

Biochemical visualization of cell surface molecular clustering in living cells

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Many plasma membrane-resident molecules cluster with other molecules to collaborate in a variety of biological events. We herein report a sensitive and simple method to identify components of cell surface molecular clusters in living cells. This method includes a recently established reaction, called the enzyme-mediated activation of radical source (EMARS), to label molecules within a limited distance (≈ 200 – 300 nm) from the probed molecule on which HRP is set. Because the size of this active area is close to that of the reported membrane clusters, it is suggested that the labeled molecules cluster with the probed molecule in the same membrane domain. A combination of the EMARS reaction and antibody array analysis demonstrated that many kinds of receptor tyrosine kinases (RTKs) formed clusters with $\beta 1$ integrin in HeLa S3 cells. A similar antibody array analysis after the EMARS reaction with three HRP-labeled antibodies against growth factor receptors showed the patterns of biotinylated RTKs to be substantially different from each other. These results suggest that different types of cell surface molecular clusters can thus be distinguished using the EMARS reaction. Therefore, the present "biochemical visualization" method is expected to be a powerful tool to elucidate molecular clustering on the cell surface of living cells in various contexts.

ganglioside | integrin | microdomain | radicals

The biological events through the plasma membranes, such as signal transmission, cell adhesion, and trafficking require the interactions between receptors, adhesion molecules, and signaling proteins. Recent studies have accumulated a line of evidence in which the functional components are distributed nonrandomly on the plasma membrane and exist as clusters in the nanometer-scale domains (1). These membrane domains are formed by the clustering of particular membrane lipids and proteins and display a dynamic property of association and dissociation between interacting molecules that occurs continuously (2). It is therefore essential to identify what functional molecules cocluster in the native membrane, and how they collaborate to create their biological output.

Among the many types of membrane domains proposed, the "lipid rafts" that are enriched in cholesterol, sphingolipid, GPI-anchored proteins, and the Src kinase family members have so far been most intensively investigated (3–5). It has been assumed that the lipid raft fractions are extracted from the rest of the plasma membrane based on the fact that the membrane domains are resistant to nonionic detergents, whereas the fluid membrane dissolves (6). However, the isolated materials are a mixture of heterogeneous microdomains that could include artificial products extracted during the process of homogenization with detergents. Therefore, it is impossible to identify what molecules cocluster in the same microdomain under the physiological conditions by means of the detergent-resistant membrane fractionation.

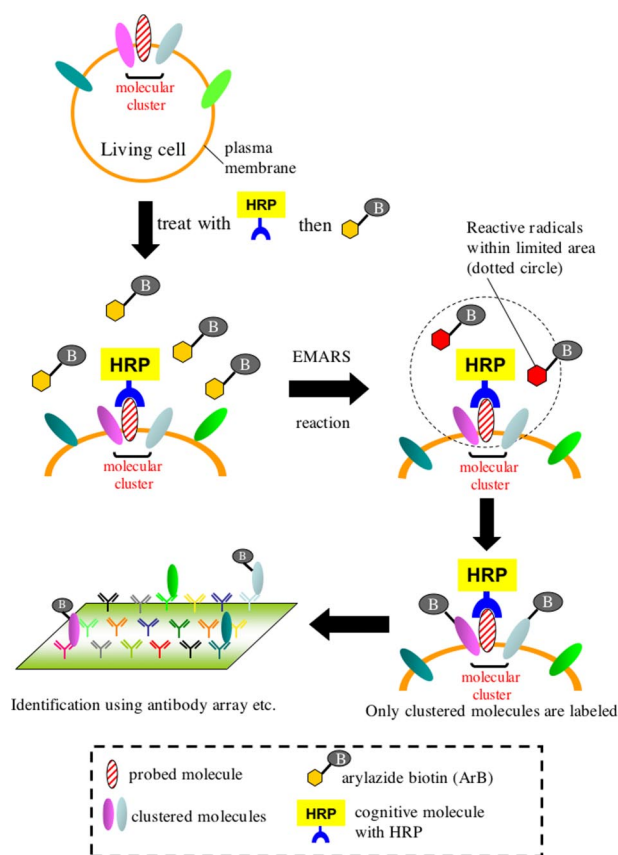


Fig. 1. Schematic diagram of the *in vivo* EMARS analysis. Living cells were treated with HRP-conjugated cognitive molecules, and subsequently treated with aryl azide-biotin. After the EMARS reaction, the membrane proteins were solubilized and the biotinylated molecules were identified by using an antibody array etc.

Until now, four analytical strategies have been developed to analyze molecular clustering on the cell surface. The first is coimmunoprecipitation, which is usually used to show direct interactions between molecules. However, this method is unable

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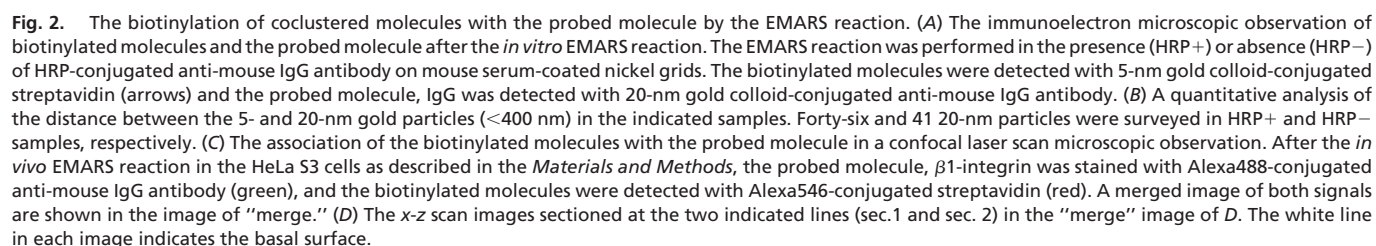
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Experimental evidence suggesting the biological functions of clustered molecules is insufficient until actual coclustering is demonstrated in living cells. We herein report a widely applicable strategy that makes it possible to visualize cell surface molecular clustering under physiological conditions.

Enzyme-Mediated Activation of Radical Sources (EMARS). In the course of an affinity-labeling experiment of biomolecules, we

Molecular Clustering Analysis Using the EMARS Reaction. Once radical species are generated, the formed radicals are supposed to be short-lived and reach within a limited area before decay. In fact, the hydroxy radical worked in the circle area with a half-maximal radius of 1.5 nm in a CALI analysis (21). Singlet oxygen affected within the range of 10–50 nm in a FALI analysis

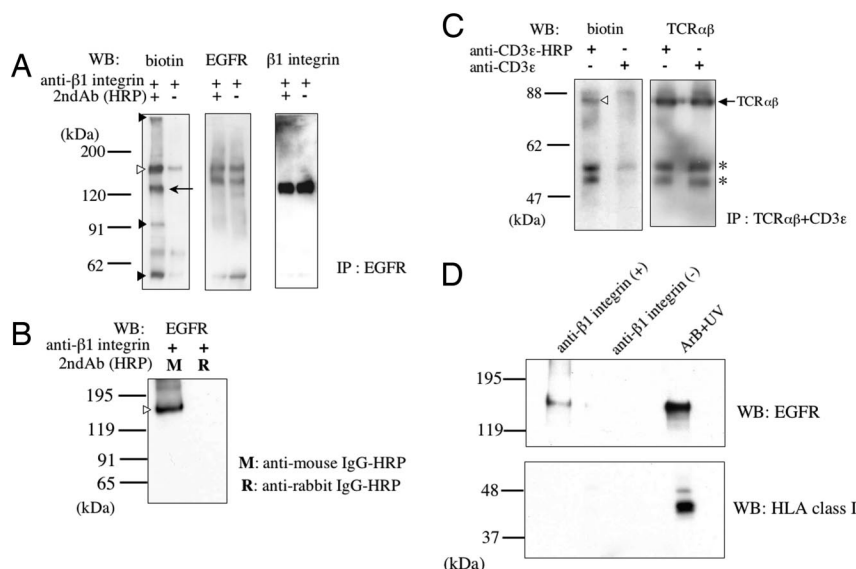


Fig. 3. Detection of known molecular assemblies on the plasma membranes by the EMARS reaction. (A) The biotinylation of EGFR by the EMARS reaction using anti- $\beta 1$ integrin as a probe. HeLa S3 cells were successively subjected to the EMARS reaction using TS2/16 and HRP-conjugated second antibody, immunoprecipitated with an anti-EGFR antibody, 6% gel SDS/PAGE under a reducing condition, blotted to a PVDF membrane, and then stained for biotinylated proteins with an ABC kit (Left). After stripping, the membrane was restained with the indicated antibodies [EGFR (Center) and $\beta 1$ integrin (Right)]. The open triangle (Left) indicates the band of biotinylated EGFR. The closed triangles (Left) indicate the bands of putative clustered molecules of $\beta 1$ integrin. The arrow indicates the band of biotinylated $\beta 1$ integrin. The upper band of EGFR (Center) is the complex-type *N*-glycosylated form (mainly located in the cell surface), and the lower is the high-mannose (hybrid)-type *N*-glycosylated form. Note that only the upper band was biotinylated (Left). (B) The streptavidin precipitation assay of the EMARS products. The EMARS reaction was performed by using TS2/16 mouse monoclonal antibody and HRP-conjugated anti-mouse or anti-rabbit (negative control) IgG, and then applied to streptavidin Sepharose. The precipitated samples were subsequently applied to Western blot analysis by using an anti-EGFR antibody. The open triangle indicates the bands of biotinylated EGFR. (C) The biotinylation of the T cell receptor (TCR $\alpha\beta$) by the EMARS reaction using the HRP-conjugated anti-CD3 ϵ antibody, 500A2, as a probe. The 2C cells expressing clonotypic TCR $\alpha\beta$ were successively subjected to the EMARS reaction using HRP-conjugated anti-CD3 ϵ antibody, immunoprecipitated with a mixture of anti-TCR $\alpha\beta$ and anti-CD3 ϵ monoclonal antibody, 8% gel SDS/PAGE under nonreducing conditions, blotted to a PVDF membrane, and stained for biotinylated proteins with an ABC kit (Left). After stripping, the membrane was restained with the anti-TCR $\alpha\beta$ antibodies (Right). The open triangle indicates the band of biotinylated TCR $\alpha\beta$. Asterisks indicate unknown bands derived from cellular component or used antibody. (D) HLA class I as a nonclustered molecule with integrin. For expression control, all cell-surface molecules were labeled by UV irradiation in the presence of arylazide-biotin as described in *Materials and Methods*. Samples of the EMARS reaction with [anti- $\beta 1$ integrin (+)] or without [anti- $\beta 1$ integrin (–)] TS2/16 antibody and the UV irradiation (ArB+UV) were applied to the streptavidin-Sepharose. The precipitated samples were subjected to Western blot analysis by using anti-EGFR and anti-HLA class I antibodies.

(20, 22, 23). Therefore, we hypothesized that the reactive radicals generated by the EMARS reaction might attack the molecules located within a limited distance from HRP set on a given molecule on the cell surface (probed molecule) and label the coclustered molecules with the probed molecule (Fig. 1).

To address this hypothesis, the range of biotinylation around the probed molecule was assessed by means of immunoelectron microscopy. When the *in vitro* EMARS reaction was carried out using HRP-conjugated anti-mouse IgG antibody on a mouse serum-coated nickel grid, a substantial number of biotinylated molecules (5-nm gold particles) were observed around the probed molecule, IgG (20-nm gold particles) (Fig. 2A, HRP+). In contrast, very few 5-nm particles were seen around the 20-nm particles in the negative control experiment (Fig. 2A, HRP-). A quantitative analysis revealed that 67 5-nm particles were seen within 400 nm of the 20-nm particles in the HRP+ sample when 46 20-nm particles were surveyed (Fig. 2B). However, only three particles were detected within 400 nm of 41 20-nm particles in the HRP- sample. In the HRP+ sample, 76% of the 5-nm particles that existed within 400 nm of the 20-nm particles were within 200 nm, 94% of them were within 300 nm. These results indicate that the molecules located within 200–300 nm around the probed molecule are most likely labeled by the EMARS reaction. The sizes of the membrane domains have been assessed in various living cells using other techniques, including FRET and a single-particle tracking analysis: 4-nm to micrometer scale (1), 325–370 nm (24), 500–700 nm (25), and 600–800 nm (26). Taken

together, the EMARS reaction can therefore be used to identify coclustering molecules in such membrane domains.

To verify this possibility, we tested the issue of whether the EMARS reaction is applicable to living cells. First, the association of the biotinylated molecules with the probed molecule was investigated by means of confocal laser scan microscopy. The HeLa S3 cells were stained with Alexa546-conjugated streptavidin after an anti- $\beta 1$ integrin antibody, TS2/16-dependent EMARS reaction. As a result, the signals of biotinylation were observed on the cell surface, after merging with a portion of $\beta 1$ integrin signals (Fig. 2C). Furthermore, the x - z scanning of these samples in a higher magnification field revealed that biotinylation predominantly occurs at a particular site of the $\beta 1$ integrin assembly, which is located on the basal surface of the cell (Fig. 2D). These results suggest that biotinylated molecules by the EMARS reaction are associated with the probed molecule. Second, the range of biotinylation around the probed molecule was assessed in living cells by means of immunoelectron microscopy (Fig. S3A). As shown in Fig. S3B, the EMARS reaction is most likely to label molecules located within 200–300 nm around the probed molecule in accordance with the findings of the *in vitro* EMARS reaction.

The EMARS Reaction Can Detect Known Molecular Assemblies. To validate the present method biochemically, two well known molecular assemblies on the plasma membranes were subjected to the EMARS reaction. One is the cluster of the epidermal growth factor receptor (EGFR) and integrin in the cell growth

process, and the other is the T cell receptor (TCR) complex in the immune system. EGFR has been reported to associate and cross-talk with an integrin in a signal transduction system (27–29). Therefore, we examined whether EGFR could be labeled or not in our method when an anti- $\beta 1$ integrin antibody was used as a probe. The cell membranes were solubilized after the EMARS reaction, and the lysate was immunoprecipitated with anti-EGFR antibody and then subjected to Western blot analysis to detect biotinylation (Fig. 3*A*). However, the biotinylated proteins that were isolated from the lysate with streptavidin-Sepharose were subjected to Western blot analysis by using an anti-EGFR antibody (Fig. 3*B*). Both experiments verified that EGFR was certainly biotinylated (open triangles in Fig. 3*A* and *B*). Upon the immunoprecipitation with the anti-EGFR antibody, EGFR was coprecipitated with putative clustered molecules that were also biotinylated (closed triangles in Fig. 3*A*).

The TCR $\alpha\beta$ complex in mature T cells is accompanied by some immunity-related molecules, such as CD3, and it also plays an important role in the immune reaction of T cells (30). We therefore tried to elucidate whether the TCR $\alpha\beta$ is biotinylated by the EMARS reaction using an HRP-conjugated anti-CD3 ϵ monoclonal antibody, 500A2 (31), which binds to the extracellular domain of CD3 ϵ . By the EMARS reaction using HRP-500A2 antibody and a monoclonal T cell line, 2C (32), which has a clonotypic TCR $\alpha\beta$, the TCR $\alpha\beta$ was certainly biotinylated (open triangles in Fig. 3*C*).

To address the question of whether irrelevant cell-surface molecules are labeled under the same conditions of an integrin partner analysis as the labeled EGFR, we examined the biotinylation of the HLA class I and EGFR after the EMARS reaction using TS2/16 as a probe (Fig. 3*D*). The cells were also UV-irradiated in the presence of arylazide-biotin to label all cell-surface proteins nonspecifically (ArB+UV). As shown in Fig. 3*D*, EGFR was biotinylated both by the EMARS reaction [anti- $\beta 1$ integrin (+)] and UV irradiation (ArB+UV). In contrast, HLA was biotinylated not by the EMARS reaction [anti- $\beta 1$ integrin (+)] but by UV irradiation (ArB+UV). These observations indicate that HLA class I is excluded from the $\beta 1$ integrin cluster even though it is an abundant cell-surface protein. These results support the validity of the EMARS method for identifying clustered molecules on the living cell surface.

The EMARS Reaction Can Distinguish Different Types of Molecular Clusters. When HeLa S3 cells underwent the EMARS reaction using the anti- $\beta 1$ integrin antibody TS2/16 as a probe, a number of biotinylated bands were detected (Fig. S4*A*), thus indicating that many kinds of molecules cocluster with $\beta 1$ integrin under physiological conditions. To study the functions of the $\beta 1$ integrin cluster, identification of the coclustered molecules is required. To this end, we used an antibody array analysis in combination with the EMARS reaction, taking account of its high sensitivity and usefulness. After the EMARS reaction, the membrane proteins were solubilized and subjected to a commercially available anti-RTK antibody array. When anti- $\beta 1$ integrin antibody was used as a probe of the EMARS reaction, many kinds of RTKs were biotinylated [Fig. 4*A*, anti- $\beta 1$ integrin (+)]. The relative signal intensity of each spot is summarized in Table S1. Among the RTKs examined, two RTKs, EGFR and hepatocyte growth factor receptor, were found to be highly expressed in HeLa S3 cells by nonspecific biotinylation using arylazide-biotin and high levels of HRP solution or UV-irradiation (Fig. 4*A*, ArB+HRP or ArB+UV). These results suggest that many kinds of RTKs actually cluster with $\beta 1$ integrin in living cells, but the probability of such coclustering is quite different among RTKs, irrespective of their expression levels.

Moreover, we investigated the coclustered molecules in regard to three growth factor receptors through the same approach. The cell lysates after the EMARS reaction using anti-EGFR, insulin-

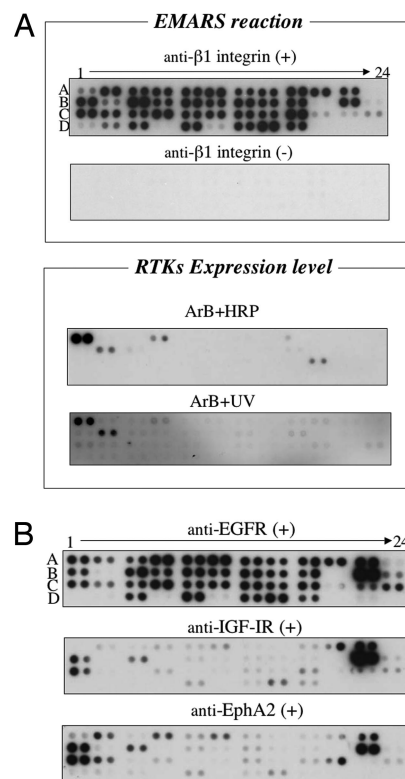


Fig. 4. Biochemical visualization of coclustering between the cell surface molecules. (*A* and *B*) The biotinylated RTKs resulting from the EMARS reaction using TS2/16 anti- $\beta 1$ integrin antibody (*A*) and anti-EGFR, IGF1R, and EphA2 antibodies (*B*) as a probe. The antibody array used in this study has 94 spots that contain antibodies against 42 kinds of RTKs (see Table S1) and control molecules with duplicates. After the EMARS reaction of the HeLa S3 cells using each antibody and HRP-conjugated second antibody [each antibody (+)], samples (TS2/16: 5 μ g, anti-EGFR, IGF1R, and EphA2 antibody: 10 μ g of total lysate proteins each) were reacted with an antibody array membrane. The control experiments were carried out without TS2/16 antibody [*A*, anti- $\beta 1$ integrin antibody (-): 5 μ g]. The cell lysates after all cell-surface molecules labeling by a high dose of HRP and UV irradiation (see *SI Materials and Methods*) were also reacted with the antibody (*A*, ArB+HRP: 30 μ g, ArB+UV: 20 μ g).

like growth factor-I receptor (IGF1R), and EphA2 receptor antibody were subjected to the RTKs antibody array in the same way as the anti- $\beta 1$ integrin antibody TS2/16 (Fig. 4*B*). The patterns of the biotinylation signals of the RTKs on the array were substantially different from each other. The pattern of $\beta 1$ integrin cluster was similar to that of the EGFR cluster but different from that of the IGF1R or EphA2 cluster. In contrast, the pattern of IGF1R was similar to that of the EphA2 cluster. These results suggest that different types of cell surface molecular clusters can be thus distinguished using the EMARS reaction.

The EMARS Reaction Reveals Alterations in Molecular Clustering. The EMARS reaction was used to investigate the effect of cellular conditions on molecular clustering. To place HeLa S3 cells into suspension, mild trypsinization (0.1% trypsin for 1 min) was carried out. After trypsinization, a part of the cells were applied to flow cytometry to confirm the retained binding of the TS2/16 antibody (Fig. S4*B* Left). The TS2/16-mediated EMARS reaction was carried out for the remaining cells and attached cells (without trypsinization). As a result, the biotinylated molecules dramatically decreased in the trypsinized cells compared with the nontreated cells in a blotting analysis (Fig. S4*B* Right). Mild

trypsinization may give rise to the dispersion of the coclustered molecules from the integrins. In addition, the EMARS products of the trypsinized cells were subjected to the RTKs antibody array. Although biotinylation was reduced in several receptors compared with the attached cells, the reduction was not so dramatic in contrast to that in the blotting analysis (Fig. S4C). Some receptors are partially dispersed from integrin clusters under mild trypsinization, whereas some are not influenced. These results suggest that some receptors are constitutively associated with integrin whereas other receptors are temporally associated with it.

The EMARS Reaction Is a Powerful Tool for a Wide Range of Research.

Finally, we performed the EMARS reaction using a different type of probe. The EMARS reaction using an HRP-conjugated cholera toxin B subunit (CTxB) probe, which binds to ganglioside GM1, was performed. Although many molecules were biotinylated (Fig. S5A), only two RTKs, EGFR and EphA2, were biotinylated in the antibody array (Fig. S5B, CTxB-HRP).

The current study is the first comprehensive approach to identify the components in the cell surface clusters under physiological conditions. The advantages of this approach are as follows: (i) easy, high throughput, and without the need for special equipment; (ii) applicable to systematic approaches such as proteomic analyses; and (iii) applicable to studies on not only proteins but also carbohydrate chains and membrane lipids. The present "biochemical visualization" although the EMARS reaction is therefore expected to become a powerful tool for a wide range of research concerning molecular clusters on the cell surface in living cells. Obtaining further comprehensive knowledge of the molecular interaction networks on the cell surface remains a challenge for the future.

Materials and Methods

For additional materials and procedures, see *SI Materials and Methods*.

The cultured cells were cooled at 4°C for 20 min, washed once with PBS, and then treated with 8 µg/ml of anti-β1 integrin monoclonal antibody TS2/16, 8 µg/ml of anti-EGFR monoclonal antibody (Abcam), 8 µg/ml of anti-IGF1R monoclonal antibody (R&D Systems), 8 µg/ml of anti-EphA2 monoclonal antibody (R&D Systems), 10 µg/ml of CTxB (SBL Vaccin), 10 µg/ml of HRP-conjugated CTxB (LIST Biological Laboratories), or 10 µg/ml of HRP-conjugated anti-CD3ε antibody in 2% BSA in PBS at 4°C for 1 h. In the case of the mouse primary antibodies, the cells were further treated with HRP-conjugated anti-mouse IgG (Promega) (10 µg/ml in 2% BSA/PBS) or HRP-conjugated anti-rabbit IgG for the negative control samples (Cappel) (10 µg/ml with 2% BSA in PBS) at 4°C for 1 h, respectively. After washing, the cells were incubated with 40 µg/ml of EZ-Link Biotin-LC-ASA or its analog, *N*-(4-azido-2-nitrophenyl)-*N'*-(*N*-D-biotinyl-3-aminopropyl)-*N'*-methyl-1,3-propanediamine (Pierce, EZ-Link Photoactivatable Biotin) in PBS at 4°C for 30 min in the dark. After washing twice with PBS, the treated cells were collected into a plastic tube with 50 mM Tris-HCl (pH 7.4) containing 5% FBS or 2% BSA and protease inhibitor mixture (Sigma). The arylazide-biotin becomes nonspecifically activated by intracellular endogenous enzymes (probably oxidases) when cells are homogenized. This activation is time-dependent, and it induces the nonspecific labeling of intracellular proteins. We can distinguish specific labeling from a nonspecific one by comparing the negative control without exogenous HRP. However, intensive nonspecific signals may disturb an accurate analysis. Therefore, we have to use complicated protocols to reduce the nonspecific labeling as much as possible. The use of homogenization through a syringe needle is a simple and speedy way to break the plasma membranes. Next, the homogenate was centrifuged at 800 × *g* for 5 min to mainly precipitate the nuclei. The supernatant was centrifuged at 20,000 × *g* for 15 min to precipitate what is generally called the "microsome fraction," containing the plasma membrane fractions. After washing the precipitates with 100 mM Tris-HCl (pH 7.4), the precipitates were then dissolved in the lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 10% glycerol, and protease inhibitor mixture]. This protocol should be slightly modified depending on the cell types.

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