A New Generation of FRET Sensors for Robust Measurement of Gi1, Gi2 and Gi3 Activation Kinetics in Single Cells


Published in:
PLoS One

DOI:
10.1371/journal.pone.0146789

Link to publication

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
A New Generation of FRET Sensors for Robust Measurement of Gαi1, Gαi2 and Gαi3 Activation Kinetics in Single Cells

Jakobus van Unen1, Anette D. Stumpf2, Benedikt Schmid2, Nathalie R. Reinhard1,3, Peter L. Hordijk1,3, Carsten Hoffmann2, Theodorus W. J. Gadella, Jr.1, Joachim Goedhart1*

1 Swammerdam Institute for Life Sciences, Section of Molecular Cytology, van Leeuwenhoek Centre for Advanced Microscopy, University of Amsterdam, P.O. Box 94215, NL-1090 GE, Amsterdam, The Netherlands, 2 Bio-Imaging-Center/Rudolf-Virchow-Zentrum and Department of Pharmacology and Toxicology, University of Wuerzburg, Versbacher Strasse 9, 97078, Wuerzburg, Germany, 3 Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, NL-1066 CX, Amsterdam, the Netherlands

* j.goedhart@uva.nl

Abstract

G-protein coupled receptors (GPCRs) can activate a heterotrimeric G-protein complex with subsecond kinetics. Genetically encoded biosensors based on Förster resonance energy transfer (FRET) are ideally suited for the study of such fast signaling events in single living cells. Here we report on the construction and characterization of three FRET biosensors for the measurement of Gαi1, Gαi2 and Gαi3 activation. To enable quantitative long-term imaging of FRET biosensors with high dynamic range, fluorescent proteins with enhanced photophysical properties are required. Therefore, we use the currently brightest and most photostable CFP variant, mTurquoise2, as donor fused to Gαi subunit, and cp173Venus fused to the Gγ2 subunit as acceptor. The Gαi FRET sensors constructs are expressed together with Gβ1 from a single plasmid, providing preferred relative expression levels with reduced variation in mammalian cells. The Gαi FRET sensors showed a robust response to activation of endogenous or over-expressed alpha-2A-adrenergic receptors, which was inhibited by pertussis toxin. Moreover, we observed activation of the Gαi FRET sensor in single cells upon stimulation of several GPCRs, including the LPA2, M3 and BK2 receptor. Furthermore, we show that the sensors are well suited to extract kinetic parameters from fast measurements in the millisecond time range. This new generation of FRET biosensors for Gαi1, Gαi2 and Gαi3 activation will be valuable for live-cell measurements that probe Gαi activation.

Introduction

The Gαi subclass of heterotrimeric G-proteins consists of 3 members in humans, Gαi1,2,3 encoded by the genes GNAI1, GNAI2, GNAI3 [1] and is activated by a wide range of G-protein coupled receptors. The Gαi family of G-proteins have been implicated in numerous pathologies, from involvement in obesity and diabetes [2], functions in the immune system [3] to their
critical roles in several stages of cancer biology [4–7]. Activation of Gαi is predominantly linked to the inhibition of adenylate cyclases, which leads to decreased cAMP accumulation in cells. However, activation of Gαi has more recently been connected to several other molecular effectors, including PI3K/Akt [8,9], ERK [10] and c-Src [5].

The measurement of Gαi activation is classically performed by measuring the inhibition of forskolin-induced cAMP production. Similar to phosphorylation assays further downstream, such measurements lack spatial resolution, have limited temporal resolution and can be influenced by considerable crosstalk and amplification or desensitization of the signal [11–13].

To investigate G-protein activation in a direct way with high spatiotemporal resolution, genetically encoded FRET (Förster Resonance Energy Transfer) or BRET (Bioluminescent Resonance Energy Transfer) biosensors can be employed [14]. These methods are based on the measurement of the non-radiative energy transfer from a donor molecule to an acceptor molecule, which only takes place when donor and acceptor are in close proximity of each other (<10nm). Changes in distance or orientation between the donor and acceptor dipole result in changes in the RET efficiency, which can be quantified.

The RET techniques allow for single cell recordings of the kinetics with millisecond resolution, which can be used to identify cell-to-cell heterogeneity and record pharmacokinetic parameters. Moreover, this approach has the potential to record GPCR activation under physiological conditions in vivo [15].

Gαi has been successfully tagged at different internal sites with luciferase and used for BRET measurements between different Gαi subunits and GPCRs [16–19] or Gγ [19]. FRET measurements between fluorescently tagged Gαi1, Gαi2 and Gαi3 and Gβ [20] or Gγ [21] have also been performed.

To perform FRET measurements, a spectrally overlapping donor and acceptor pair is necessary [24], and it was previously shown that the use of brighter fluorescent proteins can improve the sensitivity of FRET biosensor measurements [22,23]. In order to obtain robust FRET measurements that probe Gαi activation, we have made fusions of Gαi1, Gαi2, and Gαi3 with the brightest and most photostable monomeric cyan fluorescent protein (CFP) currently available, mTurquoise2 (mTq2) [25]. As acceptor we have used circular permuted Venus (cpVenus) fused to Gγ2, which has previously been used as acceptor in a single plasmid Gαq FRET sensor [26]. We use a single plasmid strategy to facilitate transfection protocols and allow a well-defined donor and acceptor expression ratio in cells [27]. This expression strategy should greatly facilitate the use and reproducibility of the results of these sensors. We present the construction strategy, validation and characterization of this new generation of FRET sensors for the activation of Gαi1, Gαi2, and Gαi3. These biosensors are very well suited for live cell microscopy and can be used for fast kinetic measurements in the millisecond range, allowing pharmacological drug characterization and determination of on- and off-kinetics for agonists and antagonists at Gαi-coupled GPCRs.

Results

Generation of constructs

The monomeric CFP variant mTurquoise2, the preferred donor in CFP-YFP FRET pairs due to its high quantum yield and photostability [25], was inserted into Gαi1 after the alanine on position 121 in the αB-αC loop. This insertion site that was previously shown to retain nucleotide exchange and GTPase reaction rates comparable to wild-type protein [20]. Gαi1-mTq2 displays plasma membrane localization when expressed in HeLa cells (Fig 1A). Trial experiments were performed to examine whether Gαi1-mTq2 is suitable for measuring, by means of FRET, the activation of the heterotrimeric G-protein complex upon GPCR activation. To this end,
Fig 1. Development and characterization of the new G_{αi1}-sensor. (A) Representative image showing the plasma membrane localization of G_{αi1} fused to mTurquoise2-Δ9, expressed in HeLa cells. (B) Schematic overview of the plasmid containing pGβ-2A-YFP-Gγ2-IRES-Gαi1-CFP, driven by a CMV promoter. The inset shows the DNA sequence encoding the end of the IRES sequence and the start of the G_{αi1} sequence. The proposed protein translation is shown in the line below the DNA sequence (single letter abbreviations of the amino acids). (C) Confocal images of the localization of G_{αi1}-mTurquoise2-Δ9 (top row) and cp173Venus-Gγ2 (bottom row) in HeLa cells, for variant 1.0 (left column) and variant 2.0 (right column) of the G_{αi1}-sensor. (D) Quantitative co-
both Gβ as well as Gγ can be tagged with an acceptor to measure heterotrimeric G-protein activation by FRET [20,21]. We have previously shown that the highest FRET contrast, for Gαq, is obtained with cpVenus-Gγ2 [26]. Since Gαi has high structural homology to Gαq and the site at which mTurquoise2 is inserted is similar, we decided to employ the same FRET acceptor here. Hence, to introduce a labeled heterotrimeric G-protein complex in cells, we co-expressed Gαi1-mTq2 together with the FRET acceptor cpVenus-Gγ2 and untagged Gβ1 [26]. Stimulation of the co-expressed α2 adrenergic receptor (α2AR) with UK14,304 shows a rapid increase in CFP fluorescence and a concomitant loss of sensitized emission from the YFP channel, reflecting a loss of FRET. The loss of FRET can be interpreted as a dissociation of the heterotrimer or a change in relative conformation of donor and acceptor. To enable robust co-expression of the different components of the multimeric FRET sensor, we introduced the subunits on the same plasmid, as we reported previously for a Gαq sensor (Fig 1B) [26]. This strategy uses a viral 2A peptide and an IRES sequence to ensure optimal relative levels of the donor (Gαi1-mTq2) and acceptor (cpVenus-Gγ2) within single cells, while minimizing cell-to-cell expression heterogeneity in the sample. After generating our first variant, we noticed that Gαi1-mTq2 was mislocalised in the cytoplasm in many cells (Fig 1C). The Gαi1 subunit is myristoylated and requires a glycine residue immediately following its starting methionine. Detailed inspection of the plasmid sequence revealed an additional starting codon for the Gαi1-mTq2 upstream of the native start-codon, generated by the IRES sequence (Fig 1B). We hypothesized that in our first variant of the sensor, most of the Gαi1-mTq2 protein produced was translated from the upstream methionine in the IRES sequence, which does not result in myristoylated protein. We removed the upstream methionine by whole-vector PCR (see material and methods), which only leaves the native starting codon of Gαi1-mTq2, followed by a Glycine providing the consensus sequence for myristoylation. Indeed, after transfecting cells with the new plasmid, we observed correctly localized Gαi1-mTq2 (Fig 1B and 1C). A Gα2-sensor and Gα3-sensor were constructed in a similar way. To examine the co-expression of the three subunits (Gαi1-mTq2, Gβ1 and cpVenus-Gγ2) from a single plasmid versus three separate plasmids, we quantified the CFP and YFP fluorescence in these two experimental conditions (Fig 1D). The CFP and YFP fluorescence in the single plasmid strategy transfection had a coefficient of determination r² of 0.64, whereas transfections with the three separate plasmids showed a coefficient of determination r² of 0.36 between CFP intensity and YFP intensity. In other words, the correlation between CFP and YFP expression is better in the single plasmid configuration, indicating a clear advantage of this design. An additional advantage of this plasmid is the 3:1 protein expression upstream and downstream of the IRES sequence, which has previously been shown to result in a preferred donor (CFP) and acceptor (YFP) expression ratio for an analogous Gαq FRET sensor [27]. Finally, the single plasmid constructs will simplify introduction into primary cells, the generation of stable cell lines or transgenic organisms with Gαi-sensors.

Performance in GPCR activation assays

To test the new Gαi1 biosensor in live cell imaging, we employed a well-characterized GPCR known to couple to Gαi1, the α2 adrenergic receptor (α2AR). HeLa cells, shown before to contain the α2AR endogenously [20], were transfected with the Gαi1 biosensor. Upon addition of 10μM UK14,304 we observed a robust loss of FRET by measuring the ratio between the YFP and CFP fluorescence of the Gαi1 biosensor, which was reversed back to baseline by the
addition of 60μM of the α₂-AR antagonist Yohimbine (Fig 2A). Pertussis toxin (PTX) has been shown to inactivate Ga₄ signaling in cells via ADP-ribosylation of the Ga₄ subunit [28], which prevents its interaction with GPCRs. The activation of the Ga₄ was completely abolished by overnight incubation with PTX, showing that Ga₁₄-mTq2 protein fusion is still PTX-sensitive (Fig 2A). To confirm that the sensor can be used to assay Ga₁₄ activation of endogenous receptors in primary cells, we repeated this experiment in HUVEC (human umbilical vein endothelial cells). Addition of a well-known stimulant for HUVECs, S1P [29], caused a sustained decrease in FRET ratio of the Ga₁₄-sensor (Fig 2B), overnight treatment with PTX completely abolished this response. Next, to investigate how robust the Ga₁₄ sensor performs on other GPCR activation assays, we tested a variety of GPCRs shown to couple to Gaᵢ. The bradykinin 2B (BK₂B) receptor [30], lysophosphatidic acid 2 (LPA₂) receptor [31,32] and muscarinic acetylcholine 3 (M₃) receptor [33,34] were co-transfected with the Ga₁₄ biosensor in HeLa cells. Upon stimulation with the relevant agonists, all three receptors showed a sustained decrease in FRET ratio of the Ga₁₄ biosensor (Fig 2C). The M₃ receptor also showed a full recovery back to baseline of the FRET ratio after addition of the antagonist atropine. The M₃ receptor is mainly known for its signaling via Gaᵢq. Still, previous studies have shown Gaᵢ activation via the M₃ receptor [19,33–36], fitting with with our observations.

In the control conditions, e.g. absence of over-expressed GPCR, we stimulated HeLa cells with the relevant agonist and antagonist, and we observed only a very minor response on the Gaᵢ₁ sensor in the case of LPA stimulation. This is most likely due to the activation of endogenous LPA receptors in HeLa cells [37]. When we co-transfected the β₂ adrenergic receptor (β₂AR), none of the cells showed Gaᵢ₁ activation in response to the agonist and antagonist treatment (Fig 2C). Of note, β₂AR is a classical activator of Gaᵢ but switching to Gaᵢ has been reported under certain conditions [38]. Our results fit with the only study that we are aware of that uses similar tools (BRET based sensors) and similar conditions (over-expressed β₂AR and heterotrimeric G-protein sensors) [19]. Also in that case no activation of Gaᵢ₁ was observed by β₂AR stimulation (and only little activation of Gaᵢ₂ and Gaᵢ₃, which was >10-fold lower than activation by the alpha-2C adrenergic receptor, a strong activator of Gaᵢq).

To verify the performance of the Gaᵢ₂ and Gaᵢ₃ biosensors, we transfected HeLa cells with their respective plasmids. Similar to Gaᵢ₁ biosensor experiment in Fig 2A we observed a robust loss of FRET after addition of 10μM UK14,304, and the signal returned to baseline upon addition of 60μM Yohimbine (Fig 2D). Under these experimental conditions we did not observe substantial differences in the activation kinetics or amplitude of the responses between the three different Gaᵢ subunits. Both Gaᵢ₂-mTq2 and Gaᵢ₃-mTq2 are still sensitive to PTX treatment, as shown by the abolishment of the FRET response after overnight incubation with PTX (Fig 2D).

**Fast kinetic measurements**

In order to look at the sub-second kinetics of Gaᵢ₁ activation in living cells in more detail, HEK293 cells were co-transfected with the Gaᵢ₁-sensor and the α₂AR or adenosine A₁ receptor, respectively. Using a fast perfusion system for ligand application, single-cell FRET measurements show a rapid loss in FRET ratio of more than 15% after short-term application of 20μM norepinephrine. After ligand washout, the FRET signal returns to baseline levels. This could be reproduced several times without any apparent loss in signal amplitude (Fig 3A). A similar response was observed for the adenosine A₁ receptor after short application of the endogenous ligand adenosine (30μM) (Fig 3B).

These fast FRET measurements can be used to estimate the on-kinetics of Gaᵢ₁ activation with sub second resolution (Fig 3C), as shown by a close-up of the first stimulation in the
Fig 2. Performance of the G\(_a\)\(_i\)-sensors in single cell GPCR signaling assays. (A) FRET ratio-imaging experiments in HeLa cells transfected with the G\(_a\)\(_i1\)-sensor. Rapid loss of FRET, observed by a decreased YFP/CFP ratio, after stimulation of the cells with 10\(\mu\)M UK-14,304, an \(\alpha_2\)AR specific agonist, addition of 60\(\mu\)M Yohimbine returns the FRET ratio towards baseline levels. Overnight treatment with (100ng/mL) PTX abolishes the response on the G\(_a\)\(_i1\)-sensor in UK-14304 stimulated cells. (B) FRET ratio-imaging experiments in Huvecs transfected with the G\(_a\)\(_i1\)-sensor. A sustained loss of FRET is observed after stimulation with 500nM S1P (Sphingosine-1-phosphate). Overnight treatment with (100ng/mL) PTX abolishes the response on the G\(_a\)\(_i1\)-sensor in S1P
experiment shown in Fig 3A. The curve was fitted to a one component exponential decay function as previously described [39], resulting in an exponential time constant ($\tau$) of 1160ms.

To assess the precise on-rate kinetics of the $\alpha_i1$-sensor, cells were stimulated with saturating ligand concentrations (100µM norepinephrine or 30µM adenosine). Each individual response was fitted to a one component exponential, this resulted in average $\tau$ values for $\alpha_2$AR of 887ms and for adenosine A1 of 963ms (Fig 3D), corresponding to half-times of 614ms and 668ms respectively. These values are in good agreement with earlier observations for G-protein activation by FRET [21,40,41].

Concluding Remarks

In this manuscript we describe the design, construction and characterization of three new FRET biosensors for the measurement of $\alpha_i1$, $\alpha_i2$, $\alpha_i3$ activation. The new sensors contain a Gq subunit fused to the donor fluorophore, mTurquoise2, and the G$i$ subunit fused to cp173Venus, as it was previously shown that this combination for a G$i$ FRET sensor provides the largest dynamic range [26]. The three subunits of the heterotrimer (Gq-mTq2, $\beta$1 and cpVen-G$i$2) were configured on a single plasmid, enabling robust co-expression with a preferred stoichiometry. We show that these sensors are well suited for live cell microscopy and extracting kinetic parameters by single-cell ratiometric FRET imaging. The standardized layout of these FRET biosensors for G-protein activation will improve reliability and reproducibility of experiments within and between laboratories. This is exemplified in this paper by the robust performance of the $\alpha_i1$ sensor in three different laboratories, without optimization of the experimental conditions.

One limitation of energy transfer based biosensors for heterotrimeric G-proteins is that they depend on overexpression of the heterotrimer, which may affect the natural preference of the GPCR for a certain class of heterotrimeric G-proteins. Tagging the endogenous subunits with fluorescent proteins can potentially alleviate this.

The exquisite sensitivity of these sensors enables the robust detection of G$i$ activation in primary cells via endogenous GPCRs. Moreover, these biosensors can be used to directly compare the preferential activation patterns of $\alpha_i1$, $\alpha_i2$ and $\alpha_i3$ between different G$i$ coupled GPCRs, which can aid the development of therapeutic strategies targeting G$i$ signaling pathways [42].

Methods

Construction of fluorescent protein fusions

To insert mTurquoise2-Δ9 (abbreviated here as mTq2) [25] in the $\alpha_i1$, $\alpha_i2$ and $\alpha_i3$ proteins, a version of mTq2 with Age1 restriction sites at its N-terminal and C-terminal was constructed by amplifying mTurquoise2 with forward primer 5’-ATaccggttctATGGTGAGCAA GGGCG-3’ and reverse primer 5’-TAaccggtGATCCCGGCAGCAA GGGCG-3’. To introduce an
Fig 3. Performance of the Gαi1-sensor in kinetic measurements. (A) HEK293 cells transfected with the Gαi1-sensor and the α2AR were repeatedly stimulated with 20μM norepinephrine during intervals that are indicated by short horizontal lines. The presented data is representative for at least six different transfections performed on six experimental days. Top panel: YFP emission, center panel: CFP emission, bottom panel: corrected and normalized FRET ratio. (B) HEK293 cells transfected with the Gαi1-sensor and the Adenosine A1-receptor were stimulated with 30μM adenosine, indicated by the short horizontal line. The presented data is representative for at least six different transfections performed on six experimental days. Top panel: YFP emission, center panel: CFP emission, bottom panel: corrected and normalized FRET ratio. (C) A close-up of the on-kinetics of Gαi1 activation, showing the normalized ratio YFP/CFP with τ = 1160 ms. (D) A scatter plot showing the distribution of Tau values for α2A-adrenoceptor and A1 receptor.
FRET ratio during the first stimulation of the experiment in (A), fitted to a one component exponential decay function with tau = 1160ms and amplitude = 0.18 (R = 0.99). (D) Scatter plot showing the average exponential time constants (tau) of pooled data from (n = 10) individual fits of HEK293 cells transfected with the Gαi1-sensor and the α2AR stimulated with 100μM norepinephrine or pooled data (n = 14) from individual fits of the Gαi1-sensor and the Adenosine A1-receptor stimulated with 30μM adenosine, respectively. Error bars indicate 95% CI.

To construct variant 2.0 of the Gαi1-sensor, we performed a mutagenesis PCR with variant 1.0 as template, by amplifying with forward primer 5'-AGGTCTATATAAGCAGAGG-3' and reverse primer 5'-TATggatccAGCCCTAGAAGAGGCCGCAGT-3' to introduce a BamHI site at the C-terminus and an Ncol site at the N-terminus. Next a triple ligation was performed with the PCR product (cut with BamHI and Ncol), a vector containing pGB1-T2A-cp173Venus-Gγ [43](cut with BamHI and SacII), and a vector containing pPRIG-IRES [44] (cut with Ncol and SacII). The resulting plasmid, pGB1-2A-γFP-Gγ-β2AR-IRES-MATT-Gαi1-mTurquoise2-Δ9 (impaired plasma membrane localization), pGB1 and cp173Venus-Gγ (Fig 1B).

To create a single plasmid sensor for Gαi2 and Gαi3, we performed an overlap extension PCR [45]. Gαi2-mTq2 was amplified with forward primer 5'-acgatgataatATGGGCTGCACGTTGA-3' and reverse primer 5'-TATttctagaAGCTCAGAAGAGGCCGCAGT-3', and Gαi3-mTq2 was amplified with forward primer 5'-acgatgataatATGGGCTGCACGTTGA-3' and reverse primer 5'-TATttctagaAGCTTTACTATCGTGTTTTTT-3'. The resulting PCR products were then ligated into the Gαi1,2,3 vectors with Age1 and subsequent ligation resulted in pGαi1-mTq2 after position 121, and HsGαi2,3 tagged with mTq2 after position 114, analogous to a previously reported functionally tagged Gαi1 [20].

To create a new generation of FRET sensors for Gαi Activation

A New Generation of FRET Sensors for Gαi Activation
Cell culture and sample preparation

HeLa cells (American Tissue Culture Collection: Manassas, VA, USA) were cultured at the University of Amsterdam (Amsterdam, the Netherlands) using Dulbecco’s Modified Eagle Medium (DMEM) supplied with Glutamax, 10% FBS, Penicillin (100 U/ml) and Streptomycin (100μg/ml). All cell culture media were obtained from Invitrogen (Bleiswijk, NL).

Cells were transfected in a 35 mm dish holding a glass 24 mm Ø #1 coverslip (Menzel-Gläser, Braunschweig, Germany), using 1–2μl Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen), 0.5–1μg plasmid cDNA and 50μl OptiMeM (Life Technologies, Bleiswijk, NL). After overnight incubation at 37°C and 5% CO₂, coverslips were mounted in an Attofluor cell chamber (Invitrogen, Breda, NL) and submerged in microscopy medium (20mM HEPES (pH = 7.4), 137 mM NaCl, 5.4mM KCl, 1.8 mM CaCl₂, 0.8mM MgCl₂ and 20mM glucose). All live cell microscopy was done at 37°C.

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured at Sanquin Blood Supply (Amsterdam, the Netherlands) on FN-coated dishes in EGM-2 medium, supplemented with singlequots (Lonza, Verviers, Belgium). HUVECs were used at passage number 4 or 5. The Neon transfection system (MPK5000, Invitrogen) and a corresponding Neon transfection kit (Invitrogen) were used as transfection method. A single pulse was generated at 1300 Volt for 30ms to microporate HUVECs with 2μg cDNA, cells were subsequently seeded on FN-coated glass coverslips.

For the rapid kinetic measurements of Gαi1 activation, HEK293 cells were cultivated at the University of Wuerzburg (Wuerzburg, Germany) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, L-Glutamine (2 mM) (PAN Biotech GmbH, Aidenbach, Germany), Penicillin (100 U/ml), and Streptomycin (100 μg/ml) and kept at 37°C in a 7% CO₂ atmosphere. Cells were harvested and seeded onto D-Polylysine-coated 24 mm glass coverslips at ~40% confluency. After three hours cells were transiently transfected with 1.0μg of receptor (α2AR or adenosine A1) and 3.0 μg pGβ1-2A-YFP-Gγ2-IRES-Gαi1-mTq2 cDNA per 6-well plate using Effectene transfection reagent (Qiagen), according to the manufacturer’s protocol. Growth medium was renewed after 24h and measurements were performed after a total incubation time of 48h. The cells were kept in microscopy medium (140 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.3) and permanently superfused with this buffer or buffer supplemented with the appropriate ligand, using a computer-assisted solenoid valve-controlled rapid superfusion device (ValveLink 8.2, Automate Scientific).

Widefield microscopy

Ratiometric FRET measurements in HeLa cells (results presented in Fig 2A, 2C and 2D) were performed using a wide-field fluorescence microscope (Axiovert 200 M; Carl Zeiss GmbH, Germany) at the University of Amsterdam (Amsterdam, the Netherlands), kept at 37°C, equipped with an oil-immersion objective (Plan-Neo-fluor 40×/1.30; Carl Zeiss GmbH) and a xenon arc lamp with monochromator (Cairn Research, Faversham, Kent, UK). Images were recorded with a cooled charged-coupled device camera (CoolSnap HQ, Roper Scientific, Tucson, AZ, USA). Typical exposure times ranged from 75ms to 150ms, and camera binning was set to 4x4. Fluorophores were excited with 420 nm light (slit width 30nm) and reflected onto the sample by a 455DCLP dichroic mirror and CFP emission was detected with a BP470/30 filter, and YFP emission was detected with a BP535/30 filter by rotating the filter wheel. In the co-expression experiments, YFP was excited with 500nm light (slit width 30nm) and reflected onto the sample by a 515DCXR dichroic and emission was detected with a BP535/30 filter. Acquisitions were corrected for background signal and, for FRET ratio imaging, bleedthrough of CFP emission in the YFP channel (55% of the intensity measured in the CFP channel).
For the FRET experiments in HUVECs (results presented in Fig 2B), a Zeiss Observer Z1 microscope was used at Sanquin Blood Supply (Amsterdam, the Netherlands) with a 40x NA 1.3 oil immersion objective and an HXP 120 V excitation light source. CFP was excited through a FRET filter cube (Exciter ET 436/20x, and 455 DCLP dichroic mirror (Chroma, Bellows Falls, Vermont, USA)). The emission was directed to an attached dual camera adaptor (Carl Zeiss GmbH, Germany) controlling a 510 DCSP dichroic mirror (Chroma, Bellows Falls, Vermont, USA). Emission wavelengths between 455–510 nm are directed to an emission filter ET 480/40 (Chroma, Bellows Falls, Vermont, USA) and then captured by a Hamamatsu ORCA-R2 camera. Emission wavelength 510 nm and higher are directed to an ET 540/40m emission filter (Ludl Electronic Products, NY, USA) and then captured by a second Hamamatsu ORCA-R2 camera. Image acquisition was performed using Zeiss-Zen 2011 microscope software. All acquisitions were corrected for background signal. Acquisitions were corrected for background signal and bleedthrough of CFP emission in the YFP channel (62% of the intensity measured in the CFP channel).

For the rapid kinetic measurements of $G_{i1}$ activation (results presented in Fig 3), imaging was performed on a Zeiss Axiovert 200 inverted microscope at the University of Wuerzburg (Wuerzburg, Germany), equipped with an oil immersion 63x objective lens and a dual-emission photometric system (Till Photonics) as described before [21]. The transfected cells were excited with light from a polychrome IV (Till Photonics). Illumination was set to 40ms out of a total integration time of 100ms. CFP (480 ± 20 nm), YFP (535 ± 15 nm), and FRET ratio (YFP/CFP) signals were recorded simultaneously (beam splitter DCLP 505 nm) upon excitation at 436 ± 10 nm (beam splitter DCLP 460 nm). Fluorescence signals were detected by photodiodes and digitalized using an analogue-digital converter (Digidata 1440A, Axon Instruments). All data were recorded on a PC running Clampex 10.3 software (Axon Instruments). Resulting individual traces were fit to a one component exponential decay function to extract the exponential time constant, tau [39]. The halftime of activation ($t_{1/2}$) is defined as $\tau \times \ln 2$. In dynamic experiments, cells were stimulated with UK14,304 (10 μM), Yohimbine (60 μM), Bradykinin (1μM), LPA (1μM), Carbachol (100μM), Atropine (10μM), Isoproterenol (10μM), Propranolol (10μM), S1P (500nM), 20 μM or 100 μM norepinephrine or 30 μM adenosine at the indicated time points. ImageJ (National Institute of Health) was used to analyze the raw microscopy images. Further processing of the data was done in Excel (Microsoft Office) and graphs and statistics were conducted using Graphpad version 6.0 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com.

**Confocal microscopy**

HeLa cells transfected with the indicated constructs were imaged using a Nikon A1 confocal microscope equipped with a 60x oil immersion objective (Plan Apochromat VC, NA 1.4). The pinhole size was set to 1 Airy unit (<0.8μm).

Samples were sequentially excited with a 457nm and a 514nm laser line, and reflected onto the sample by a 457/514 dichroic mirror. CFP emission was filtered through a BP482/35 emission filter; YFP emission was filtered through a BP540/30 emission filter. To avoid bleed-through, images were acquired with sequential line scanning modus. All acquisitions were corrected for background signal.

**Supporting Information**

S1 Data. The compressed file contains all the data that was used in this manuscript. (ZIP)
Acknowledgments
We thank Eelco Hoogendoorn (University of Amsterdam) and Marten Postma (University of Amsterdam) for assisting in data analysis. We thank Jorrit Broertjes (MsC student at University of Amsterdam) and Dennis Botman (MsC student at University of Amsterdam) for generating Go_{i1}mTurquoise2 and Go_{i2}mTurquoise2, respectively. We thank Merel Adjobo-Hermans, Anna Pietraszewska and Scott Gibson for providing plasmids.

Author Contributions
Conceived and designed the experiments: JvU PLH CH JG TWG. Performed the experiments: JvU ADS BS NRR. Analyzed the data: JvU ADS BS NRR CH JG. Wrote the paper: JvU ADS BS NRR PLH CH TWG JG.

References


34. Akam EC, Challiss RA, Nahorski SR. G(q/11) and G(i/o) activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor subtype. Br J Pharmacol. 2001; 132: 950–958. doi: 10.1038/sj.bjp.0703892 PMID: 11181437


