

# A modified application of the luciferase immunoprecipitation systems for detecting antibodies to the G protein-coupled receptors

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**Background:** When multipass transmembrane molecules are located on the cell surface, there may be interaction with not only bioactive molecules but also pathogenic molecules in areas protruding outside the cell. In antibody-mediated autoimmune disorders, it has been found that the autoantibodies occasionally attack membrane molecules on the cell surface, thus causing the disease such as myasthenia gravis. In such cases, highly sensitive autoantibody detection technology is required for early diagnosis. However, autoantibody analysis technology that is specialized for membrane molecules is still under development. Here we demonstrate a novel method for detecting of antibodies against the extracellular portions of multipass transmembrane molecules.

**Methods:** Antibodies for muscarinic acetylcholine receptor type3 (M3R) were detected with two kinds of luciferase immunoprecipitation systems (LIPS), conventional LIPS (cLIPS) and its modified application, termed modified LIPS (mLIPS). In mLIPS, antibodies against extracellular portions of membrane molecules could be preferentially detected.

**Results:** An antibody to the amino-terminal portion of human M3R was detected with modified LIPS with a high sensitivity. In contrast, an antibody to the carboxyl-terminal portion was not detected with mLIPS, because it did not interact with intracellular portions of M3R in living cells. We also found antibodies for M3R in a patient serum with Sjögren's syndrome.

**Conclusion:** Our technology has a promising future, and we hope that it will be applied in the analysis of antibodies against a diverse range of multipass transmembrane molecules, including GPCRs.

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**Key words:** GPCR, LIPS, AChR, luciferase, autoantibody, and autoimmune disease

## Introduction

In the human genome, G-protein coupled receptors (GPCRs) are encoded by approximately 826 genes, forming the largest family of membrane molecules. They have been

found to be associated with various diseases and exert various types of bioactivity by binding to endogenous ligands including hormones, growth factors, and neurotransmitters (1). With regard to their involvement in autoimmune diseases, autoantibodies against a rhodopsin subfamily in GP-

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CRs have been found to be associated with the molecular condition of several diseases. For example, there have been reports on the involvement of autoantibodies against thyroid-stimulating hormone receptors in Graves' disease (2), autoantibodies against adrenalin beta-1 receptors and muscarinic acetylcholine receptor (AChR) type 2 in cardiovascular disease (3-5), antibodies against muscarinic AChR type 1 in chronic fatigue syndrome (6,7), as well as autoantibodies against muscarinic AChR type 3 in Sjögren's syndrome (SS) and in acquired idiopathic generalized anhidrosis (8,9).

GPCRs consist of an extracellular-facing amino-terminal (N-terminal) region and an intracellular-facing carboxyl-terminal (C-terminal), with seven transmembrane regions. Therefore, to analyze the epitope of these antibodies, the extracellular facing N-terminal and three extracellular loops (ECL1-3) are usually examined. However, given the spatial structure of the extracellular region and post-translational modification, antigens should ideally be used under conditions in which they adopt structures close to those occurring naturally. In this respect, the cell-based assay (CBA) is a suitable method to detect antibodies against multipass transmembrane molecules, such as GPCRs (10-12). CBA has the highest sensitivity for antibody detection among conventional diagnostic methods because of the following reasons: 1) it is used with living cells that express full-length antigen-encoding genes, thereby ensuring that the spatial structure and post-translational modification is very similar to the natural membrane molecule, i.e., the antigen, 2) it enables selective detection of antibodies that interact in the extracellular region, and 3) it utilizes a measuring device equipped with a fluorescence detector. Therefore, among existing antibody measurement technologies, CBA is particularly outstanding in the analysis of antibodies that target membrane molecules. Two principal types of fluorescence detectors are used in CBA. These are fluorescence microscope and flow cytometer. The former is used to determine the presence or absence of antibodies upon imaging, making it unsuitable for quantitative analysis, while the latter enables quantitative analysis. However, both devices are extremely expensive laboratory equipment and are not readily available to general laboratories. Furthermore, a certain level of expertise is required to operate such equipment.

An antibody detection technique that has gained recent attention is the luciferase immunoprecipitation system (LIPS) (13, 14). In LIPS, antibodies are detected with a luciferase reporter in which an antigenic polypeptide is fused to a luciferase at its amino- or carboxyl-terminus, and because it is used for luciferase reporter genes expressed in

cells, it ensures correct post-translational modification of antigenic membrane molecules. Furthermore, as the luminescence-measuring device (luminometer) is less expensive than fluorescence microscopes and flow cytometers and can be operated easily, it is a promising technological substitution for CBA. It has been reported that LIPS is capable of detecting various types of autoantibodies; however, very few reports have mentioned antibodies that target membrane molecules such as GPCR. Therefore, in the present study, we thoroughly examined the application of LIPS in the detection of GPCR antibodies with the view of optimization.

## Materials and Methods

### *Ethics*

The study was approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (Nagasaki, Japan). Written informed consent was obtained from each subject, in person, prior to participation in the study. This process was documented by means of an approved consent form.

### *Patients and serum samples*

Serum samples were available for 39 (2 males and 37 females) patients with definite SS (mean age,  $58.4 \pm 11.8$  years old) recruited through Nagasaki Kawatana Medical Center and Nagasaki University Graduate School of Biomedical Sciences. These samples represented both primary SS ( $n = 31$ ) and secondary SS ( $n = 8$ ). We also tested 39 serum samples from healthy volunteers (mean age, 46 years old; male: female ratio, 2:37). They were also recruited from Nagasaki Kawatana Medical Center and Nagasaki University Graduate School of Biomedical Sciences.

The diagnosis of SS was established by the criteria proposed by the Diagnostic Committee of Health and Welfare of Japan (15) or by the American-European Consensus Group (16).

### *Cells, DNA, and antibodies*

FreeStyle 293 cells (293F) were obtained from Invitrogen. Cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Human muscarinic AChR type 3 (M3R) cDNA was obtained from Promega (FHC10778). Gaussia luciferase (GL) cDNA was purchased from pBasic-Gluc (New England Biolabs). GL<sup>8990</sup> (F89W and I90L) is one of the GL mutants generated by site-direct-

ed mutagenesis and emits 10 times stronger and/or prolonged bioluminescence than intact GL (17). pcDNA3.1-Myc/His (A) was obtained from Invitrogen. Antibodies recognizing the N- and C-terminal portions of human M3R, H-20 (goat IgG), and H-210 (rabbit IgG), were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Alexa Fluor<sup>®</sup>488 sheep anti-goat IgG antibody and Alexa Fluor<sup>®</sup>488 goat anti-rabbit IgG antibody were acquired from Molecular Probes.

#### *Construction of M3R-GL<sup>8990</sup> expression plasmids*

The human M3R-GL<sup>8990</sup> plasmid was constructed by cloning the full-length of human M3R cDNA upstream of the GL<sup>8990</sup> using BamHI/AgeI restriction sites in pcDNA3.1-Myc/His (A). Construction of luciferase reporter expression plasmids of ganglionic acetylcholine receptor (gAChR) has been described elsewhere (18). All plasmids were purified using the Labo Pass<sup>™</sup> Mini Kit (Hokkaido System Science).

#### *Conventional LIPS (cLIPS)*

293F cells were transfected with M3R-GL<sup>8990</sup> expression plasmids using FuGENE6 (Promega). Two days later, transfected cells were solubilized with Tris-based saline containing 1% Triton<sup>™</sup> X-100. A total of 50  $\mu$ l of the soluble fraction was mixed with anti-M3R antibodies (H-20 or H-210, Santa Cruz Biotechnology), 15  $\mu$ l of protein G-sepharose (PGS, GE Healthcare), and 600  $\mu$ l phosphate-buffered saline with 3% bovine serum albumin (BSA) and 0.05% Tween<sup>®</sup> 20, and incubated overnight at 4  $^{\circ}$ C with a rotator. Following precipitation and washing of PGS, bioluminescence activity of the luciferase reporters in the PGS was measured using the BioLux<sup>™</sup> *Gaussia* Luciferase Assay Kit (New England Biolaboratories) and a Lumat LB 9507 luminometer (Berthold Technologies). The luminometer output was measured in relative luminescence units (RLU). 293F cells expressing luciferase reporters for two different subunit of gAChR (AChR  $\alpha$  3-GL<sup>8990</sup> and AChR  $\beta$  4-GL<sup>8990</sup>) were used as a negative control in cLIPS.

#### *Cell-based assay (CBA) using fluorescence microscope*

To detect serum antibodies binding to M3R on living cells, a CBA using fluorescence microscope was designed. In brief, 293F cells were cultured on 6-well multidish collagen (Nalge Nunc International) in 1 ml DMEM supplemented with 10% FBS. Cells were transfected with M3R expression plasmid (0.8  $\mu$ g per well) using FuGENE6 (Promega; 3  $\mu$ L per well) and were used for immunostaining after 18 h. Cells

expressing M3R were incubated with H-20 or H-210 anti-M3R antibody for 1 h in a CO<sub>2</sub> incubator; they were then washed once with the Opti-MEM medium (Gibco). Further, cells were incubated with 1  $\mu$ L of Alexa Fluor<sup>®</sup>488 donkey anti-goat IgG or goat anti-rabbit IgG antibody for 30 min in a CO<sub>2</sub> incubator; they were then extensively washed with Opti-MEM medium. Immunofluorescence images were analyzed using the EVOS<sup>®</sup> Flouid<sup>®</sup> Cell Imaging Station (Life Technologies).

#### *Modified LIPS (mLIPS)*

293F cells were inoculated in 6-well multidish collagen and cultured in 1 ml of DMEM supplemented with 10% FBS. 293F cells were transfected with 0.5  $\mu$ g of luciferase expression plasmids by 2  $\mu$ l of FuGENE6 (Promega) per well. Eighteen hours later, antibody or antiserum was added to culture wells and incubated for 1 h at 37  $^{\circ}$ C in a CO<sub>2</sub> incubator. Cells were washed twice with 1 ml PBS and lysed with 200  $\mu$ l cell-lysis buffer (20 mM Tris-HCl, pH 7.5; 100 mM NaCl, 1% Triton X-100). Cell lysates were centrifuged at 21,130  $\times$  g at 4  $^{\circ}$ C for 15 min, after which, soluble fractions were collected. A total of 170  $\mu$ l of the soluble fraction was mixed with 500  $\mu$ l of PBS with 3% BSA, 0.05% Tween 20, and 15  $\mu$ l (volume of resin) PGS, and the mixture was incubated for several hours or overnight at 4  $^{\circ}$ C with a rotator. The PGS was precipitated at 9,391  $\times$  g for a few sec and extensively washed with PBS supplemented with 0.05% Tween20 (PBST) up to two times. Luminescence activity was measured as described above. 293F cells expressing luciferase reporters for two different subunit of gAChR (AChR  $\alpha$  3-GL<sup>8990</sup> and AChR  $\beta$  4-GL<sup>8990</sup>) were used as a negative control in mLIPS. To normalize the transfection efficiency of M3R-GL<sup>8990</sup> in each well, a net of luciferase activity in 20  $\mu$ l of the soluble fraction was measured. Antibody levels were finally expressed as arbitrary units (A.U.) calculated as follows:

$$\text{A.U.} = [\text{mLIPS measurement value of sample (RLU)}] / [\text{measurement value of 20 } \mu\text{l of soluble fraction value (RLU)}].$$

#### *mLIPS for human sera*

mLIPS was performed as described above. A total of 15  $\mu$ l human serum was used in this experiment. Based on the data from 39 human control sera for anti-M3R antibodies, cut-off values were calculated as the mean + four standard deviations (SD) from the mean. In this study, antibody titers were expressed as an antibody index (A.I.) calculated as follows:

A.I. = [measurement value of the sample serum (RLU)]/[the cut-off value (RLU)].

The normal value established in this study from healthy individuals was <1.0 A.I. The autoimmune autonomic ganglionopathy patient serum has been tested positive for anti-gAChR antibody in the previous study (18).

### Statistical analysis

Commercially available statistics software was used for data analysis (SigmaPlot®). A.I. data that were normally distributed were analyzed by one-way analysis of variance (ANOVA). For data that were not normally distributed, the one-way ANOVA of ranks was employed. The level of statistical significance was set at a probability (*P*) value < 0.05.

## Results

### cLIPS

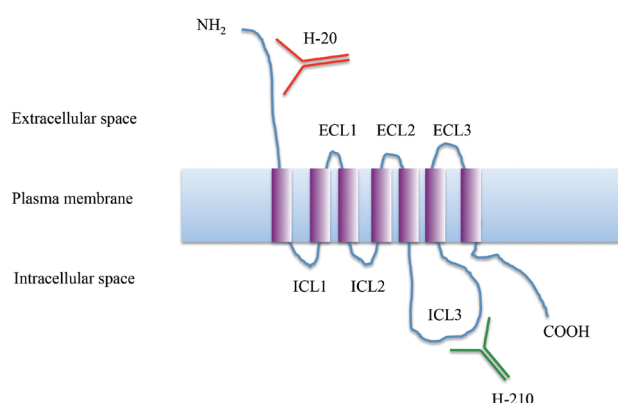
To know whether M3R-GL8990 works in a conventional LIPS (cLIPS), we first used two ready-made antibodies for human M3R. H-20 and H-210 antibodies are generated against the N-terminal portion and ICL3 of human M3R, respectively (Figure 1). As shown in Figure 2a, both H-20 and H-210 antibodies were adequately detected. On the other hand, when using the soluble fraction prepared from cells with gAChR reporters that have low amino sequence homology with M3R, we were unable to detect either antibody (Figure 2a). These data demonstrated that M3R-GL8990 was applicable to cLIPS.

### mLIPS

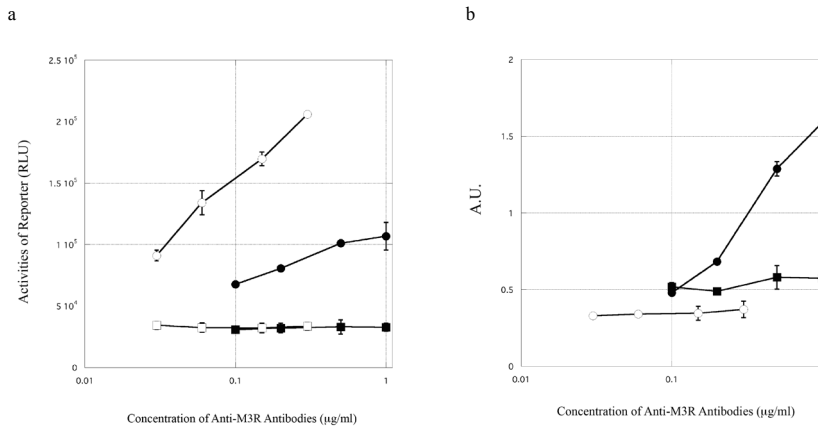
We next tried a modified application of the cLIPS, termed modified LIPS (mLIPS). In brief, serum antibodies bind to M3R-GL8990 in living cells in a new technique (Supplementary Figure 1). H-20 antibodies were detected at 0.1-1.0  $\mu$ g/ml in a dose-dependent manner, but H-210 antibodies were not detected even at 0.3  $\mu$ g/ml. In mLIPS using cells that expressed gAChR-GL<sup>8990</sup> as a control, H-20 antibodies were not detected at all. We then performed CBA with a fluorescence microscope to compare antibody detection sensitivity. The fluorescent signals of H-20 antibody were obviously visible 0.06-0.6  $\mu$ g/ml (Figure 3a). In contrast, H-210 antibody was not detected even at 0.6  $\mu$ g/ml in mLIPS (Figure 3b).

### Screening of autoantibodies for M3R in human sera

It is known that M3R antibodies can be detected in the blood of patients with SS and acquired idiopathic generalized anhidrosis. In the present study, we performed mLIPS using the serum of 39 SS patients enrolled through the Nagasaki University First Department of Internal Medicine. As a result, we identified one patient with antibody-positive serum exceeding the cut-off value based on analysis of serum from healthy individuals (Figure 4). We also found that the increase in reporter activity was proportional to the increase in antibody-positive serum (Figure 5a). To test the specificity of antigen of SS patient serum, gAChR-GL expressing cells were used. As shown in Figure 5b, the reporter activity of gAChR-GL was increased in dose-dependent-manner of anti-gAChR antibody-positive autoimmune autonomic ganglionopathy (AAG) patient serum, but not anti-M3R antibody-positive SS serum.

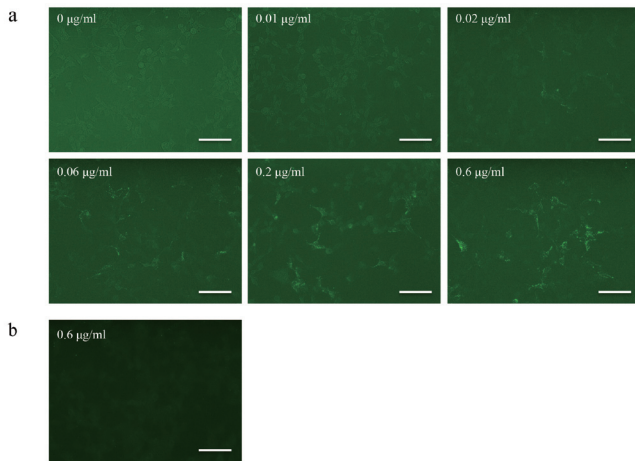


**Figure 1.** Schematic representation of human muscarinic acetylcholine receptor type-3. Muscarinic acetylcholine receptor type-3 (M3R) has seven membrane spanning domains (purple bars) and localizes at the cell plasma membrane (blue bar). The amino-terminal portion and the three extracellular loops (ECL1–3) are in the extracellular space. Carboxyl-terminal portions and the three intracellular loops (ICL1–3) are in the intracellular area. M3R interacts with G-protein at ICL3. Two ready-made antibodies, H-20 (red Y-shaped line) and H-210 (green Y-shaped line), have epitopes in the amino-terminal portion and ICL3 of human M3R, respectively.



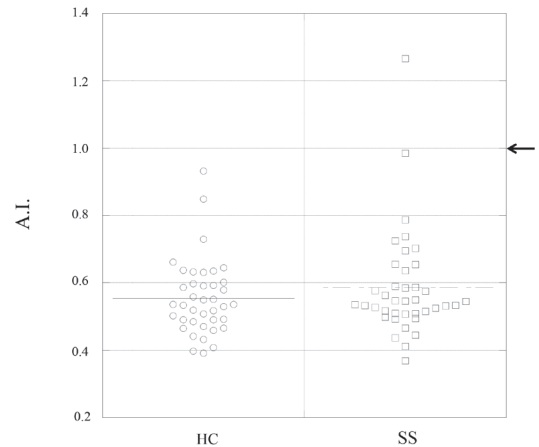
**Figure 2.** Detection of anti-M3R antibodies with conventional LIPS and modified LIPS.

(a) Reporter-specific detection of anti-M3R antibodies by the conventional luciferase immunoprecipitation systems (cLIPS). Detection of two-types of anti-M3R antibodies by cLIPS with M3R-GL<sup>8990</sup> and ganglionic acetylcholine receptor (gAChR)-GL<sup>8990</sup>. Circles and squares indicate M3R-GL<sup>8990</sup> and gAChR-GL<sup>8990</sup>, respectively. Closed and open symbols indicate H-20 and H-210 antibodies, respectively. The y-axis indicates luciferase reporter activity (relative luminescence units, RLU). The x-axis indicates concentrations of anti-M3R antibodies (μg/ml) in log scale. Error bars indicate standard deviations (SD) from the mean (n = 3). We evaluated that two lines with circle symbols shows dose-response manner (one way-ANOVA comparisons were performed using the Student-Newman-Keuls method in each reaction between reporter gene and antibody: P < 0.001), but not two lines with square symbols. (b). Specific detection of the antibody that binds the amino-terminal portion of M3R in the modified luciferase immunoprecipitation systems (mLIPS). Detection of anti-M3R antibodies by mLIPS with M3R-GL<sup>8990</sup> and gAChR-GL<sup>8990</sup>. Closed and open symbols indicate H-20 and H-210 antibodies, respectively. Circles and squares indicate M3R-GL<sup>8990</sup> and gAChR-GL<sup>8990</sup>, respectively. The y-axis indicates arbitrary units (A.U.; see “Materials and Methods”). The x-axis indicates concentrations of anti-M3R antibodies (μg/ml) in log scale. Error bars indicate standard deviations (SD) from the mean (n = 3). We evaluated that a line with closed circles shows dose-response manner (one way-ANOVA comparisons were performed using the Student-Newman-Keuls method in each reaction between reporter gene and antibody: P < 0.001), but not two lines with open circles and closed squares.



**Figure 3.** Detection of anti-muscarinic acetylcholine receptor type-3 (M3R) antibodies in the cell-based assay (CBA) using fluorescence microscopy.

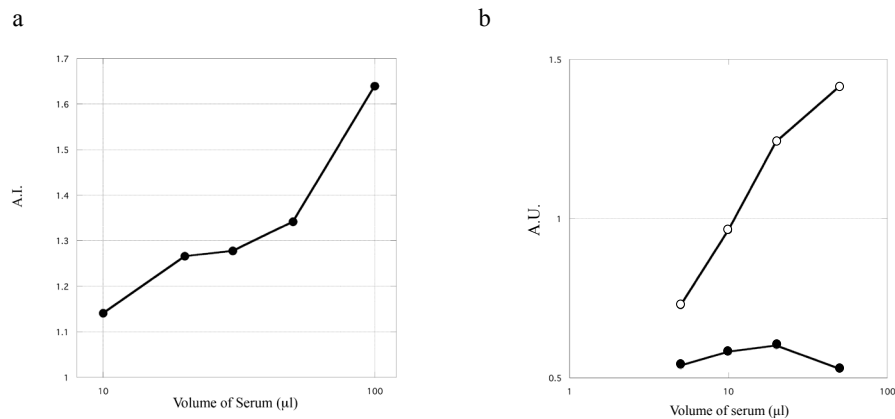
Living cells expressing M3R-GL<sup>8990</sup> were stained with H-20 (a) or H-210 antibody (b) and secondary antibody-conjugated with Alexa488. The concentrations of H-20 antibody were as indicated. The concentration of H-210 antibody was 600 ng/ml. Scale bars indicate 100 μm.



**Figure 4.** Anti-muscarinic acetylcholine receptor type-3 (M3R) antibody in Sjögren's syndrome (SS) patient sera samples, as determined by the modified luciferase immunoprecipitation systems (mLIPS).

One anti-M3R antibody-positive serum sample was identified by mLIPS among 39 SS patient samples. Open circles and open squares indicate data from healthy controls (HC) and from SS patients (SS), respectively. Solid and broken lines indicate the means of data from HC and SS, respectively. The y-axis indicates antibody index (A.I.; described in “Materials and Methods”). Arrow indicates the cut-off value of 1.0.



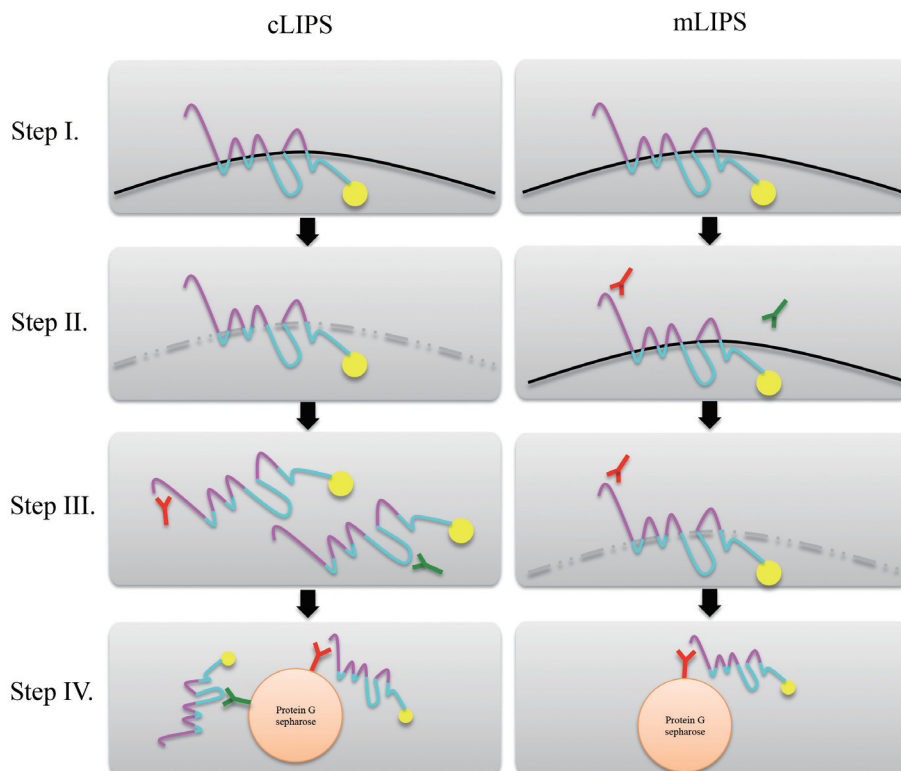


**Figure 5.** Examination of the antigen specificity of SS patient serum in mLIPS.

Luciferase activity of M3R-GL<sup>8990</sup> increased in a dose-dependent manner of anti-M3R antibody-positive serum. The y-axis indicates antibody index (A.I.; described in “Materials and Methods”). The x-axis indicates the volume of anti-M3R antibody-positive serum (μl) in log scale. b) No increase of the luciferase activity of gAChR-GL<sup>8990</sup> was observed in mLIPS with anti-M3R antibody-positive serum (Open circles). In contrast, the luciferase activity of gAChR-GL<sup>8990</sup> increased in a dose-dependent manner of an AAG patient serum (Closed circles). y-axis indicates arbitrary units (A.U.; see “Materials and Methods”). The x-axis indicates the volume of human serum (μl) in log scale.

Supplementary Figure 1. Contrast between conventional luciferase immunoprecipitation systems (cLIPS) and modified LIPS (mLIPS).

In cLIPS (the left panel), M3R-GL<sup>8990</sup> is expressed at the plasma membrane (Step I) and then is solubilized with a cell lysis buffer containing a detergent (Step II). Magenta and cyan colors indicate the extracellular and intracellular portions, respectively. Yellow color indicates luciferase. Solubilized M3R-GL<sup>8990</sup> is mixed with antibodies (Step III). Red and green Y-shaped lines indicate H-20 and H-210 antibodies, respectively. In step IV, anti-M3R antibodies binding M3R-GL<sup>8990</sup> are concentrated using protein G-sepharose (Orange circle). In contrast, in mLIPS (the right panel), luciferase reporters are bound to antibodies before cell solubilization (Step II). As a result, antibodies recognizing extracellular portions, but not intracellular regions, are selectively concentrated using protein G-sepharose (Step IV).



## Discussion

In this study, we demonstrated that mLIPS was one of the most suitable tools for selective detection of antibodies that recognizes extracellular portions of antigen. As reporters are solubilized after expression on the cell membrane, there is the risk of destroying the spatial structure of antigen proteins when using cLIPS. There is also the possibility that antigens bound to the intracellular region will also be detected (Supplementary Figure 1, left panel). Therefore, based on CBA application, we devised a modified protocol for LIPS, mLIPS, in which cells producing reporters on the membrane and antibodies were left to react in advance; then, after the antibody–antigen complex had formed, the cells were solubilized (Supplementary Figure 1, right panel). Several existing autoantibodies against GPCR target muscarinic AChRs (5–9). Therefore, in the present study, we chose to target muscarinic AChR type 3 (M3R). M3Rs have seven transmembrane regions and are located on the cell membrane with four extracellular-facing regions, including the N-terminal portion and ECL 1–3 (Figure 1). On the other hand, the C-terminal portion and intracellular loop (ICL) 1–3 are intracellular-facing (Figure 1). In cLIPS, H-210 antibody with an epitope in ICL3 was detected (Figure 2a), whereas the antibody was not detected in mLIPS (Figure 2b). The large number of existing autoantibodies target intracellular antigens, such as antinuclear antibodies. However, the underlying pathogenicity remains unclear. Antibodies with extracellular epitopes include those that cause V-type allergies (Graves' disease-related autoantibodies) and those that cause complement-mediated tissue destruction (myasthenia gravis-related autoantibodies), which have a direct interaction with the onset of diseases (2, 19). Consequently, a development of diagnostic method to detect autoantibodies binding the extracellular regions of membrane proteins with a high sensitivity has been desired intensely. Our mLIPS enables selective detection of such pathogenic autoantibodies (Figure 2b). Furthermore, mLIPS and CBA possibly have equal sensitivity in terms of antibody detection, at least for H-20 antibodies (Figures 2b and 3). mLIPS is easier to operate and is a less expensive measurement device with quantitative capacity and in these respects has the potential to be an alternative to CBA in the near future.

The detection of antibodies that recognize the spatial structure of membrane molecules using cLIPS remains controversial. Compared with the detection sensitivity in the conventional radioimmunoassay (RIA) for myasthenia gravis-related autoantibodies against muscular nicotinic AChRs, that of cLIPS was very low (20). Muscular nicotinic AChR

forms a pentamer composed of four different subunits (alpha 1, beta 1, delta, and epsilon/gamma). In general, a pentamer form of AChR is used in the RIA for detecting of anti-muscular nicotinic AChR antibody. In brief, AChRs bound with  $^{125}\text{I}$ -bungarotoxin (Btx) are first mixed with human serum. If there is anti-AChR antibody in human serum, it could form an immunocomplex with AChRs. The radioactivity of immunocomplex indicates a level of anti-AChR antibody in human serum (21). In the LIPS performed by Ching et al, the alpha1 subunit fused to luciferase was used as a luciferase reporter in the absence of other subunits (20). They claimed that this might be attributed to the fact that antibodies against spatial structures formed between other subunits in the pentamer cannot be detected in cLIPS using a luciferase reporter of alpha 1 subunit. In contrast, Nakane et al. used cLIPS using a luciferase reporter of ganglionic nicotinic acetylcholine receptor (gAChR) for detecting of autoantibodies in autoimmune autonomic ganglionopathy (AAG) (18). It is known that gAChR is mainly composed of two subunits (alpha 3 and beta 4) (22). A single subunit (alpha 3 or beta 4 subunit) is used in their study. However, they observed no major differences of the prevalence of antibody in AAG relative to the conventional RIA (18). Therefore, the applicability of cLIPS for antibodies to multispan membrane proteins can be described on a case-by-case basis because only a small number of cases have been examined (18, 20). However, when analyzing antibodies to multispan membrane proteins expected to have a complex spatial structure, mLIPS should be chosen as the first-line tool to minimize the risk of destroying the spatial structure of the target antigen as much as possible.

For detection sensitivity of antibody in mLIPS, an effect of the internalization of M3R following to antibody binding remains elusive. It has been reported that a half of M3R on cell surface was internalized after attacking of anti-M3R antibody to cells (23). In this study, we performed a binding reaction between antibody and antigen at 37°C for one hour, whereas a possibility that an internalization of antigen at cell surface decreases a sensitivity of antibody in mLIPS is not excluded. However, we think that a part of antibodies internalized could be detected by mLIPS if before internalized antibodies are degraded in cellular organelle such as lysosome.

Finally, one SS patient serum containing anti-M3R antibody was identified with mLIPS, demonstrating that this new technique is applicable to human biospecimens. In the near future, mLIPS may prove to be a powerful diagnostic tool to detect pathogenic autoantibodies for GPCRs and contribute to screening of therapeutic antibodies directed at GPCRs (24).

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## References

1. Stevens RC, Cherezov V, Katritch V, Abagyan R, Kuhn P, Rosen H, et al. The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. *Nat. Rev. Drug Discov.* 2013; 12: 25-34.
2. Menconi F, Marcocci C, Marino M. Diagnosis and classification of Graves' disease. *Autoimmun. Rev.* 2014; 13: 398-402.
3. Wallukat G, Wollenberger A, Morwinski R, Pitschner HF. Anti-beta 1-adrenoceptor autoantibodies with chronotropic activity from the serum of patients with dilated cardiomyopathy: mapping of epitopes in the first and second extracellular loops. *J. Mol. Cell Cardiol.* 1995; 27: 397-406.
4. Fu ML, Herlity H, Schulze W, Wallukat G, Micke P, Eftekhari P, et al. Autoantibodies against the angiotensin receptor (AT1) in patients with hypertension. *J. Hypertens.* 2000; 18: 945-53.
5. Matsui S, Fu ML, Hayase M, Katsuda S, Yamaguchi N, Teraoka K, et al. Active immunization of combined beta-1 adrenoceptor and M2-muscarinic receptor peptides induces cardiac hypertrophy in rabbits. *J. Card. Fail.* 1999; 5: 246-54.
6. Tanaka S, Kuratsune H, Hidaka Y, Hakariya Y, Tatsumi KI, Takano T, Kanakura Y, et al. Autoantibodies against muscarinic cholinergic receptor in chronic fatigue syndrome. *Int. J. Mol. Med.* 2003; 12: 225-30.
7. Yamamoto S, Ouchi Y, Nakatsuka D, Tahara T, Mizuno K, Tajima S, et al. Reduction of [<sup>11</sup>C](+)-3-MPB binding in brain of chronic fatigue syndrome with serum autoantibody against muscarinic cholinergic receptor. *PLoS One.* 2012; 7: e51515.
8. Bacman S, Sterin-Borda L, Camusso JJ, Arana R, Hubscher O, Borda E. Circulating antibodies against rat parotid gland M3 muscarinic receptors in primary Sjögren's syndrome. *Clin. Exp. Immunol.* 1996; 104: 454-59.
9. Asahina M, Sano K, Fujinuma Y, Kuwabara S. Investigation of anti-muscarinic receptor autoantibodies in patients with acquired idiopathic generalized anhidrosis. *Intern. Med.* 2013; 52: 2733-7.
10. Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J. Exp. Med.* 2005; 202: 473-7.
11. Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. *Ann. Neurol.* 2007; 61: 25-36.
12. Vincent A, Waters P, Leite MI, Jacobson L, Koneczny I, Cossins J, et al. Antibodies identified by cell-based assays in myasthenia gravis and associated diseases. *Ann. N.Y. Acad. Sci.* 2012; 1274: 92-8.
13. Burbelo PD, Ching KH, Bren KE, Iadarola MJ. Searching for biomarkers: humoral response profiling with luciferase immunoprecipitation systems. *Expert. Rev. Proteomics* 2011; 8: 309-16.
14. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann. Neurol.* 2011; 69: 418-22.
15. Fujibayashi T, Sugai S, Miyasaka N, Hayashi Y, Tsubota K. Revised Japanese criteria for Sjögren's syndrome (1999): availability and validity. *Mod. Rheumatol.* 2004; 14: 425-34.
16. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann. Rheum. Dis.* 2002; 61: 554-8.
17. Kim SB, Suzuki H, Sato M, Tao H. Superluminescent variants of marine luciferases for bioassays. *Anal. Chem.* 2011; 83: 8732-40.
18. Nakane S, Higuchi O, Koga M, Kanda T, Murata K, Suzuki T, et al. Clinical features of autoimmune autonomic ganglionopathy and the detection of subunit-specific autoantibodies to the ganglionic acetylcholine receptor in Japanese patients. *PLoS One* 2015; 10: e0118312.
19. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J. Autoimmun.* 2014; 52: 90-100.
20. Ching KH, Burbelo PD, Kimball RM, Clawson LL, Corse AM, Iadarola MJ. Recombinant expression of the AChR-alpha1 subunit for the detection of conformation-dependent epitopes in Myasthenia Gravis. *Neuromuscul. Disord.* 2011; 21: 204-13.
21. Vincent A and Newsome-Davis J. Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. *J. Neurol. Neurosurg. Psychiatry* 1985; 48: 1246-52.
22. Vernino S, Low PA, Fealey RD, Stewart JD, Farrugia G, Lennon VA. Autoantibodies to ganglionic acetylcholine receptors in autoimmune autonomic neuropathies. *N. Engl. J. Med.* 2000; 343: 847-55.
23. Kim N, Shin Y, Choi S, Namkoong E, Kim M, Lee J, et al. Effect of antimuscarinic autoantibodies in primary Sjögren's syndrome. *J. Dent. Res.* 2015; 94: 722-8.
24. Hutchings CJ, Koglin M, Marshall FH. Therapeutic antibodies directed at G protein-coupled receptors. *MAbs* 2010; 2: 594-606.