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.
Protocol for visualizing conditional interaction between transmembrane and cytoplasmic proteins

Takuya Ooki¹,² and Masanori Hatakeyama¹,³,*

¹Division of Microbiology, Graduate School of Medicine, the University of Tokyo, Tokyo 113-0033, Japan
²Technical contact
³Lead contact
*Correspondence: mhata@m.u-tokyo.ac.jp
https://doi.org/10.1016/j.xpro.2021.100430

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...
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 H₂O 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 ddH₂O by stirring overnight (12–16 h), (ddH₂O 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**
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 (DBH1X) 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 #A-12379 |
| **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 |
| KH2PO4 | Wako | Cat #169-04245 |
| MgCl2 | Wako | Cat #135-00165 |
| NaCl | Wako | Cat #195-01663 |
| Na2HPO4 | Wako | Cat #197-02865 |
| NaN3 | 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)
MATERIALS AND EQUIPMENT

**Note:** Prepare all solutions using 18.2 Ω MilliQ sterilized H₂O.

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

### Materials

| Material                  | Recipes                                                                 | Preservation conditions                                    |
|---------------------------|-------------------------------------------------------------------------|------------------------------------------------------------|
| 1x PBS                    | Add 50 mL, 20x PBS in 950 mL ddH₂O and then perform autoclave (121°C, 20 min). | Room temperature (23°C–26°C, approximately 1 month).       |
| 1x PBS/MgCl₂              | Add 50 μL 1 M MgCl₂ in 50 mL 1x PBS.                                    | Room temperature (23°C–26°C, approximately 1 month).       |
| 1x PBS/0.1% Tween 20      | Add 50 μL Tween 20 in 50 mL 1x PBS.                                     | Room temperature (23°C–26°C, approximately 1 month).       |
| 1x PBS/0.25% triton X-100 | Add 125 μL Triton X-100 in 50 mL 1x PBS.                                | Room temperature (23°C–26°C, approximately 1 month).       |
| 90% Glycerol/PBS          | Add 5 mL 1x 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 ddH₂O 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 20x PBS in 5.5 mL ddH₂O.                     | Prepare before using.                                     |
| 5% NaN₃                   | Add 0.5 g NaN₃ in 10 mL ddH₂O                                            | Room temperature (23°C–26°C, approximately 3 months).     |
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 $1.0 \times 10^4$ 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% CO₂.

   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 × 10³ cells/cm² for low density condition. We also used cells cultured at high density (6.25 × 10⁴ cells/cm²) as a positive control of Hippo signal activation.

4. After seeding, culture cells for 16 h in a 5% CO₂ condition at 37°C (until the cells are attached well to the cover 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.
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% CO₂ 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% CO₂ 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 MgCl₂ at room temperature (23°C–26°C).
11. Add 1 mL of PBS containing 1 mM MgCl₂ 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 MgCl₂ 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.
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 ddH₂O (Contamination of nuclease interferes with ligation and amplification.).

30. Rinse the cells with 1 mL of PBS containing 1 mM MgCl₂ at room temperature (23°C–26°C).

31. Add 1 mL of PBS containing 1 mM MgCl₂ 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 MgCl₂ for 5 min ×2 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](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 cm² 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=11232138). 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 ddH₂O).
   a. Dilute the 5× ligation stock 1:5 in ddH₂O.
   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 ddH₂O.
   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 ddH₂O.

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 ddH₂O.

Note: To make 50 mL of wash buffer B (1:100), add 500 µL of wash buffer B to 49.5 mL of ddH₂O.

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
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...
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 cm² 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 to 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.

**ACKNOWLEDGMENTS**

This work was supported by Grant-in-Aids for Scientific Research on Innovative Area (16H06373; 16K15273) from Japan Society for the Promotion of Science (JSPS), Japan (to M.H.) and by Project for Cancer Research and Therapeutic Evolution (P-CREATE) (160200000291) from Japan Agency for Medical Research and Development (AMED), Japan (to M.H.).

**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.

**REFERENCES**

Chakravarty, D., Pedraza, A.M., Cotari, J., Liu, A.H., Punko, D., Kokroo, A., Huse, J.T., Altan-Bonnet, G., and Brennan, C.W. (2017). EGFR and PDGFRA co-expression and heterodimerization in glioblastoma tumor spheres lines. Sci. Rep. 7, 9043.

Damiano, V., Spessotto, P., Vanin, G., Perin, T., Maestro, R., and Santarosa, M. (2020). The autophagy machinery contributes to E-cadherin turnover in breast cancer. Front. Cell Dev. Biol. 8, 545.

Fuji, Y., Fuji, K., Nakano, K., and Tanaka, Y. (2003). Crosslinking of CD44 on human osteoblastic cells upregulates ICAM-1 and VCAM-1. FEBS Lett. 539, 45–50.

Hegazy, M., Cohen-Barak, E., Koetsier, J.L., Najar, N.A., Arvanitis, C., Sprecher, E., Green, K.J., and Godsel, L.M. (2020). Proximity ligation assay for detecting protein-protein interactions and protein modifications in cells and tissues in situ. Curr. Protoc. Cell Biol. 89, e115.

Ibuka, S., Matsumoto, S., Fuji, S., and Kikuchi, A. (2015). The P2Y6 receptor promotes Wnt3a- and EGF-induced epithelial tubular formation by IEC6 cells by binding to integrins. J. Cell Sci. 128, 2156–2168.

Ooki, T., Murata-Kamiya, N., Takahashi-Kanemitsu, A., Wu, W., and Hatakeyama, M. (2019). High-molecular-weight hyaluronan is a hippo pathway ligand directing cell density-dependent growth inhibition via PAR1b. Dev. Cell 49, 590–604.

Rawat, S.J., Araiza-Olivera, D., Arias-Romero, L.E., Villamar-Cruz, O., Prudnikova, T.Y., Roder, H., and Cheroff, J. (2016). H-ras inhibits the hippo pathway by promoting Mst1/Mst2 heterodimerization. Curr. Biol. 26, 1556–1563.

Toki, M.I., Carvajal-Hausdorf, D.E., Altan, M., McLaughlin, J., Henick, B., Schaper, K.A., Syrigos, K.N., and Rimm, D.L. (2016). EGFR-GRB2 protein colocalization is a prognostic factor unrelated to overall EGFR expression or EGFR mutation in lung adenocarcinoma. J. Thorac. Oncol. 11, 1901–1911.

Tsutsumi, R., Masoudi, M., Takahashi, A., Fuji, Y., Hayashi, T., Kikuchi, I., Satou, Y., Taira, M., and Hatakeyama, M. (2013). YAP and TAZ, Hippo signaling targets, act as a rheostat for nuclear SHP2 function. Dev. Cell 26, 658–665.

Turutin, D.V., Kubareva, E.A., Pushkareva, M.A., Ulrich, V., and Sud’ina, G.F. (2003). Activation of NF-kappa B transcription factor in human neutrophils by sulphatides and L-selectin cross-linking. FEBS Lett. 536, 241–245.

Yang, C., Cao, M., Liu, H., He, Y., Xu, J., Du, Y., Liu, Y., Wang, W., Cui, L., Hu, J., and Gao, F. (2012). The high and low molecular weight forms of hyaluronan have distinct effects on CD44 clustering. J. Biol. Chem. 287, 43094–43107.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., and et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. 21, 2747–2761.