Reversible G Protein $\beta_9\gamma$ Distribution-Based Assay Reveals Molecular Underpinnings in Subcellular, Single-Cell, and Multicellular GPCR and G Protein Activity

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Supporting Information

ABSTRACT: Current assays to measure the activation of G protein coupled receptors (GPCRs) and G proteins are time-consuming, indirect, and expensive. Therefore, an efficient method which directly measures the ability of a ligand to govern GPCR-G protein interactions can help to understand the molecular underpinnings of the associated signaling. A live cell imaging-based approach is presented here to directly measure ligand-induced GPCR and G protein activity in real time. The number of active GPCRs governs G protein heterotrimer (alpha subunit) dissociation, thereby controlling the concentration of free beta gamma subunits. The described $\beta_9\gamma$ assay measures the GPCR activation-induced extent of the reversible $\beta_9\gamma$ subunit exchange between the plasma membrane (PM) and internal membranes (IMs). Confocal microscopy-based $\beta_9\gamma$ assay quantitatively determines the concentration dependency of ligands on GPCR activation. Demonstrating the high-throughput screening (HTS) adaptability, the $\beta_9\gamma$ assay performed using an imaging plate reader measures the ligand-induced GPCR activation. This suggests that the $\beta_9\gamma$ assay can be employed to screen libraries of compounds for their ability to activate GPCRs. Together with subcellular optogenetics, the spatiotemporal sensitivity of the $\beta_9\gamma$ assay permits experimental determination of the limits of spatially restricted activation of GPCRs and G proteins in subcellular regions of single cells. This assay works effectively for GPCRs coupled to alpha/o and alpha/s heterotrimers, including light-sensitive GPCRs. In addition, computational modeling of experimental data from the assay is used to decipher intrinsic molecular details of the GPCR-G protein activation process. Overall, the $\beta_9\gamma$ assay provides a robust strategy for quantitative as well as qualitative determination of GPCR and G protein function on a single-cell, multicell, and subcellular level. This assay not only provides information about the inner workings of the signaling pathway, but it also strengthens GPCR deorphanization as well as drug discovery efforts.

G protein coupled receptors (GPCRs) and their interacting partners, heterotrimeric G proteins, are universal controllers of cellular signaling. These are implicated in a majority of pathological conditions, ranging from heart diseases to cancer. Although ~30% of all drugs on the market act on GPCRs, the molecular mechanisms of their actions are just coming to light. In response to external stimuli, ranging from small molecules, lipids, peptides, and hormones to light, GPCRs control a variety of cellular and physiological processes. Although the name implies that GPCRs are coupled to G protein heterotrimers ($\alpha$(GDP)/$\beta\gamma$) through their cytosolic domains, it is controversial whether the heterotrimer is precoupled to the inactive receptor or the receptor ($R$)-ligand (L) complex (RL) formation induces the coupling.1-4 Activation of a GPCR by its cognate ligand results in activation and dissociation of the G protein heterotrimer into its subunits $\alpha$ and $\beta\gamma$, transducing the signal into the cell interior.5-6 In a G protein heterotrimer, the $\alpha$ subunit is in the GDP bound state: $\alpha$(GDP), which possesses higher affinity to $\beta\gamma$, GPCRs and guanine nucleotide exchange factors (GEFs) induce the conformational changes required for the $\alpha$ subunit in the RL-$\alpha$G($\alpha$(GDP))$\beta\gamma$ ternary complex to exchange its GDP to a GTP molecule.7 The lower affinity of G($\gamma$(GTP)) for $\beta\gamma$ promotes the heterotrimer dissociation. The free G($\gamma$(GTP)) that is generated has a limited lifetime, because of its intrinsic GTPase activity, which is accelerated by GTPase-accelerating proteins (GAPs). This results in the termination of heterotrimeric G protein signaling through sequestration of $\beta\gamma$ to regenerate the heterotrimer.8,9

Measuring a ligand’s ability to induce conformational changes in a GPCR-induced heterotrimeric G protein activation is important in pharmacology, and this action is required to identify potent and selective modulators of signaling. In early GPCR assays, radioisotope-labeled ligand binding to isolated cell membranes has been used, and, in addition to the risks of radiation exposure, the lack of dynamic signaling information has been an impediment.10,11 As a direct measure of GPCR activation and heterotrimer dissociation, the GTPase binding assay was developed. Although it measures G protein activation in multiple types of GPCRs and has been developed into a HTS assay,
extensive protein purification requirements and radioactive material usage have diminished its wide applicability.\textsuperscript{12,13} Fluorescence- and bioluminescence-based methods that have been developed later possess the ability to acquire information on ligand-GPCR interactions and second messenger activities in living cells in a high-throughput fashion. Among these, the FRET-based ePAC sensor has been frequently used to measure the activities of both \( \alpha \) and \( \alpha_{i/o} \) (activation and inhibition of cAMP production).\textsuperscript{14,15} Furthermore, several assays have been developed to measure FRET between G proteins–G proteins and G proteins–GPCRs to measure GPCR activation and heterotrimer dissociation.\textsuperscript{16–18} However, these assays have several limitations, including inadequate sensitivity, the requirement of multiple genetically encoded protein expression, and the design of specific assay components for every ligand or GPCR. They are also susceptible to interference from other signaling entities, especially when detecting downstream signaling molecules.\textsuperscript{12,19,20} In contrast, the C-terminal polybasic regions, allowing multiple genetically encoded protein expression, and the design of specific assay components for every ligand or GPCR. They are also susceptible to interference from other signaling entities, especially when detecting downstream signaling molecules.\textsuperscript{12,19,20} In recent years, there has been a substantial interest in developing label-free live cell assays, in which GPCR activity-induced morphological, as well as electrical changes of cells have been measured.\textsuperscript{21} However, label-free assays are hampered by the lack of specificity, matrix interference, and their limited sensitivity. In contrast, the \( \gamma_9 \) assay described below has overcome these limitations through its higher sensitivity, selectivity, and HTS adaptability gained by measuring the common signaling element for all GPCR pathways: the heterotrimer dissociation.

G protein \( \alpha \) subunits at the N-terminus and \( \gamma \) subunits at the C-terminus are post-translationally modified with a diverse group of lipids allowing them to interact and remain bound to the plasma membrane (PM) lipid bilayer when they are in the heterotrimeric form. The \( \alpha \) subunits are N-myristoylated for the membrane targeting, which is further strengthened by N-palmitoylation.\textsuperscript{22} Free \( \beta \gamma \) has been thought to be restricted to the PM, although it transiently interacts with the PM through C-terminal prenyl moiety of the \( \gamma \) subunit.\textsuperscript{23} Depending on the CaaX box in the C terminus of the \( \gamma \), the lipid modification can either be farnesyl or geranylgeranyl.\textsuperscript{24} However, this prenylation is not sufficient for its membrane targeting and, therefore, support from the C-terminal polybasic peptide region is required.\textsuperscript{25} Mammalian cells express 12 \( \gamma \) subunits and they possess different C-terminal polybasic regions, allowing \( \beta \gamma \) subunits to have different membrane affinities.\textsuperscript{26,27} Interestingly, all of the \( \gamma \) subunits are capable of reversibly moving between the PM and IMs upon activation of \( \alpha_{i/o} \) or \( \alpha_9 \), and \( \alpha_q \) coupled receptors with distinctly different rates,\textsuperscript{26,28} while activated \( \alpha \) subunits remain bound to the PM.\textsuperscript{28,29} Among the 12 \( \gamma \) subunits, \( \gamma_3 \) shows the slowest translocation rate (translocation half-time: \( t_{1/2} \geq 250 \) s), while \( \gamma_5 \) subunits with \( \gamma_9 \) possess the fastest shuttling between the PM and IMs with the forward translocation of \( t_{1/2} \approx 10 \) s.\textsuperscript{28} Here, the ability of free \( \beta \gamma \) subunits to reversibly distribute between the PM and IMs was employed as an assay (\( \gamma_9 \) assay) to detect ligand-concentration-dependent GPCR/G protein activation—deactivation in living cells.

\section*{MATERIALS AND METHODS}

\textbf{Constructs, Cell Culture, and Transfections.} Constructs, GFP–\( \gamma_9 \), mCherry–\( \gamma_9 \), and blue opsin–GFP have been described previously.\textsuperscript{28,30–32} J1-AR-CFP was a gift from N. Gautam. M4 muscarinic–mTurquoise was created by PCR amplification of M4 with NotI and XbaI from M4-CFP and subcloning to corresponding sites of blue opsin–mTurquoise, after restriction digestion. This construct was in pcDNA3.1 (Invitrogen). HeLa cells (ATCC) were cultured in minimum essential medium (CellGro) containing 10% dialyzed fetal bovine serum (Atlanta Biologicals), in the presence of 1% penicillin–streptomycin in 60 mm tissue culture dishes. At 75% confluency, cells were lifted after incubating with versene-EDTA (CellGro) for 3 min at 37 °C, centrifuged at 1000g for 3 min, and versene-EDTA was aspirated before resuspending in the regular culture media at a cell density of \( 1 \times 10^6 \) mL. One day before the transfection of DNA into cells, \( 1 \times 10^5 \) cells were seeded on 35 mm glass-bottomed dishes (In Vitro Scientific). The transfection was performed using the transfection reagent PolyJet (SignaGen), according to the manufacturer’s protocol.

\textbf{Time-Lapse Imaging.} These experiments were performed with a 60X, 1.4 NA oil objective or a 10X, 0.3 NA objective in a spinning-disk XD confocal TIRF imaging system that is composed of a Nikon Ti-R/B inverted microscope, a Yokogawa CSU-X1 spinning disk unit (5000 rpm), an Andor FRAP-PA (fluorescence recovery after photobleaching and photoactivation) module, a laser combiner with 40–100 mW 445, 488, 515, and 594 nm solid-state lasers and iXon ULTRA 897BV back-illuminated deep-cooled EMCCD camera. Fluorescently tagged–\( \gamma_9 \) (FP–\( \gamma_9 \)) translocation in cells, cultured on glass-bottomed imaging dishes, was examined by imaging either GFP or mCherry, using 488 nm excitation–515 nm emission or 594 nm excitation–630 nm emission settings, respectively. During time lapse imaging at a frequency of 1 Hz, ligands were added at 2X concentration to activate corresponding GPCRs in appropriate volumes to achieve efficient diffusion and 1X final concentration in the imaging media. Imaging was continued until fluorescence intensities of the PM and IMs reached the final equilibrium.

\textbf{Optogenetic Control of GPCR Signaling and Imaging of G Protein Dynamics.} HeLa cells expressing blue opsin–GFP and mCherry–\( \gamma_9 \) were cultured on 35 mm glass-bottomed imaging dishes, as described above. Using a 445 nm optical stimulus, opsins in cells were activated globally or locally using a computer-steered galvo device. Details are given in the Supporting Information.

\textbf{Image Analysis, Data Processing, and Simulation of Laser Power Distribution.} Time-lapse images were analyzed using the analytical tools accompanied by Andor iQ3.1 software (Andor Bioimaging). Additional image analysis was performed using ImageJ (National Institute of Health),\textsuperscript{35} and custom-built Python algorithms (Python Software Foundation). The details of processes are given in the Supporting Information.

\textbf{Computational Model.} The details of the computational model and differential equations (eqs 1–13) are given in the Supporting Information.
Table 1. Group of Reactions Describing the Process of G Protein γ Subunits That Are Capable of Translocating as βγ Dimers from the Plasma Membrane (PM) to Internal Membranes (IMs)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
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<tbody>
<tr>
<td>$R + L \xrightarrow{k_1} RL$</td>
<td>(a)</td>
</tr>
<tr>
<td>$\alpha(GDP)\beta\gamma + RL \xrightarrow{k_2} \alpha(GDP)\beta\gamma RL$</td>
<td>(b)</td>
</tr>
<tr>
<td>$\alpha(GDP)\beta\gamma RL + GTP \xrightarrow{k_{in}} \alpha(GTP) + \beta\gamma GTP + GDP + RL$</td>
<td>(c)</td>
</tr>
<tr>
<td>$\beta\gamma_{IM} \xrightarrow{k_{out}} \beta\gamma_{PM}$</td>
<td>(d)</td>
</tr>
<tr>
<td>$\alpha(GTP) \xrightarrow{k_{GAP}} \alpha(GDP)$</td>
<td>(e)</td>
</tr>
<tr>
<td>$\alpha(GDP) + \beta\gamma_{IM} \xrightarrow{k_{PM}} \alpha(GDP)\beta\gamma$</td>
<td>(f)</td>
</tr>
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The higher affinity of $\alpha(GDP)$ for $\beta\gamma$. Even as the $\alpha(GDP)\beta\gamma$ heterotrimer, IM photobleaching and fluorescence recovery data show that G proteins continually shuttle between the PM and IMs (see Figure S1A in the Supporting Information, as well as Movie S1). This transient interacting nature of G proteins prevents the detection of their subcellular locations using immunofluorescence. HeLa cells expressing GFP-γ9 show a distinct PM distribution and, after activation of endogenous CXCR4 receptors, γ9 subunits translocated to IMs (Golgi and Endoplasmic Reticulum-ER), until equilibrium was reached (see Figures 1A–C, as well as Figure S2 in the Supporting Information). The addition of the CXCR4 antagonist, AMD3100, completely reversed this process, suggesting that the free γ9 subunit continuously shuttles between the PM and IMs. (See Figure 1B). This allows free γ9 to continuously sense the PM and reverse upon $\alpha(GDP)$ formation. The plot shows GFP dynamics on the PM (black), in IMs (red) and the IM/PM ratio (blue) before and after the ligand (SDF-1α) addition and after the antagonist addition (Figure 1B). Since GPCR activation results in GFP-γ9 fluorescence reduction on the PM and an increase in IMs, either the fluorescence in IMs ($F_{IM}$) or the ratio ($F_{IM}/F_{PM}$) can be considered as the extent of heterotrimer dissociation, as well as GPCR activation in living cells (see Figure S1B in the Supporting Information, as well as Movie S2).

Using the fast activation and deactivation kinetics of a light sensing GPCR (blue opsin) from the vision system, the time constant of signal detection in the γ9 assay, defined as the “time delay from the stimulus to the appearance of the first detectable signal” ($\tau_{SD}$) was calculated with a statistical certainty. From a flash of light to the G protein activation by opsins, ~7.8 ms is required, with a time constant of 2.1 ms, and this is due to the formation of short-lived meta-II opsin.34 In meta-II opsin, photoconverted all-trans retinal remains bound to the opsin and the opsin-retinal conjugate activates G proteins. Opsi activation resulted in a fast βγ translocation, which reached a steady state within $t_{1/2} < 10$ s at which the rates of $\alpha(GTP)$ generation and its hydrolysis (to form $\alpha(GDP)$) became equal (see Figure S3A in

Figure 1. Receptor activation- and deactivation-induced reversible distribution of βγγ9, as a reporter of GPCR-G protein activity. (A) Four-dimensional (4D) confocal live cell images of HeLa cells expressing YFP-γ9 before and after activation of endogenous CXCR4 receptors with 100 ng/mL SDF-1α. Note the robust accumulation of GFP fluorescence in IMs (yellow arrow). (B) Plot showing the intensity of YFP-γ9 on the PM (black), in IMs (red), and the IM/PM intensity ratio (blue) during the process above. The βγ accumulated in IMs was completely reversed to the PM when CXCR4 was inhibited with its antagonist, AMD3100 (10 μM). Images on the plot show CXCR4 activation induced translocation of YFP-γ9 from the PM to IMs, while the PM-bound CFP-αo remained stationary. (C) Extent of loss and gain of FP-γ9 fluorescence by the PM and IMs. (D) Calculation of response time delay of γ9 assay determining the time from stimulation to the first detectable response. Images of mCherry-γ9 were acquired at 20 Hz and after 200 captures, a 20 ms 445 nm flash of light was used to activate blue opsin, and imaging was continued. Images of a HeLa cell at various time intervals after optical activation of blue opsin, which induced translocation of mCherry-γ9 from PM to IMs. Note the detectable appearance of mCherry in IMs in the image at 477 ms. (E) Plots showing (left) $F_{IM/PM}$ (black) and $F_{PM}$ (red) vs time (in milliseconds) and (right) an expanded section of the first plot, showing the time of stimulus and the increase in $F_{IM/PM}$ above the baseline. The earliest detectable signal (SD) was reached within 491 ± 65 ms. Scale bars = 10 μm (n = 7) (mean ± standard error (SEM)).

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the Supporting Information, as well as Movie S3). Once opsin activation is terminated, the reversal of βγ9 to the PM should therefore be dictated by the rates of βγ9 shuttling and α(GDP) generation (Figure S3A). To overcome the insufficiency of temporal resolution at 1 Hz time-lapse imaging, a fast image acquisition-optical activation protocol was adopted to image mCherry-γ9 and optically activate blue opsin at 20 Hz (see Figures 1D and 1E). A 10 ms, 445 nm light pulse (500 nW/μm²) resulted in a tSD value of 491 ± 65 ms, where signal-to-noise ratio is given as FIM/PM/(FIM/PM (before activation)) = 1.25 (see Figure 1E). This suggests that GPCR and G protein activation can be detected using the γ9 assay within a subsecond tSD. Considering the lifetime of an active meta-II opsin (~40 ms), within 40 ms of the last 445 nm pulse, the opsin should become completely inactive.35,36 Therefore, together with additional experiments, kinetics of βγ9 reversal can be used to examine the rates of α(GTP) hydrolysis and specific GAP activities in living cells.

Quantification of Ligand-Concentration-Dependent Activation of GPCRs Using the γ9 Assay. Both αi/o and αs coupled receptors can induce a profound βγ9 redistribution upon activation, while αq-coupled receptors produce only a marginally detectable signal (see Figure S3B). In addition, αq-coupled receptor activation induces a significant change in cell morphology, introducing artifacts in live cell assays (see Movie S4). To examine the ligand-concentration-dependent activation of endogenous α2 adrenergic receptors (α2-ARs), 37 HeLa cells expressing GFP-γ9 were activated with norepinephrine (NE), carbachol, and isoproterenol (Iso), respectively (see Figure 2, as well as Figure S3C). As the concentrations of these ligands increase, a gradual increase in the fluorescence in IMs and a decrease in the PM, compared to the basal level, was observed (see Figure 2A, as well as Movie S5). The plots show that FIM/PM is sensitive to a broad range of concentrations, ranging from the nanomolar level to the micromolar level [NE]-s. Despite the
transient expression of γ9 and subsequent heterogeneous GFP expression among cells, once normalized to a basal level fluorescence (before NE addition), all of the cells showed a uniform dose–response relationship. Regardless of having a slightly different expression of GFP-γ9 in the two cells in Figure 2A, a clear GFP intensity change on the PM and IMs can be observed in both cells. The FIM/PM data, at steady state with no further accumulation of βγ in IMs, were used to construct the dose–response curve (see the plot in Figure 2A). Fitting the experimental data with the DoseResp function (OriginLab) resulted in a submaximal concentration of 2.13 ± 0.88 μM, which is equivalent to the EC50 value of NE in this single cell assay. In vitro ligand response studies with mesenteric vascular smooth muscle contraction in rats have shown that these tissues respond to NE with an EC50 value of 400 nM.38 Given that the γ9 assay is performed in cultured single cells, there are several reasons that can cause this discrepancy in the EC50 values, such as (i) the limits of detection of the imaging sensor for fluorescently tagged free γ9, (ii) receptor concentration on the PM, and (iii) molecular considerations inherent to βγ generation, such as its limited lifetime governed by the lifetime of α(GTP) and the diffusion time of NE in the media. After each experiment, the NE-containing media was replaced with fresh imaging buffer and a complete reversal of γ9 back to the PM was observed, indicating that no significant receptor desensitization occurred during the experiment.

**Dynamic Sensing of the Environment by the Receptor-Bound Ligand.** Equation (a) in Table 1 shows the equilibrium in RL complex formation, indicating the reversible exchange of the ligand in the bulk media with the binding pocket of the receptor. This environment sensing mechanism was examined using the γ9 assay to determine if the gradual reduction of ligand concentration allows the reaction to reach a steady state with a new equilibrium. HeLa cells expressing GFP-γ9 were exposed to a saturating concentration of the ligand, and allowed 200 s to reach steady state before stepping down the concentration. Images of the subcellular regions show the return of IM-bound GFP-γ9 to the PM, once the [NE] reaches a subnanomolar value (~0.5 nM) (see Figure 2B). The kymograph of a one-pixel-wide horizontal cross section (orange line) of the cell shows the change in GFP intensity during the gradient dilution of NE. The scale below the image shows [NE] at different time points. The plot shows the corresponding changes in FIM/PM during the gradual dilution of 10 μM NE. During this process, the dose–response curve of NE is shifted to the left, resulting in a lower EC50 value (859 ± 79 nM), compared to that of the gradient addition, suggesting a bistable receptor activation–deactivation behavior (Figure S4 in the Supporting Information). A similar observation has been made in previous studies, which explained using the ability of ligands to move among receptors, as a result of the dilution.39,40 The data from mathematical modeling also indicate a bistable dose–response relationship, which can be attributed to differences in the “on” and “off” rate constants of the ligand–receptor interaction (see Figure 2C).

The sensitivity of the γ9 assay to detect the gradual inhibition of activated α2-AR was tested in HeLa cells expressing GFP-γ9 treated with 10 μM NE. Cells with fully activated α2-AR were exposed to 0.5 nM to 20 μM yohimbine at intervals of 200 s (see Figure 2D, as well as Movie S6). In order to visualize the cumulative fluorescence changes on the PM and in IMs (orange and blue regions of interest (ROIs)), time stacks of the cropped ROIs were created (Figure 2D). The time stack image of IMs show a gradual reduction, while that of the PM shows a gradual increase in GFP as [yohimbine] increases. Subcellular confocal images show the distribution of GFP fluorescence before and after NE and after the addition of 20 μM yohimbine. Average dose response curve from multiple cells shows an effective inhibitory action of yohimbine, ranging from 1 nM to 1 μM with the IC50 value of 59 ± 4 nM (see plot in Figure 2D). In the presence of an agonist, using an in vitro assay, it was reported that yohimbine is effective in the concentration range from 10 pM to 100 nM, with an IC50 value of ~5 nM.41 While the higher IC50 value of yohimbine observed in single cells can be explained by the same reasons discussed above, the sigmoidal dose–response curve has the same 100-fold range as reported in the in vitro assay.41 Therefore, the higher range for the signaling inhibition appears to be physiological and not due to the lack of sensitivity in the γ9 assay.

**Reversible γ9 Distribution as a Universal Assay for αi/o- and αs-Coupled GPCR Activity.** GPCR and G proteins appear to form overlapping microdomains on the PM, indicating that they either are precoupled or exist in close proximity (Figure S5 in the Supporting Information). This observation is common for a wide variety of αi/o-, αs-, or αtq-coupled receptors. In order to examine if γ9 can be used to measure the activation and deactivation of αi/o- or αs-coupled GPCRs in general, a series of experiments with heterologously expressed M4 muscarinic receptors and β1 adrenergic receptors (β1-ARs) were conducted. In cells, expressing M4-mTurquoise and GFP-γ9 showed a dose-dependent redistribution of γ9 between the PM and IMs upon gradient-addition (Figure S6A in the Supporting Information), as well as dilution of carbachol (Figure S6B). Similar to a typical dose response curve, plots show that the γ9 response is sensitive to carbachol concentrations ranging from 10 nM to 10 μM with a nonlinear relationship. The plots were fitted with the DoseResp equation with a nonlinear Hill slope, as described in Figures 2A–C. During the activation and deactivation of M4-muscarinic receptors, the dose–response behavior observed was similar to that of NE (see Figures S6A and S6B). The curve fitting resulted in a EC50 value of 378 ± 65 nM for carbachol additions, which was shifted to the left with an EC50 value of 71 ± 12 nM during dilution experiments. An in vitro study conducted on tissues extracted from guinea-pig ileum has shown a EC50 value of 177 nM for carbachol, indicating the comparable sensitivity of the γ9 assay to that of the in vitro assay.42 Similarly, the activation of β1-AR in HeLa cells expressing β1-AR-CFP and GFP-γ9 with Iso concentrations from 0.1 nM to 16 μM produced a dose-dependent FIM/PM response with an EC50 value of 158 ± 60 nM (see Figure S6D). A chemiluminescence assay to examine the ability of Iso to induce the luciferase expression conducted in cardiomyocytes has resulted in an EC50 value of ~400 nM, indicating that, for certain ligand–receptor systems, γ9 assay can outperform the conventional methods.43 Serial dilution of Iso from 10 μM to 0.5 nM and examination of GFP-γ9 return from IMs to the PM resulted in a slightly left-shifted dose–response curve with an EC50 value of 120 ± 6 nM (see Figure S6E). Similar to the modeled dose–response curves for NE, the simulation conducted assuming equations in Table 1 has resulted in bistable dose–response curves for carbachol and Iso, suggesting a conservation of molecular mechanisms among different receptor families (see Figures S6C and S6F). Every αi/o- or αs-coupled receptor tested so far with the γ9 assay, including the μ and κ opioid, C5a and D2 dopamine showed a robust γ9 translocation upon receptor activation (see Figure S7 in the Supporting Information). The crystal structure of beta 2 adrenergic receptor...
(β2-AR) (Protein Data Bank No. 3SN6) with Gαs heterotrimer shows that only Gα interacts with the receptor, while neither Gβ nor Gγ show physical interactions with the GPCR. Collectively, these data suggest that the heterotrimers that contain γ9 universally interact with all GPCRs, and this assay is universally sensitive to ligand-concentration-dependent αi/o- and αs-coupled GPCR activation.

The ability of γ9 assay to distinguish differences in the ligand strength was tested using agonists for α2-ARs; Tizanidine and NE. A study conducted using rabbit aortic strips showed that NE is more efficacious than Tizanidine.45 The γ9 assay shows that 10 μM NE can increase the FIM/PM response in cells that are already activated with 10 μM Tizanidine, while 10 μM Tizanidine reduces the FIM/PM response of cells that are preactivated with 10 μM NE (see Figure S8 in the Supporting Information). These data demonstrate that the γ9 assay can distinguish between strong and weak agonists for the same receptor.

### Computational Prediction of the Behavior of G Protein Species during GPCR Activation.

The simulated dose–response curves show that the predicted free βγγ generation precisely follows the experimental FIM/PM value, indicating that the model may accurately predict the signaling in vivo (Figure 2C, as well as Figures S6C and S6F). Additional simulations were conducted to capture the dynamics of molecular entities in the reactions in Table 1. As justified above, while FIM/PM reflects free βγγ generation, FPM provides a cumulative measure of FP-γ9 (βγγ in the heterotrimer + βγγ interacting with effectors + free βγγ). When heterotrimer dissociation results in free βγγ generation, a loss of FPM was observed, which was due to βγ9 translocation to IMs (see black trace in Figure 1B), while both FIM and FIM/PM were increased (see red and blue traces, respectively, in Figure 1B). Interestingly, the model shows a rapid increase in free βγγ on the PM immediately after GPCR activation, while βγ9 in IMs gradually increases (see Figure 3A). At low ligand concentrations (100–1000 nM), a relatively slow heterotrimer generation may allow free βγγ on the PM to reach steady state, because Rtrans = Rass + Rtrans (eq 1). However, βγγ in IMs reaches the same steady state 35–40 s later, possibly due to the lag time in translocation. At higher ligand concentrations (2500–100 000 nM), excessive heterotrimer dissociation and free βγγ overloading can result in an abrupt increase in free βγγ on the PM within the first few seconds (3–8 s). This short-lived peak of free βγγ is followed by a ligand-concentration-dependent decay with the decay constant (t1/2) of 10 s−1 (at 100 000 nM), 12 s−1 (at 2500 nM), and 13 s−1 (at 1000 nM), until the βγγ concentrations on the PM and IMs reach the same value. Simulation of the heterotrimer dissociation (Figure 3B) and α(GTP) generation (Figure 3C) shows a tight relationship between the increase in α(GTP) and corresponding heterotrimer dissociation at higher ligand concentrations. However, when [L] < 1000 nM, a slight deficit in α(GTP) concentration, compared to the amount of heterotrimer dissociation, is observed. This may be a result of GTP hydrolysis, because of the maximum GAP activity on a limited [α(GTP)]. This further can lead to an almost-zero [α(GTP)] at low ligand concentrations. Therefore, such a specific ligand concentration may not show signaling associated with α(GTP), although the receptors are active. For instance, at a ligand concentration of 1000 nM, the model shows that, within 5 s, the amount of heterotrimer consumption, as well as [α(GDP)], reach ~90 nM, while [α(GTP)] only reaches ~7 nM (see Figures 3C and 3D). At the same time, the PM appears to have ~10-fold more free [βγγ], compared to [α(GTP)], biasing the pathway toward βγγ with only a minor α(GTP) activity. This observation can be explained by assuming an optimum GAP activity, where Rcycle > Rdis and further assuming that the generated free βγγ subunits are still in complex with effectors or in IMs, preventing heterotrimer regeneration (eqs 1–5). Simulated dynamics of βγγ, α(GTP), α(GDP) and the heterotrimer after activation of β1-AR and M4 muscarinic receptors also show analogous behaviors (see Figure S9 in the Supporting Information). Three-dimensional (3D) molecular entity concentrations–time–[L] plots can be used to further analyze the cross talk between associated molecules for further interpretation of the process (see Figure S10 in the Supporting Information).
Qualitative Multicellular Screening of αi/o- and αs-Coupled GPCR Activation. High-magnification fluorescence imaging of FP-γ9 and monitoring of the entire-cell fluorescence change allowed determination of GPCR activation in single cells (see Figures S11A–C in the Supporting Information). Using the same images, calculation of the cumulative fluorescence change in multiple cells also showed a robust increase in fluorescence upon GPCR activation (Figure S11C). Using these results as the basis, the feasibility of adopting γ9 assay for HTS to detect GPCR activation using epifluorescence microscopy was tested. Time-lapse images of HeLa cells expressing GFP-γ9 and appropriate GPCRs (when applicable) were captured using an epifluorescence microscope with a 10× objective, before and after the agonist addition. Images were digitally magnified (by a factor of 5) and IMs of single cells were selected as ROIs to examine the βγ9 redistribution (Figure 4A). A significant increase in fluorescence in IMs was observed upon addition of NE, indicating the corresponding GPCR activation, as well as heterotrimer dissociation (black trace in Figure 4A). However, the heat map images clearly show an increase in IM fluorescence, and, without ROI selection, no change was observed in the whole-cell fluorescence (red trace in Figure 4A). Similarly, cumulative time averages of the entire-field fluorescence showed no intensity change upon agonist addition (Figure 4B). The large depth of focus that covers the entire height of cells in epifluorescence imaging may have prevented detection of the changes in cumulative fluorescence in GFP-γ9 in cells. Therefore, confocal imaging was used to observe an optical cross section across the girth of the cell, only to capture the GFP fluorescence of the entire cell (Figure 4B). The large depth of focus that covers the entire height of a HeLa cell, even with confocal imaging, detection of cumulative fluorescence in IMs was observed upon addition of NE, indicating the corresponding GPCR activation (Figure S11C). Using these results as the basis, coupled GPCR activation (Figure S11C). Using these results as the basis, confocal imaging was unable to capture a change in whole-cell fluorescence upon GPCR activation of M4-muscarinic and β2-ARs, using 10× objective, without ROI selection. Images were digitally magnified and IMs were imaged under a 10× objective, using a DAPI cube before and after ligand addition. Figure S12 shows the corresponding data for endogenous α2-AR-activation-induced GFP-γ9 redistribution can be clearly seen in subcellular regions. The plot shows no change in the cumulative fluorescence of the entire cell (red). However, ROI-based analysis (the yellow region) shows that receptor activation induces an increase in fluorescence in the IMs (black). The heat map images show the increase in IM fluorescence after NE addition. (B) Raw (unprocessed) images of the entire field of vision under a 10× objective before and after NE. The ligand addition only induces a barely visible fluorescence intensity change, while the plot shows that the NE addition does not change the cumulative fluorescence. (C) Intensity-thresholded images of the entire field of vision before and after exposure to 10 μM NE. Yellow arrows show a visible increase in single-cell fluorescence after NE. The corresponding plots show that the thresholding filters out the noise, as shown in Figure S11D, and show an increase in the signal after ligand addition. Figure S12 shows the corresponding data for activation of M4-muscarinic and β1-ARs. (D) Plate reader experiments show HTS capabilities of the γ9 assay. A 12-well glass-bottomed plate, each well-seeded with 0.1 million HeLa cells, were transfected with GFP-γ9. Cells were imaged using a Cytation 5 Cell Imaging Multi-Mode Reader with a 20× objective, using a DAPI cube before and after activation of endogenous α2-ARs, using 10 μM norepinephrine (NE). The top row shows images before and after ligand addition. The bottom row shows the thresholded images used to quantify the GPCR-activation-induced fluorescence intensity changes. Note the increase in fluorescent intensity in IMs after ligand addition (white arrows). The plot shows the normalized cumulative fluorescence, indicating a ~60% increase in intensity after NE addition (n = 6, *p = 0.001).
intensities capturing the activation of all three receptors examined; α2-AR, M4 muscarinic, and β1-AR (as shown in Figure 4C, as well as Figure S12 in the Supporting Information).

**γ9 Assay Is HTS-Capable.** Pre- and post-agonist GFP-γ9 images captured using a Cytation 5 Cell Imaging plate reader show a clear accumulation of GFP in IMs after activation of endogenous α2-AR with 10 μM NE (Figure 4D, top row). Upon thresholding, the post-agonist images show a significant intensity increase (see Figure 4D, bottom row and the plot). This demonstrates that the γ9 assay can be used for HTS of compounds for their ability to activate GPCRs.

**γ9 Acts as a Spatiotemporal Sensor for Subcellular GPCR and G Protein Activation.** Once exposed to a spatially restricted extracellular ligand, it is not clear how GPCRs in a cell confine the activities of G proteins to govern asymmetric signaling and behaviors such as cell migration and neuron development. A dearth of approaches to spatiotemporally control signaling in single cells hinders mapping the information flow from the stimulus onset to the GPCR, G protein, and effector activation, as well as the reversal of the processes after stimulus termination. Therefore, such events have mostly been explored using computational models. Using the γ9 assay, the accessibility of activated receptors in a localized region to the heterotrimer pool and the span of the G protein activation envelope has been explored. A diffraction-limited line optical input of 445 nm described in the Methods section was used to induce the confined activation of GPCRs in a selected PM region. Time-lapse images of a HeLa cell expressing mCherry-γ9 show that localized opsin activation results in a confined loss of mCherry (at 42 s) (Figures 5A and 5B). Termination of the opsin activation results in the recovery of mCherry-γ9 on the PM (Figure 5B, recovery). To reduce the effect of inherent heterogeneity in mCherry-γ9 expression, the pixel intensities were baseline-normalized prior to the kymograph generation. The kymograph was generated using the line ROI (Figure 5C). The 3D plot of the kymograph (Figure 5D, left) show that confined GPCR activation results in an envelope of G protein loss around the active site, which is broader than the width of the diffraction-limited line optical stimuli (Figure 5C). To understand this broadening, the energy distribution around the optical stimulus was calculated (Figure 5D, right). The middle plot shows the scaled, superimposed simulated light energy distribution (d = 6 μm) and the extent of mCherry loss (~8 μm) around the activated region (Figure 5D, middle). The additional ~2 μm expansion beyond the calculated light spread reaches steady state while the localized optical activation continues. This suggests that cells can continuously maintain the confined GPCR and G protein activation for a prolonged period of time as long as the stimulus is stationary. Furthermore, transmembrane GPCRs laterally move on the PM at a velocity of ~2–3 μm/min, which is sufficiently slower than the “on” and “off” reaction rates for GPCRs. Therefore, during prolonged activations, cells can maintain a steady concentration of active GPCRs at the site of activation. Global G protein activation data show that, after termination of opsin activation, γ9 takes a relatively long time to reverse to the PM than its forward movement to IMs, likely due to the rate-limiting kinetics of GTP hydrolysis on the α subunit (see Figures S1A and S1B). This can also lead to the expansion of the region of γ9 loss beyond the activated region due to lateral movement of α(GTP) on the PM (Figure 5D). In addition, extended scattering of the light stimulus beyond estimated limits can result in the same observation.

![Figure 5. Estimation of G protein activation envelope using subcellular optogenetics and γ9 assay. (A) A HeLa cell expressing blue opsin-GFP and mCherry-γ9 shows a spatially confined activation of G proteins; the white line indicates the region of the 445 nm stimulus pulsed at 0.5 Hz (scale bar = 5 μm). (B) Magnified view of the region within the white box in panel (A). Upon optical activation (OA) of blue opsin in the line region, mCherry-γ9 on the PM translocates to IMs, resulting in a transient mCherry loss at the activated PM region. The distribution of mCherry on the PM before OA (t = 35 s), during OA (t = 42 s), and the recovery after termination of the activation pulse. (C) Kymograph generated in ImageJ, using the multiple kymograph tool, showing mCherry-γ9 loss on PM and plotted with distance on the PM versus time. Red lines indicate the start and end of OA (from t = 42 s to t = 100 s). Yellow dashed line indicates the location of OA on the PM region over time. (D) Three-dimensional (3D) surface plot generated (in ImageJ) using the kymograph shown in panel (C). Pixel intensities were normalized to the initial intensities. The higher loss of mCherry-γ9 is shown by the blue–violet area. The plot in the middle shows the distribution profile of laser light energy, which is aligned with the area of mCherry-γ9 loss. This plot shows that the radius of the envelope of γ9 loss exceeding only ~1 μm beyond the opsin-activating light distribution. The panel on the right shows the simulated light energy distribution of a 1 μm point optical stimuli of 445 nm laser (blue line). Note the distribution of light from the stimuli on the PM area with a diameter of ~8 μm, because of the scattering and diffraction limit considerations of light. Such an extended light distribution can activate blue opsin, resulting in a broader-than-expected envelope of G protein activation.](image-url)

### CONCLUSION

Contrary to limitations in current assays to detect GPCR-G protein activities, the γ9 assay described here provides (i) an enhanced detection ability of G protein activation, even at low ligand concentrations (higher detection limit); (ii) a fast sub-second rSD2; (iii) monitoring of native GPCR-G protein interactions; (iv) direct detection of G protein activation and deactivation by a GPCR, (v) detectability of spatially and temporally confined GPCR, G protein activation; and (vi) adaptability for HTS. Therefore, a combination of these characteristics in the γ9 assay allows quantitative measurement of receptor–ligand interactions and their dose–response relationships. Data further show that cells can achieve a series of graded G protein activation states by reaching multiple dose-dependent steady states. This may allow cells to control a series of unique dose-dependent signaling events for a single ligand. The computational model...
also concur with the existence of these multiple steady states. Furthermore, both experimental and modeling data suggest a bistable dose—response behavior in G protein activation—deactivation process. Bistability in the GPCR activation—deactivation process can help cells to sustain responses by tolerating the temporal fluctuations and switch between states when only significant changes in the ligand concentration occur. Measurement of the G protein activation envelope suggests that, during spatially restricted GPCR activation, cells have mechanisms in place to contain the localized active regions, so that cells can continually maintain internal signaling gradients and direct polarized cell behaviors. Overall, the studies presented here conclusively demonstrate that, reversible polarized cell behaviors. Overall, the studies presented here continually maintain internal signaling gradients and direct place to contain the localized active regions, so that cells can continually maintain internal signaling gradients and direct polarized cell behaviors. Overall, the studies presented here continually maintain internal signaling gradients and direct

**REFERENCES**


**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02512.

Detailed experimental methods, differential equations and parameters used in the computational model, and Figures S1–S12 (PDF)

IM photobleaching and fluorescence recovery data show that G proteins continually shuttle between the PM and IMs (Movie S1) (AVI)

The fluorescence in IMs or the ratio can be considered as the extent of heterotrimer dissociation as well as GPCR activation in living cells (Movie S2) (AVI)

Opsin activation resulted in a fast βγ9 translocation, which reached a steady state within t1/2 < 10 s, at which the rates of α(GTP) generation and its hydrolysis (to form α(GDP)) became equal (Movie S3) (AVI)

αq-coupled receptor activation induces a significant change in cell morphology, introducing artifacts in live cell assays (Movie S4) (AVI)

As the concentrations of these ligands increase, a gradual increase in the fluorescence in IMs and decrease in the PM is observed, compared to the basal level (Movie S5) (AVI)

Cells with fully activated α2-AR, exposed to 0.5 nM to 20 μM yohimbine at intervals of 200 s (Movie S6) (AVI)

**NOTE ADDED AFTER ASAP PUBLICATION**

This paper originally published ASAP on November 14, 2016. Due to a production error, an incorrect graphic was placed for Figure 3. The correct graphic was placed, and the paper was reposted on November 15, 2016.