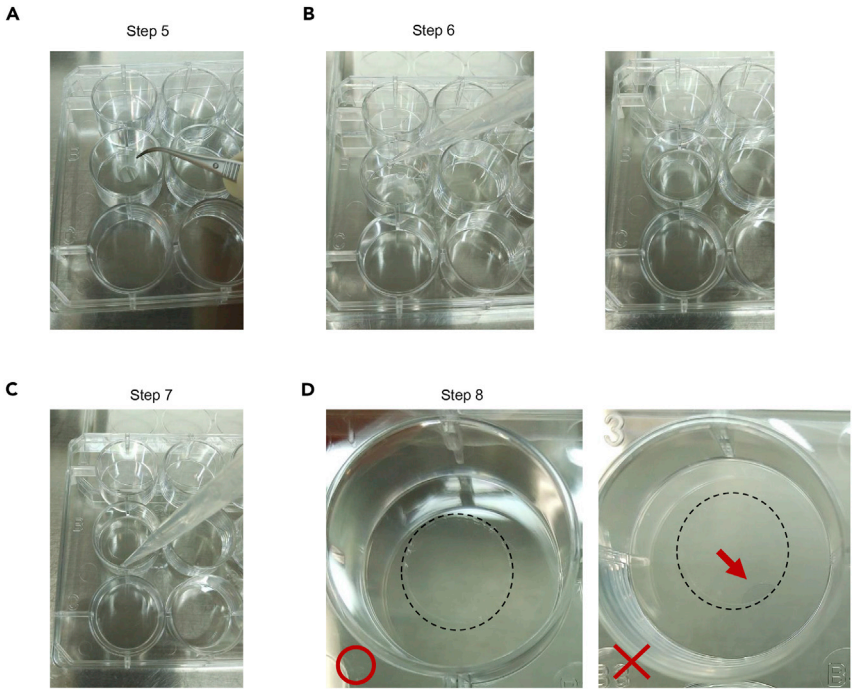

Figure 2. Preparation of collagen-coated cover slips

(A) Put cover slips onto a 12-well plate.

(B) Add 1 mL of collagen solution (*left* panel). After having removed air bubbles (*right* panel).

(C) Remove the residual collagen solution using an appropriate size of the pipet.

(D) After coating with collagen solution. Good coating (*left* panel) and bad coating (*right* panel). Red arrow indicates contamination of air bubbles.

5. Keep the 12-well dish and the cover slips in a clean bench with UV light for 30–60 min to maintain sterility and put the cover slips into a 12-well plate (Figure 2A).

   Note: You can choose the scale of the cell culture dish as needed (e.g., 12-well plate, 24-well plate, 35 mm dish or 6 cm dish). The volume of the solution will change depending on the well size/coverslip size.

6. Add 1 mL of collagen (Cell matrix Type I-C (3 mg/mL), Nitta Gelatin, Cat #631-00771, purified from pig skin was used), into each well and then spread the solution (Figure 2B).

   Note: When air bubbles are present between the well (or dish) and cover slip, push out the air bubble using tips.

   Alternatives: Instead of Cell matrix Type I-C (3 mg/mL), Nitta Gelatin, Cat #631-00771, you can use collagen type I solution that was produced by another company (e.g., collagen, Type I solution from rat tail, SIGMA, cat# C3867. When you use it, see Collagen Coating Protocol | Sigma-Aldrich).

7. Immediately after spreading the collagen solution, recover the residual collagen solution using a p1000 pipet (Figure 2C).

   Note: The removed collagen solution can be reused. If you want to reuse the solution, store the solution at 4°C.

8. Wait for 30–60 min until the cover slips dry up (Figure 2D).
9. Repeat steps 6–8 at least 3 times (usually 3–5 times). Troubleshooting problem 2 or 3
10. Store the collagen-coated cover slips at room temperature (23°C–26°C, approximately 25°C).

Note: If collagen interferes with your experimental process, you may alternatively use poly-L-lysine (PLL) or another coating solution. Troubleshooting problem 2

Note: If you use chamber slides (e.g., 8-well Chamber Slide w/removable wells, Thermo Fisher Scientific, Cat #177402) instead of the cover slips, you can skip steps 1–10. However, if you use chamber slides, this would be a very expensive assay as you would need a large amount of the reagents (At least 200 μL of solution would be required per well.).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-CD44 (1:500)	Cell Signaling Technologies	Cat #5640 (8E2) RRID: AB_10547133
Mouse monoclonal anti β-Actin (1:1000)	Cell Signaling Technologies	Cat #3700 (8H10D10) RRID: AB_2242334
Rabbit monoclonal anti-YAP (1:500)	Cell Signaling Technologies	Cat #14074 (D8H1X) RRID: AB_2650491
Rabbit monoclonal anti-MARK2/PAR1b (1:500)	Abcam	Cat #ab133724 (EPR8553)
Rat monoclonal anti-CD44 (final concentration: 10 μg/mL)	Thermo Fisher Scientific	Cat #14-0441-86 (IM7) RRID: AB_467248
Normal rat IgG (final concentration: 10 μg/mL)	Santa Cruz	Cat #sc-2026 RRID: AB_737202
Alexa-Fluor 488 mouse anti-IgG (1:1000)	Thermo Fisher Scientific	Cat #A-11029 RRID: AB_2534088
Alexa-Fluor 546 mouse anti-IgG (1:1000)	Thermo Fisher Scientific	Cat #A-11030 RRID: AB_2534089
Alexa-Fluor 488 rabbit anti-IgG (1:1000)	Thermo Fisher Scientific	Cat #A-11034 RRID: AB_2576217
Goat anti-Rat IgG Fc secondary antibody (final concentration: 1 μg/mL)	Thermo Fisher Scientific	Cat #31226 RRID: AB_228348
Goat anti-mouse IgG-Fc fragment antibody (final concentration: 1 μg/mL)	Bethyl	Cat #A90-131A
Alexa-Fluor 488 Phalloidin (1:50)	Thermo Fisher Scientific	Cat #A12379
Chemicals, peptides, and recombinant proteins		
Bovine Serum Albumin (BSA)	Sigma	Cat #A7030
Collagen; Cell matrix Type I-C (3 mg/mL)	Nitta Gelatin	Cat #631-00771
DAPI	Wako	Cat #043-18804
Glycerol	Wako	Cat #075-00616
HCl (35.0%–37.0% mass/mass)	Wako	Cat #080-01066
Hyaluronan Low MW	R&D	Cat #GLR001
Hyaluronan High MW	R&D	Cat #GLR002
KCl	Wako	Cat #163-03545
KH_2PO_4	Wako	Cat #169-04245
$MgCl_2$	Wako	Cat #135-00165
NaCl	Wako	Cat #195-01663
Na_2HPO_4	Wako	Cat #197-02865
NaN_3	Wako	Cat # 198-14902
NaOH	Wako	Cat #198-13765
Paraformaldehyde (PFA)	Nacalai Tesque	Cat #26126-25
Penicillin and streptomycin	Gibco	Cat #15140-122
MEGM	Lonza	Cat #CC-3150

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris base	Wako	Cat #201-06273
Tris-HCl	TCI	Cat #T0740
Triton X-100	Wako	Cat #A16046
Tween-20	Nacalai Tesque	Cat #28353-85
Critical commercial assays		
Duolink In situ PLA kit: Duolink In Situ Detection Reagents Orange	Sigma	Cat #Duo92007
Duolink In situ PLA kit: Duolink In Situ PLA Probe Anti-Rabbit PLUS	Sigma	Cat #Duo92002
Duolink In situ PLA kit: Duolink In Situ PLA Probe Anti-Mouse MINUS	Sigma	Cat #Duo92004
Duolink In Situ Mounting Medium with DAPI	Sigma	Cat #Duo82040
Experimental models: cell lines		
MCF10A (female)	ATCC	Cat #CRL-10317 RRID: CVCL_0598
Software and algorithms		
Excel	Microsoft Office	Excel 2013
FV10-ASW	Olympus	N/A
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism	GraphPad software	Version 6
Other		
Confocal laser scanning microscope	Olympus	FLUOVIEW FV1200
Micro Cover Glass (12 mm, No.1) (Cover slip)	Matsunami	Cat #C012001
Micro Slide Glass	Matsunami	Cat #S2215
0.22 μm Filter	Thermo Fisher Scientific	Cat #566-0022

MATERIALS AND EQUIPMENT

Note: Prepare all solutions using 18.2 Ω MilliQ sterilized H_2O.

Note: Room temperature is approximately 25°C (temperature range: 23°C–26°C).

Materials	Recipes	Preservation conditions
1× PBS	Add 50 mL 20× PBS in 950 mL ddH_2O and then perform autoclave (121°C, 20 min).	Room temperature (23°C–26°C, approximately 1 month).
1× PBS/$MgCl_2$	Add 50 μL 1 M $MgCl_2$ in 50 mL 1× PBS.	Room temperature (23°C–26°C, approximately 1 month).
1× PBS/0.1% Tween 20	Add 50 μL Tween 20 in 50 mL 1× PBS.	Room temperature (23°C–26°C, approximately 1 month).
1× PBS/0.25% triton X-100	Add 125 μL Triton X-100 in 50 mL 1× PBS.	Room temperature (23°C–26°C, approximately 1 month).
90% Glycerol/PBS	Add 5 mL 1× PBS in 45 mL Glycerol	Room temperature (23°C–26°C, approximately 3 months).
Collagen solution	Add 5 mL Collagen type I-C (3 mg/mL) in 45 mL pH 3 HCl solution.	Store at 4°C (approximately 3 months).
10% PFA (paraformaldehyde)	Add 2 g PFA in 20 mL ddH_2O and then stir on hotplate (60°C–70°C, at this time PFA is not dissolved). Drop 10N NaOH until PFA dissolve.	Store at 4°C in dark (1–2 weeks).
4% PFA/PBS	Add 4 mL 10% PFA and 0.5 mL 20× PBS in 5.5 mL ddH_2O.	Prepare before using.
5% NaN_3	Add 0.5 g NaN_3 in 10 mL ddH_2O	Room temperature (23°C–26°C, approximately 3 month).

20× PBS		
Reagent	Final concentration	Add to 500 mL
NaCl	2.74 M	80 g
KCl	54 mM	2 g
Na_2HPO_4	200 mM	14.4 g
KH_2PO_4	36 mM	2.4 g
ddH_2O		Up to 500 mL
Store at 23°C–26°C. The storage time is approximately 6 months.		

Blocking solution		
Reagent	Final concentration	Add to 40 mL
BSA	5% (w/v)	2 g
Tween 20	0.1% (v/v)	40 μl
5% NaN_3	0.05% (w/v)	400 μl
1× PBS		Up to 40 mL
Store at 4°C. The storage time is usually within 1 month.		

Wash buffer A		
Reagent	Final concentration	Add to 500 mL
NaCl	0.15 M	4.4 g
Tris base	0.01 M	0.6 g
Tween 20	0.05% (v/v)	250 μl
HCl (35.0%–37.0% mass/mass)		Adjust the pH to 7.4
ddH_2O		Up to 500 mL
Filter the solution through a 0.22 μm filter. Store at 4°C in dark. The storage time is usually within 6 month.		

Wash buffer B		
Reagent	Final concentration	Add to 500 mL
NaCl	0.1 M	2.92 g
Tris base	0.2 M (as Tris)	2.12 g
Tris-HCl		13.0 g
HCl (35.0%–37.0% mass/mass)		Adjust the pH to 7.5
ddH_2O		Up to 500 mL
Filter the solution through a 0.22 μm filter. Store at 4°C. The storage time is usually within 6 month.		

Alternatives: You can use Duolink In Situ Wash Buffer A (Sigma; DUO82047) and Duolink In Situ Wash Buffer B (Sigma; DUO82048) instead of wash buffers A and B, respectively.

STEP-BY-STEP METHOD DETAILS

Seeding and culturing of the cells

Timing: 1 day

Depending on the aim of your experiment, seed the cells you will need. Since the Hippo signal is regulated by cell confluency (Zhao et al., 2007), to inhibit the Hippo signal, cells should be seeded in a low-cell density condition such as 1.0×10^4 cells/well in a 24-well plate (Tsutsumi et al., 2013). If confluency does not interfere with or have an effect on your experimental process, approximately 50% confluency will give good staining images.

⚠ CRITICAL: When you have a positive or negative control for your experiments, you should simultaneously prepare the samples to know whether clustering or the PLA has succeeded or not (for preparation of the control, see individual sections and Troubleshooting problem 5).

⚠ CRITICAL: Please confirm mycoplasma contamination before starting this experiment and use mycoplasma-eliminated cells (It is possible that contamination of mycoplasma disturbs the physiological intracellular signaling pathways. Such signal perturbation might substantially influence protein-protein interaction of interest, thereby leading to incorrect conclusions.).

1. Sterilize the cover slips by UV irradiation in a clean bench (tissue culture hood) for 30–60 min (if collagen solution is sterilized, you can skip UV irradiation).
2. Wash the cover slips twice using a medium (your cell culture medium with antibiotics) by gently shaking.
3. Seed the cells on collagen-coated cover slips and culture at 37°C in 5% CO_2.

Note: MCF10A cells were cultured in Mammary Epithelial Cell Growth Medium (MEGM) (Lonza Cat. No. CC-3150) with penicillin (final concentration: 100 units/mL) and streptomycin (final concentration: 100 μg/mL) instead of GA-1000 (gentamycin-amphotericin B mix), and they were used in experiments at less than 7 passages. We seeded cells at 6.25×10^3 cells/cm^2 for low density condition. We also used cells cultured at high density (6.25×10^4 cells/cm^2) as a positive control of Hippo signal activation.

4. After seeding, culture cells for 16 h in a 5% CO_2 condition at 37°C (until the cells are attached well to the over slips) and then subject the cells to a cross-linking step. Troubleshooting problem 2 or 3

Note: If you have control samples for clustering of the transmembrane protein (positive or negative control for clustering), you should prepare these samples.

Clustering of the transmembrane proteins by antibody-mediated cross-linking

Timing: 2 h

For cross-linking, selection of the antibody is the most critical point.

Note: Recognition of the extracellular domain is required for cross-linking.

Note: Decrease nonspecific signals as much as possible. Before using the antibody, check the specificity of antibody by immunofluorescence staining or immunoblotting (Knockdown or knockout of your targeted-protein will give you information on the specificity of your primary antibodies.).

Note: Consider the appropriate concentration, incubation time, incubation temperature and solution for antibody dilution.

Note: When you performed a PLA after antibody-mediated cross-linking, you must use antibodies (both for primary and secondary antibodies) made by animal species that are different from those used for the PLA reaction. (e.g., in this protocol, mouse and rabbit primary antibody for the PLA, rat antibody (primary) and goat antibody (secondary) for cross-linking).

Note: An overview is shown in Figures 3A–3C.

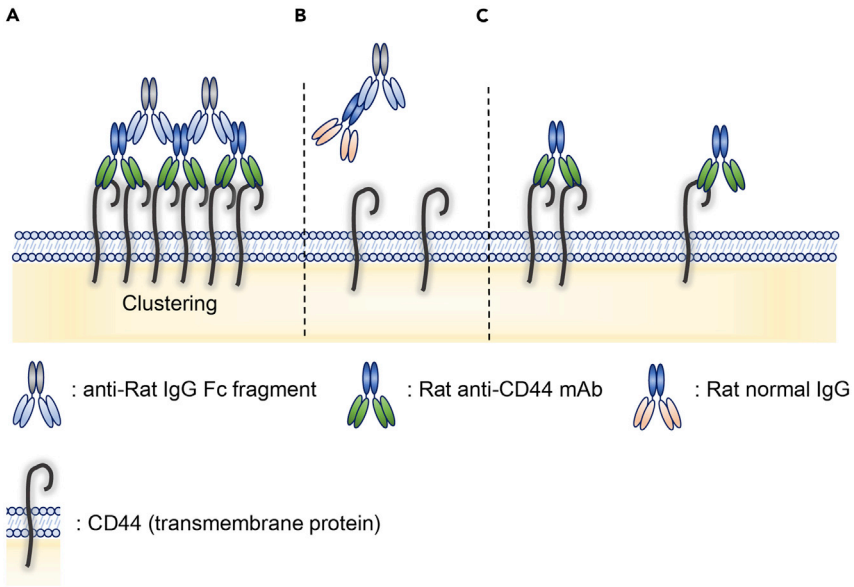

Figure 3. Overview of the antibody-mediated CD44 clustering

(A) CD44 clustering by cross-linking of the anti-CD44 antibody.

(B) Negative control of cross-linking.

(C) Without cross-linking (control for ignoring the effect of the primary antibody).

5. Prepare the primary reaction solution (medium containing the primary antibody).
   a. Add the primary antibody to the culture medium (final concentration: usually 0.5–20 μg/mL, please optimize the proper concentration). Troubleshooting problem 4

   Note: In this protocol, we added a rat monoclonal anti-human CD44 antibody (RRID: AB_467248) or normal rat IgG (as a negative control of cross-linking, RRID: AB_737202) to MEGM with penicillin and streptomycin (The final concentration of the primary antibody was 10 μg/mL.).

   Alternatives: Anti-hCD44s pan specific antibody (mouse monoclonal; clone: 2C5, #BBA10, R&D) and anti-mouse IgG-Fc fragment antibody (goat polyclonal; #A90-131A, Bethyl) can also be used for clustering of CD44.

6. Incubate the cells for 90 min at 37°C in 5% CO_2 with 1 mL of the primary reaction solution.
7. Wash the cells with a culture medium or PBS three times.
8. Prepare the secondary reaction solution (medium containing the secondary antibody).
   a. Add the secondary antibody into the culture medium (final concentration: 1–10 μg/mL, please optimize the concentration). Troubleshooting problem 4

   Note: In this protocol, we added a goat anti-rat IgG-Fc (RRID: AB_228348) to MEGM with penicillin and streptomycin (The final concentration of a secondary antibody was 1 μg/mL.).

9. Incubate the cells for 60 min at 37°C in 5% CO_2 with 1 mL of the secondary reaction solution. Then subject the cross-linked cells to an immunofluorescence staining (move to step 10) or a PLA (move to step 30). (If you prepare two cover slips for each sample, you can do both of PLA and IF in parallel.)

   Note: To confirm the effect of the primary antibody, prepare an additional control sample (primary antibody (+)/secondary antibody (-)). (See Figure 3C).

Immunofluorescence staining (IF)

Timing: 6 h – 18h

Note: To test whether the clustering of a targeted membrane protein is successful or not, you should check the localization of the targeted protein or examine changes in the downstream signals by appropriate experiments before conducting the PLA (In this protocol, confirmation of the CD44 or YAP localization by immunofluorescence staining (Figure 5).

Note: Please optimize the conditions (concentration of the antibodies, incubation time, medium and temperature) for your experiments. To decrease non-specific PLA signals, optimization of the staining condition of your antibodies should be performed by immunofluorescence staining before conducting the PLA.

⚠ CRITICAL: To decrease nonspecific signals or background, avoid drying the cover slips.

10. Rinse the cells with 1 mL of PBS containing 1 mM $MgCl_2$ at room temperature (23°C–26°C).
11. Add 1 mL of PBS containing 1 mM $MgCl_2$ to a new 12-well plate and then transfer the cover slips into the new 12-well plate.
12. Wash the cells with 1 mL of PBS containing 1 mM $MgCl_2$ for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
13. Fix the cells with 1 mL of 4% PFA/PBS for 15 min at room temperature (23°C–26°C).
14. Wash the cells with 1 mL of PBS for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
15. Permeabilize the cells with 1 mL of 0.25% TritonX-100/PBS for 10 min at room temperature (23°C–26°C).
16. Wash the cells with 1 mL of PBS for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
17. Perform blocking using 1 mL of proper blocking solution (this protocol used blocking solution which was described in "materials and equipment") for 60 min at room temperature (23°C–26°C).
18. Wash the cells with PBS containing 0.1% tween 20 for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
19. Incubate the cells overnight (12–16 h) at 4°C or for 60 min at room temperature (23°C–26°C) with a primary antibody (you could speed up the reaction of the primary antibody by incubating at 37°C).
    a. Dilute the primary antibody in PBS or blocking solution (usually 1:100–1:1000, concentration should be optimized).
    b. To make a humidified chamber, place a parafilm and wet paper on to 10 cm dish (Figure 4A).
    c. Tap off the PBS containing 0.1% tween 20 and transfer the cover slips into the humidified chamber (Figure 4B).
    d. Drop 50 μL of the antibody solution onto the cover slip (Figure 4C).

Note: In our experiments, to confirm CD44 clustering and re-localization of YAP, we used a mouse anti-CD44 antibody (CST, 8E2; 1:500 in PBS) and a rabbit anti-YAP antibody (CST, D8H1X; 1:500 in PBS), respectively.

20. Transfer the cover slips into a 12-well plate and wash the cells with 1 mL of PBS containing 0.1% tween 20 for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
21. Prepare a secondary antibody solution.
    a. Dilute a fluorophore-conjugated secondary antibody (please optimize the proper concentration) in PBS or blocking solution.

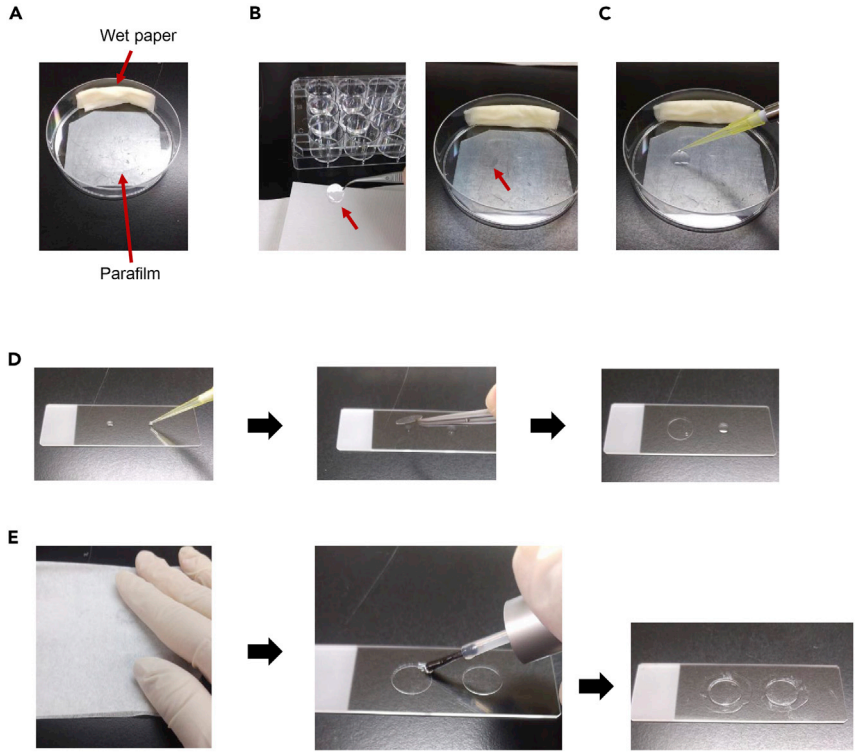

Figure 4. How to handle cover slips

(A) To make the humidified chamber, cut the parafilm to an appropriate size and moisten the paper (e.g., KimWipes) with 18.2 Ω MilliQ sterilized H_2O. Then put the parafilm and the wet paper onto a dish.

(B) Tap off the PBS (*left* panel) and transfer the cover slips into a humidified chamber (*right* panel). Red arrows indicate the cover slip.

(C) Drop 50 μL of the antibody solution onto the cover slip.

(D) Drop 5–10 μL of mount medium onto the slide glass (*left* panel). Tap off PBS (as in Figure 3B) and then transfer the cover slips upside down onto the slide glass (*middle* and *right* panels).

(E) Gently wipe the extra amount of medium (*left* panel) and then seal the edges of the cover slips using nail polish (*middle* and *right* panels). (Be sure the nail polish is cured in the dark before imaging.)

Note: Choose an appropriate fluorophore-conjugated secondary antibody for your experiment. In our case, we used an Alexa-Fluor 488 mouse anti-IgG for CD44 (1:1000) and an Alexa-Fluor 488 rabbit anti-IgG for YAP (1:1000).

22. Incubate the cells for 60 min with a secondary antibody solution at room temperature (23°C–26°C) in the dark.
    a. Tap off the PBS containing 0.1% tween 20.
    b. Transfer the cover slips into the humidified chamber and drop 50 μL of the secondary antibody solution onto the cover slip.
23. Wash the cells with 1 mL of PBS containing 0.1% tween 20 for 10 min by gently shaking at room temperature (23°C–26°C) in the dark.
24. Incubate the cells for 15 min with DAPI solution at room temperature (23°C–26°C) in the dark.
    a. Dilute the DAPI in PBS (1:1000).
    b. Tap off the PBS containing 0.1% tween 20.
    c. Transfer the cover slips into the humidified chamber and drop 50 μL of the DAPI solution onto the cover slip.
25. Wash the cells with 1 mL of PBS containing 0.1% tween 20 for 10 min by gently shaking at room temperature (23°C–26°C) in the dark.

26. Wash the cells with 1 mL of PBS for 10 min by gently shaking at room temperature (23°C–26°C) in the dark.
27. Drop 5–10 μL of 90% glycerol/PBS onto the slide glass (Figure 4D *left* panel).
28. Tap off the PBS and transfer the cover slips upside down onto the slide glass (Figure 4D *middle* and *right* panels).

Note: Avoid the presence of air bubbles.

29. Wipe the extra amount of medium (Figure 4E *left* panel), seal the edges of the cover slips using nail polish and then move to step 56 to take images (Be sure the nail polish is cured before imaging to avoid damage to the objective.) (Figure 4E *middle* and *right* panels).

Pause point: You can store the slides at 4°C in the dark for several days.

Proximity ligation assay (PLA)

Timing: 24 h

Note: An antibody that recognizes the intracellular domain of the target transmembrane protein is required for detection of the interaction between transmembrane protein and intracellular protein using PLA.

Note: Since the PLA is a highly sensitive assay that detects protein-protein interaction, several negative control experiments should be required (e.g., knockout of targeted protein with PLA and PLA performed without a primary antibody are shown in Rawat et al., 2016.).

Note: This step was referred to in the sigma protocol for Proximity Ligation Assay (Sigma-Aldrich; https://www.sigmaaldrich.com/life-science/cell-biology/antibodies/duolink-and-pla-technology.html) and was optimized for our experiments (Ooki et al., 2019). When you observe inadequate protein-protein interactions, additional optimization of the PLA conditions may be required (see Troubleshooting problem 5).

Note: Bring the wash buffers A and B to room temperature (23°C–26°C) before use.

⚠ CRITICAL: To decrease nonspecific signals or background, avoid drying the cover slips.

⚠ CRITICAL: To avoid contamination of nuclease, use autoclaved PBS and ddH_2O (Contamination of nuclease interferes with ligation and amplification.).

30. Rinse the cells with 1 mL of PBS containing 1 mM $MgCl_2$ at room temperature (23°C–26°C).
31. Add 1 mL of PBS containing 1 mM $MgCl_2$ to a new 12-well plate and then transfer the cover slips into the new 12-well plate.
32. Wash the cells with PBS containing 1 mM $MgCl_2$ for 5 min ×x2 by gently shaking at room temperature (23°C–26°C).
33. Fix the cells with 1 mL of 4% PFA/PBS for 15 min at room temperature (23°C–26°C).
34. Wash the cells with 1 mL of PBS for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
35. Permeabilize the cells with 1 mL of 0.25% TritonX-100/PBS for 10 min at room temperature (23°C–26°C).
36. Wash the cells with 1 mL of PBS for 5 min ×2 by gently shaking at room temperature (23°C–26°C).

37. Perform blocking using 1 mL of proper blocking solution (this protocol used blocking solution which was described in "materials and equipment") for 60 min at room temperature (23°C–26°C).

Alternatives: You can use Duolink Blocking Solution. Duolink PLA Probes kit includes Duolink Blocking Solution and Duolink Antibody Diluent.

38. Wash the cells with PBS containing 0.1% tween 20 for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
39. Incubate the cells overnight (12–16 h) at 4°C or for 60 min at room temperature (23°C–26°C) with a primary antibody.
    a. Dilute the primary antibody (usually 1:100–1:1000, concentration should be optimized). If two primary antibodies are used, dilute them in the same diluent.
    b. Tap off the PBS containing 0.1% tween 20 and transfer the cover slips into the humidified chamber.
    c. Drop 50 μL of antibody solution onto the cover slip.

Note: In our PLA experiment, to observe the interaction between CD44 and PAR1b, we used a mouse anti-CD44 antibody (CST, 8E2; 1:500) and rabbit anti-MARK2/PAR1b antibody (Abcam; 1:500). The antibodies were diluted in PBS.

40. Transfer the cover slips into a 12-well plate and then wash the cells with 1 mL PBS containing 0.1% tween 20 for 5 min ×2 by gently shaking at room temperature (23°C–26°C).
41. Incubate the cells for 15 min with DAPI solution at room temperature (23°C–26°C) in the dark.
    a. Dilute DAPI in PBS (1:1000).
    b. Tap off the PBS containing 0.1% tween 20.
    c. Transfer the cover slips into the humidified chamber and drop 50 μL of DAPI solution onto the cover slip.

Optional: If you use Duolink In Situ Mounting Medium with DAPI, skip the DAPI staining steps (40 and 41).

Note: Choose an appropriate PLA probe. (https://www.sigmaaldrich.com/life-science/molecular-biology/molecular-biology-products.html?TablePage=112232138). In our experiment, we used an anti-mouse MINUS (Duo92004) and an anti-rabbit PLUS (Duo92002).

42. During step 41, prepare the PLA probe solution (for a 40 μL reaction, take 8 μL of PLA probe MINUS, 8 μL of PLA probe PLUS and 24 μL of the antibody diluent).
    a. Dilute the PLUS and MINUS PLA probes 1:5 in the Duolink Antibody Diluent or an appropriate diluent for your antibody combinations. Allow the PLA probe mixture to sit for 20 min at room temperature (23°C–26°C).

Note: Recommended total reaction volume: 40 μL per 1 cm^2 cover slip.

43. Transfer the cover slips into a 12-well plate and then wash the cells with 1 mL of PBS containing 0.1% tween 20 for 5 min by gently shaking at room temperature (23°C–26°C).
44. Incubate the cells for 60 min with the PLA probe solution (prepared step 42) at 37°C.
    a. Tap off the PBS containing 0.1% tween 20.
    b. Transfer the cover slips into the humidified chamber and drop 40 μL of PLA probe solution onto the cover slip.
45. Transfer the cover slips into a 12-well plate and then wash the cells with 1 mL of wash buffer A for 5 min ×2 by gently shaking at room temperature (23°C–26°C).

Note: Choose appropriate PLA detection reagents. (https://www.sigmaaldrich.com/life-science/molecular-biology/molecular-biology-products.html?TablePage=112232138). Each kit includes 5× Ligation stock, Ligase (1 U/μL), 5× Amplification stock and polymerase (10 U/μL). In our experiment, we used Duolink In Situ Detection Reagents Orange (Duo92007).

46. During step 45, prepare the ligation-ligase solution (if you make a 40 μL ligation-ligase solution, take 8 μL of 5× Ligation stock, 1 μL of ligase and 31 μL of ddH_2O).
    a. Dilute the 5× ligation stock 1:5 in ddH_2O.
    b. Add ligase (stock concentration: 1 U/μL).
47. Incubate the cells for 30 min with the ligation-ligase solution at 37°C.
    a. Tap off wash buffer A.
    b. Transfer the cover slips into the humidified chamber and drop 40 μL of the ligation-ligase solution (prepared step 46) onto the cover slip.
48. Transfer the cover slips into a 12-well plate and then wash the cells with 1 mL of wash buffer A for 5 min by gently shaking at room temperature (23°C–26°C).
49. During step 48, prepare the amplification-polymerase solution.
    a. Dilute the 5× Amplification stock 1:5 in ddH_2O.
    b. Add polymerase (stock concentration: 10 U/μL).

Note: if you make a 40 μL amplification-polymerase solution, use 8 μL of 5× Amplification stock, 0.5 μL polymerase and 31.5 μL of ddH_2O.

50. Incubate the cells for 100 min with the amplification-polymerase solution at 37°C in the dark.
    a. Tap off wash buffer A.
    b. Transfer the cover slips into the humidified chamber and drop 40 μL of the amplification-polymerase solution (prepared step 49) onto the cover slip.
51. Transfer the cover slips into a 12-well plate and then wash the cells with 1 mL of wash buffer B for 5 min ×2 by gently shaking at room temperature (23°C–26°C) in the dark.
52. Rinse the cells with 1 mL of diluted wash buffer B (1:100).
    a. Dilute wash buffer B 1:100 in ddH_2O.

Note: To make 50 mL of wash buffer B (1:100), add 500 μL of wash buffer B to 49.5 mL of ddH_2O).

53. Drop 5–10 μL of 90% glycerol/PBS onto the slide glass.

Alternatives: You can use Duolink In Situ Mounting Medium with DAPI or another mounting solution.

54. Tap off diluted wash buffer B (1:100) and let the cover slips dry at room temperature (23°C–26°C) in the dark. Then transfer the cover slips upside down onto the slide glass.

Note: Avoid the presence of air bubbles.

55. Wipe the extra amount of medium and seal the edges of the cover slips using nail polish (Be sure the nail polish is cured in the dark before imaging.).

Pause point: You can store the slides without decreasing the signal at 4°C in the dark for several days.

Taking images by a confocal laser microscope

Timing: 1 day

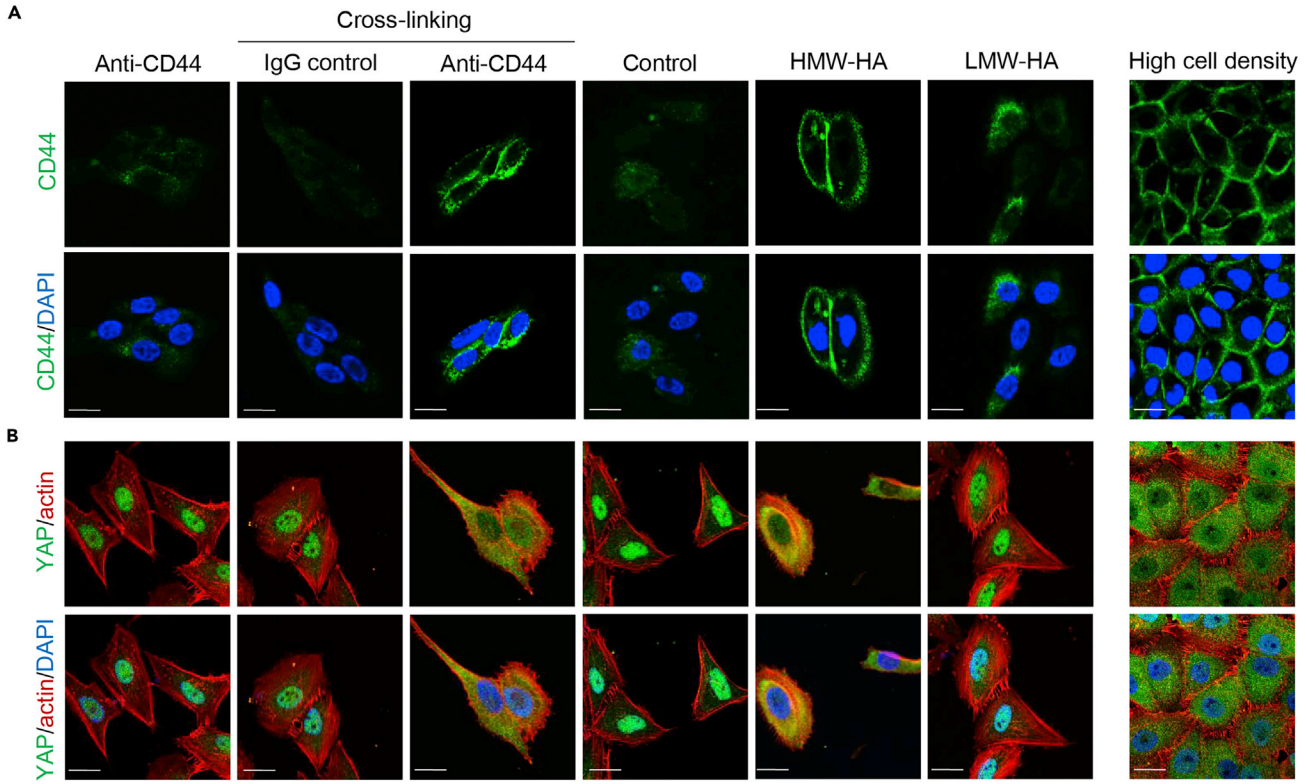

Figure 5. Cross-linking of the anti-CD44 antibody mimicked HMW-HA treatment and activated Hippo signal

For CD44 clustering, we prepared two control samples. Specifically, cells were treated with MEGM containing HMW-HA (1000 ng/mL) and with medium containing LMW-HA (1000 ng/mL) for 20 min as a positive and a negative control, respectively (Yang et al., 2012). As a positive control of activation of the Hippo signal, MCF10A cells were seeded in a high cell density condition (6.25×10^4 cells/cm^2) and then cultured for 16 h. Cultured cells were fixed with 4% PFA and then used for IF staining (Figure. 5) or a PLA (Figure 6).

(A) Cells were cultured in the indicated conditions and stained with the anti-CD44 antibody (green). Nuclei were stained by DAPI (blue). Cross-linking of the anti-CD44 antibody increased clustering of CD44 as well as in HMW-HA-treated cells (positive control of CD44 clustering). On the other hand, LMW-HA treatment (negative control of CD44 clustering) failed to induce CD44 clustering. Clustering of CD44 was also increased in cells cultured in a high cell density condition. Scale bars indicate 20 μm.

(B) To confirm whether the Hippo signal was activated or not by cross-linking of the anti-CD44 antibody, we observed localization of YAP (effector molecule of the Hippo signal). Cultured cells were stained with an anti-YAP antibody (green) and an anti-actin antibody (red). Nuclei were stained by DAPI (blue). Cross-linking of the anti-CD44 antibody activated the Hippo signal (induction of cytoplasmic accumulation of YAP) as well as a high density condition (positive control of activation of the Hippo signal) and HMW-HA treatment. In terms of activation of the Hippo signal via CD44 clustering, cross-linking of the anti-CD44 antibody mimicked HMW-HA treatment. Scale bars indicate 20 μm. (Figures reprinted with permission from Ooki et al., 2019.)

56. Set the imaging parameters of the confocal laser microscope (e.g., Olympus, FLUOVIEW FV1200 confocal microscope systems).
    a. Equipped with excitation and emission filters that are compatible with the fluorophore and nuclear stain.
57. Take images (Results of IF, see Figures 5A and 5B. Results of PLA, see Figures 6A and 6B). Troubleshooting problem 5

Optional: To visualize cell morphology, you can take DIC (differential interference contrast) images or you can stain with F-actin using fluorophore-conjugated phalloidin (Figure 6B). Alternatively, you can choose a bright-field PLA detection reagent (DUO92012) instead of the fluorophore-conjugated PLA detection reagent. If you choose the bright-field PLA detection reagent, hematoxylin staining is required for visualization of the nucleus instead of DAPI staining (Please see the protocol for bright-field PLA detection

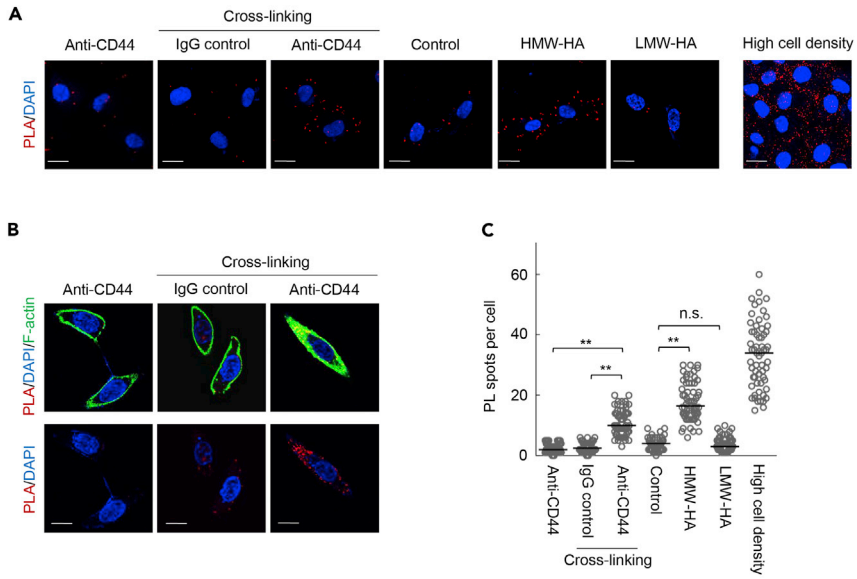

Figure 6. Antibody-mediated CD44 clustering increased interaction between CD44 and PAR1b

(A) Cells were cultured in the indicated conditions. The interaction between CD44 and PAR1b in cultured cells was analyzed by PLA (red puncta). Nuclei were stained by DAPI (blue). Cross-linking of the anti-CD44 antibody increased CD44-PAR1b interaction as well as positive control samples (HMW-HA treatment and cultured in a high density condition) but not LMW-HA treatment (negative control). Scale bars indicate 20 μm.

(B) F-actin was co-stained with phalloidin (green). Alexa fluorophore-488-conjugated phalloidin was diluted (1:50) in PBS containing the primary antibodies (described as step 39). CD44-PAR1b interaction was increased at the peripheral membrane (red) in a cross-linking sample (*right* panel). Scale bars indicate 20 μm.

(C) Dots indicate the number of PL spots per cell. Bars indicate median. n = 60 cells. **p < 0.01 (Mann-Whitney U test). PL spots were counted using ImageJ software and analyzed by PRISM GraphPad software. (Figures reprinted with permission from Ooki et al., 2019.)

reagent: https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-brightfield-user-manual.html.).

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 1 day

58. Open the images using image J software (File -> Open -> select your image).
59. Set the image J software.
    a. Select "Multi-points".
    b. Analyze -> Tools -> ROI manager.
60. Count the PL spots per cell manually (in our research: n = 60 cells).
    a. Count the PL spots using "Multi-points".
    b. Add ROI (ROI manager -> Add).
    c. Save the ROIs (ROI manager -> More -> Save).

Alternatives: When you have a lot of PL spots (you may not be able to separate each PL spot), you can measure the signal intensity of the PL spots per cell instead of counting the number of PL spots per cell.

Alternatives: An automatic method is shown in Hegazy et al., 2020.

61. Perform statistical analysis using GraphPad Prism (Figure 6C).

a. Input your data according to the instructions.
b. Select the appropriate statistical method (e.g., Mann-Whitney U test).
c. Choose the type of graph.
d. Save the graph.

EXPECTED OUTCOMES

Extracellular ligand-mediated clustering (or multimerization) of transmembrane proteins could activate or inactivate intracellular signaling pathways that are important for physiological and/or pathological cell functions. However, in many cases, physiological ligands or mechanisms that mediate membrane clustering are still unknown. This protocol will allow for the induction of artificial clustering of transmembrane proteins without known ligands by antibody-mediated cross-linking and may be used for investigating novel functions of the transmembrane proteins. In addition, interaction between transmembrane proteins and intracellular proteins can be visualized more by the PLA than by co-immunoprecipitation. Generally, transmembrane proteins are more difficult than cytoplasmic proteins to extract by weak lysis buffers, which are used to preserve protein-protein interaction, and stoichiometry of the protein-protein interaction is thereby usually low as determined by co-immunoprecipitation experiments. Since cell lysis is not required for the PLA, this procedure avoids the problem of destroying the interaction between the two proteins. This protocol may be used as a tool to investigate novel interactions between transmembrane and cytoplasmic proteins that are induced by clustering/multimerization of the transmembrane protein by extracellular ligands.

LIMITATIONS

This protocol has been designed for imaging the interaction between the CD44 cytoplasmic domain and an intracellular protein(s) and for activation of the Hippo signal by CD44 clustering in mammary epithelial cells, especially MCF10A cells. If you are planning to conduct antibody-mediated cross-linking and to observe an interaction between a transmembrane protein and an intracellular protein(s) by clustering of the transmembrane protein, optimization of the antibodies for cross-linking and for PLA is required (See troubleshooting problems 4 and 5.). Furthermore, since the quality of the PLA is dependent on the distance of antibodies, you may not be able to observe PL spots even if a protein-protein interaction is indeed generated (see troubleshooting problem 5). Thus, to maintain the quality of your experiments, you should prepare several control samples (For example, knockdown or knockout of your targeted protein will give you information on the specificity of your primary antibodies, and omitting the primary antibodies will give you a hint of how the PLA probe background looks like in your experimental system.). Furthermore, if possible, you should check alteration of the assumed downstream events (e.g., localization of YAP in our work) or biological phenotypes upon clustering of the transmembrane protein of interest.

TROUBLESHOOTING

Problem 1

The cover slips are cracked when burning off ethanol using a gas burner (step 4 in Preparation of collagen-coated cover slips).

Potential solution

Very little ethanol must remain when flaming cover slips.

Bake the cover slips for 1 sec (less than 1 s).

Alternatively, after step 3 in "Preparation of collagen-coated cover slips", dry up the cover slips at room temperature (23°C–26°C) and then you can sterilize the cover slips by UV irradiation instead of baking.

Problem 2

Cells are not attached to the cover slips (steps 6–9 in Preparation of collagen-coated cover slips, step 4 in step-by-step method details).

Potential solution

Collagen is expired. Cover slips are not sufficiently coated. Repeat steps 6–8 (prepare collagen-coated cover slips) at least 5 times. Alternatively, try to use PLL-coated cover slips or commercially available chamber slides.

If you use chamber slides (e.g., 8-well Chamber Slide w/removable wells, Thermo Fisher Scientific, Cat #177402), you will need a larger volume of antibody-diluted solution or PLA solution (e.g., 200–300 μL per well).

Problem 3

Cover slips float in the culture medium (steps 6–9 in Preparation of collagen-coated cover slips, step 4 in step-by-step method details).

Potential solution

Coating is insufficient or air bubbles are present between plate and cover slips. Repeat steps 6–8 (prepare collagen-coated cover slips) at least 5 times and avoid the presence of air bubbles.

When you add or change the medium, gently add or gently aspirate the medium.

Avoid strongly shaking the plates/dishes and vibration.

Problem 4

Clustering does not occur (steps 5, 6, 8, and 9 in step-by-step method details).

Potential solution

Confirm the target site of the antibody (The primary antibody must target the extracellular domain.) or subtype of immunoglobulin of the primary antibody (The specificity and efficiency of the secondary antibody are important for cross-linking.). Consider the cell line and the reaction conditions (time, antibody concentration, medium and temperature). Recommended ranges of concentrations: primary: 0.5–20 μg/mL, secondary: 1–10 μg/mL. (e.g., CD44: Fujii et al., 2003; L-selectin: Turutin et al., 2003).

Problem 5

There are no PL spots, there is a weak signal or there are too many background signals (steps 39, 44, 47, and 50 in step-by-step method details).

Potential solution

The reaction volume is insufficient (recommended total reaction volume: 40 μL per 1 cm^2 cover slip).

Optimization of the reaction conditions (specificity of the antibodies, concentration of the antibodies, reaction time and temperature) is required. However, other than these conditions, there are many reasons for the lack of PLA signal. Please refer the PLA Troubleshooting Guide (https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-troubleshooting-guide.html). This web site includes more detailed information on the troubleshooting of PLA.

You can search for the primary antibodies that are optimized for the PLA (https://www.sigmaaldrich.com/life-science/cell-biology/antibodies/duolink-and-pla-technology/pla-antibodies.html).

PLA reagents are deactivated. The reagents should be stored in recommended preservation conditions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masanori Hatakeyama (mhata@m.u-tokyo.ac.jp).

Materials availability

This study did not generate any unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

M.H. supervised the project. T.O. conducted the experiments and data analysis. T.O. wrote the manuscript. M.H. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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