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Characterization of Genetically Encoded FRET Biosensors for Rho-Family GTPases

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Abstract

Genetically encoded FRET-based biosensors are increasingly popular and useful tools for examining signaling pathways with high spatial and temporal resolution in living cells. Here, we show basic techniques used to characterize and to validate single-chain, genetically encoded Förster resonance energy transfer (FRET) biosensors of the Rho GTPase-family proteins. Methods described here are generally applicable to other genetically encoded FRET-based biosensors by modifying the tested conditions to include additional/different regulators and inhibitors, as appropriate for the specific protein of interest.

Keywords

Rho GTPases; FRET; Biosensors; Fluorometry; Validation; Ratiometric analysis

1 Introduction

Fluorescent biosensors for Rho-family GTPases [1–19] have revealed new aspects of biology that had been inaccessible in the past, because of their superior ability to report

dynamic changes in living cells and within subcellular compartments that render traditional approaches suboptimal. Biosensor designs have also evolved to optimize for better response, better stability, and improved optophysics. As end users, it is often easy and tempting to treat these biosensors as a monolith, without questioning the specific considerations that went into the design, validation, and characterization of these molecular constructs. As biosensor makers, we often fall into a situation whereby we pursue increased dynamic range of FRET response under very specific conditions without considering other minute molecular nuances, which may be even more important in understanding the biology of our target molecule. In order to reconcile these issues, we describe here basic guidelines for characterizing and validating these biosensor constructs. These methods are useful to not only biosensor designers but also the users of these tools. After obtaining a new biosensor designed by someone else, we often subject the sensor to these types of assays to validate in our own hands prior to performing biological experiments. This ensures a clearer understanding of how and what the sensor actually senses, and provides increased reliability for the data gathered using such tools. Biosensors are often engineered to achieve specific goals that may not be compatible with what is required in each individual circumstance, unless the limitations and features of specific biosensor are clearly understood.

In this chapter we describe these validation guidelines using Rac/Cdc42 family of GTPase biosensors as models [3, 4, 20]. These are monomeric, single-chain biosensors that contain FRET donor monomeric Cerulean1 [21], a tandem binding domain from PAK1 (PBD) that allows autoinhibitory control, circularly permuted monomeric Venus [22,23], and a C-terminally attached GTPase (see Fig. 1) [3,20]. These biosensors have been designed with good sensitivity, selectivity, ability to interact with appropriate upstream regulators, and ability to insert into correct membrane domains upon activation due to their intact C-terminal lipid modification motif. The validation and characterization approaches presented here will allow an appreciation of what exactly these sensor constructs are responding to in cells and their utilization will enable appropriate biological interpretations to be made based on their fluorescence readouts.

2 Materials

2.1 Cell Culture and Transfection

1. LinXE cell line, a derivative of HEK293T [24].
2. RAW/LR5, a monocyte/macrophage cell line [25].
3. DMEM (4.5 g/mL glucose with 110 mg/L sodium pyruvate) supplemented with 10% fetal calf serum, penicillin (100 IU)/streptomycin (100 µg/mL), and 2 mM Glutamax: Used to cultivate LinXE cells using standard cell culture procedures.
4. RPMI (with 300 mg/L L-glutamine) supplemented with 10% newborn calf serum, penicillin (100 IU)/streptomycin (100 µg/mL): Used to cultivate RAW/LR5 cells using standard cell culture procedures.
5. Opti-MEM (Invitrogen).

6. Plasmids: The biosensor constructs in pTriEX backbone or in other mammalian expression plasmid systems. Rho GTPase biosensors and their mutants are available from the author's laboratory upon inquiry. Expression constructs for various Rho GTPase upstream regulators including GEF, GAP, and GDI can be obtained from sources such as Addgene.
7. Lipofectamine 2000 reagent (Invitrogen).
8. Eugene HD reagent (Roche).
9. Dulbecco's phosphate-buffered saline (DPBS), calcium and magnesium free: 0.2 g/L KCl, 0.2 g/L KH_2PO_4 , 8 g/L NaCl, 1.15 g/L Na_2HPO_4 (anhydrous).
10. 0.01% (w/v) Poly-L-lysine stock solution: Dilute 1:10 in DPBS for use.
11. 6-Well tissue culture plates.

2.2 Fluorometry Assay

1. 6-Well tissue culture plates.
2. 3.7% Formaldehyde solution: Prepare from 37% formaldehyde stock solution in DPBS.
3. 0.25% Trypsin.
4. Spectrofluorometer with quartz cuvette and plate reader capability.

2.3 GST-PBD Pull-Down Assay

1. PAK1-PBD-agarose slurry (Cytoskeleton, Inc.).
2. Plasmids: pTriEX-EGFP-Rac1 (Q61L and T17N mutants; can be labeled with any fluorescent protein, i.e., EGFP, mCerulean, mVenus, mCherry).
3. Cell lysis buffer for pulldown: 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 50 mM MgCl_2 , 1% (w/v) Triton X-100, protease inhibitor cocktail (Sigma), 1 mM phenylmethanesulfonyl fluoride (PMSF) (add at the very last minute).
4. 5× Gel loading buffer with dithiothreitol (DTT): 0.25 M Tris-Cl pH 6.8, 10% (w/v) sodium dodecylsulfate (SDS), 0.25% (w/v) bromophenol blue, 0.5 M DTT, 50% glycerol.
5. 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting supplies/apparatus.
6. Anti-GFP antibody (Roche, clones 7.1 and 13.1).
7. Anti- β -actin antibody (Santa Cruz Biotechnology, Clone AC-15).
8. Ponceau S solution.

2.4 Imaging Experiments for In-Cell Validation

1. 25 mm Round coverslips #1.5 thickness (Warner Instruments).

2. 3.7% Formaldehyde solution made from 37% stock formaldehyde solution and DPBS.
3. BWD buffer for live-cell experiments [20, 25]: 20 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM NaHCO₃, 1 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4.
4. Ham's F-12K medium (phenol red free) (Crystalgen).
5. FluoroBrite DMEM without phenol red (Invitrogen).
6. Attotfluor chamber system or other compatible live-cell chamber system that can accommodate 25 mm coverslips, or Cell-View glass-bottom culture dishes (Greiner Bio-One).
7. Fluorescence inverted microscope for imaging FRET in living cells [26].

3 Methods

3.1 Transfection of Biosensor and Regulators

For validation and characterization, it is critical to achieve the best possible transfection efficiency and cell health.

1. Treat 6-well plates with the diluted poly-L-lysine solution for 5 min at 24 °C and aspirate prior to plating cells (no need to rinse).
2. Plate LinXE cells on poly-L-lysine-pretreated 6-well dishes at 1.25×10^6 cells per well in normal culture medium, 1 day before transfection (*see* Note 1). Plate as many wells as needed for different conditions plus one additional well for the negative control. Wells are typically set up in triplicates.
3. For transfection, follow the manufacturer's protocols for Lipofectamine 2000 reagent. We use 4 µg total DNA per well together with 10 µL per well of Lipofectamine 2000 reagent, in 100 µL Opti-MEM solutions each for dispersing the DNA and dispersing the Lipofectamine 2000 reagent. The amount of biosensor DNA as well as regulator DNA must be optimized based on titration, ensuring that the total amount of DNA is kept the same per well (using empty pcDNA3.1 vector to make up the deficit as required) (*see* Fig. 2) (*see* Note 2). The total volume of medium plus the transfection mix in wells should be 2 mL. We do not change the medium following transfection.

¹-Cells should be monodispersed for best transfection efficiency. LinXE or any other HEK293T derivative detaches easily even if no trypsin is used. However, without trypsin, the cells tend to come off in clumps, which significantly reduces transfection efficiency following replating. Thus, always trypsinize them carefully and sufficiently so that cells are detached and monodispersed.

²-Within the 4 µg DNA per well specified, one must account for different amounts and mixtures of DNA components to achieve the desired combinations of biosensors and regulators. Typically, a titration experiment of the biosensor DNA concentration should be performed first to determine the minimal concentration of biosensor DNA needed to overcome endogenous cellular GTPase regulators, especially the GDI. In the example given in Fig. 2, a Cdc42 biosensor-GDI interaction [1] is used to illustrate the saturation point of endogenous GDI binding as a function of the biosensor DNA concentration being transfected. This is always an important consideration if the biosensor is not a strong mutant version (i.e., constitutive active or dominant negative) but rather wild-type or other weaker mutants such as effector-binding mutations.

4. 24 h following transfection cells are ready for pull-down experiments (*see* Subheading 3.3). 48 h following transfection cells are ready for fluorometric assays (*see* Subheading 3.2) (*see* Note 3).

3.2 Fluorometric Analysis of Rho GTPase Biosensors

Testing biosensor mutants and responses to upstream regulators *in vitro* in fluorometry assays is absolutely critical to characterize how the sensor responds (*see* Fig. 3) (*see* Note 4). If biosensor responses are not carefully and exhaustively tested in this manner, the biosensor cannot be trusted to provide biologically meaningful readouts. We suggest that the following versions of the biosensors should be tested (Rac GTPase mutations are quoted as an example (*see* Fig. 3), analogous mutations should be used for other family members of GTPases): (1) wild-type version; (2) Q61L and G12V constitutively activated mutants (F28L can also be a good choice as a fast-cycling activated mutant); (3) T17N dominant negative mutant; (4) effector-binding mutants T35S and/or Y40C; and (5) if interested in showing the effect of GEF interaction G15A and/or D118A mutants in addition to the T17N mutant.

These versions of the biosensor should be expressed together with exogenous, excess GDI (2–4-fold excess by DNA quantity) to determine if GDI binds and reduces FRET as expected in some conditions (i.e., WT, G12V, effector-binding mutants) but not in conditions that are known not to bind GDI (i.e., Q61L, T17N for Rac1/2, R66E). The co-expression of GEF-DHPH domains (active, catalytic fragments) that are either targeting or non-targeting should also be shown. This experiment should be done with or without exogenous GDI to show (1) rescue above and beyond GDI-mediated repression of activity, and (2) in the absence of excess GDI the recovery of FRET up to similar levels as the constitutively activated mutant versions of the biosensor. Similarly, targeting and non-targeting GAPs should be co-expressed to show the attenuation of FRET in response to GAPs. *See* Note 4 for further details. In these experiments include a negative control transfected with empty pcDNA3.1 vector or equivalent for background autofluorescence correction.

3.2.1 Adherent Cell Analysis

1. Directly fix cells in 6-well dishes using 3.7% formaldehyde in DPBS, 2 mL per well. Leave at room temperature in a dark location for 20 min (*see* Note 5).

³Prior to pull-down experiments or fluorometric analysis, it is good practice to image the biosensors in fluorescence under 20–40 × magnification to observe the expression patterns. This should be used to confirm if the patterns of localizations are appropriate to the particular mutant versions (and/or with upstream regulators) being expressed. The observations are best performed using the yellow fluorescent protein (YFP) excitation/emission settings, which directly excites the FRET acceptor. Direct excitation/emission of FRET acceptor is not affected by presence or absence of FRET; thus, this can be used to track the localization of biosensors in cells.

⁴Additional GTPase mutations that may be useful can be found in Takai et al. (1994) [28]. In addition, versions of the biosensor in which a constitutively activated mutation (e.g., Q61L) is present but with H83/86D (GTPase-binding deficient mutations) in the PAK1 PBD domain [29] should be used to show the binding specificity. The H83/86D mutation within the PBD domain together with an additional Q61L constitutively activating mutation in the GTPase is also important in the pull-down experiment to determine effector competition. For the upstream regulators, exogenous GDI at 2–4-fold excess should be tested. In the regulator co-expression experiments, each GEF/GAP should be titrated to achieve the best response while determining the toxicity limits (GEF/GAP overexpressions are highly toxic). Typical range for GEF/GAP co-expression is 1:0.5–10, biosensor DNA:regulator DNA [20]. In all cases, total DNA quantity should be 4 µg per a 6-well plate, if using the Lipofectamine 2000 protocol. In the case of a deficit, the balance is achieved by using an empty, pcDNA3.1 vector.

⁵For the Rac biosensors, cellular signaling pathways are such that Rac activity is dependent on cell adhesion [30, 31]. This means that by trypsinizing and detaching cells, the signaling pathways are altered and the resulting measurements of biosensor may not be reliable. We have found this to be the case for the wild-type and other weaker mutant versions of the biosensor and when expressing the wild-type sensor together with various upstream mutants. This effect is not as obvious when dealing with the constitutively active

2. Gently rinse cells three times with DPBS, add 1 mL of DPBS, and immediately scan the plate in a fluorometer (*see* Note 6).
3. The fluorometry conditions are set up as follows: excitation wavelength = 433 nm; emission wavelengths scanned between 450 and 600 nm at 3 nm increments; and 1-s integration at the photomultiplier tube (PMT) per increment, with appropriate correction factors for the signal and the reference channels that are provided by the manufacturer. For adhered cell scanning using plate reader, the entry, intermediate, and exit slits are all set to 5 nm (*see* Note 7).

3.2.2 Detached Cell Analysis

1. Rinse cells once gently with DPBS, suction out, and add 1 mL of 0.25% trypsin per well. Ensure that the trypsin wets the entire surface of the well, then suction out excess trypsin, and incubate at room temperature for 1–2 min.
2. Using 500 μ L ice-cold DPBS, detach and resuspend cells by using a vigorous pipetting action, collect into 1.5 mL microcentrifuge tubes, and place on ice. Transfer the detached cell suspension to a 500 μ L quartz fluorescence cuvette and immediately perform an emission scan (*see* Note 8).
3. The basic setting for the detached measurement conditions is identical to adherent cell condition (*see* Subheading 3.2.1), the only difference being that slit widths of 1–2 nm are used instead of 5 nm to cut down on the incident light intensity and attenuate brightness (*see* Note 9).

3.2.3 Analysis of the Results

1. Export the data in such a way to obtain the signal/reference output (S/R). A typical reference is the photodiode output but in older fluorometer models signal from a concentrated rhodamine solution may be used as well.
2. Subtract the background autofluorescence S/R obtained from the “mock”-transfected negative control (*see* Note 10) at each emission wavelength from the raw data.

and/or dominant negative mutants as their signaling capacity is strong enough that they appear to overwhelm this effect. We have found this not to be a problem with our Rho biosensors [7, 10]. Therefore, depending on what proteins are being tested and what their native signaling pathways require, one must decide on how to measure the biosensor readout (detached or attached). The contra argument for the adhered cell measurement is a possible reduction in the signal-to-noise ratio of measurements in some cases, as compared to direct measurement of concentrated cell suspensions in quartz cuvettes, which produce the maximum signal-to-noise ratio.

⁶Most plate readers are designed to accommodate 96 wells or greater number of wells per plate. When scanning 6-well plates, we normally set up multiple sampling positions (typically 7–8) within a well of a 6-well plate and take an average of these measurements.

⁷Our fluorometer is configured with a set of double-grating-based monochromators for the excitation and emission paths; thus, an intermediate slit is needed. If a single grating system is used, entry and exit slits should be set at 5 nm.

⁸The detached cell suspensions, as a function of time on ice, will begin to aggregate and form stringy cell clusters. This will differentially affect light scattering during fluorescence emission scans. It is therefore important to pipette the samples vigorously to resuspend and break up any clusters and aggregates quickly just prior to placing the sample into the cuvette for measurement. Thus, processing one 6-well plate at a time is highly recommended.

⁹For detached cell measurements, an appropriate quartz cuvette should be used (cuvette should be able to hold 500 μ L). The slit widths of the fluorometer should be adjusted so that one does not saturate the PMT and stays within the linear range of signal measurement. Consult the manufacturer's instructions for the specific fluorometer to determine the linear limit of detection of the particular PMT.

3. Normalize the emission spectrum against the intensity at the peak of FRET donor (CFP emission at 470 nm) fluorescence emission. The FRET peak is at 525 nm and an example scan is shown in Fig. 3a (*see Note 11*).
4. Compare the resulting FRET/donor peaks at 525 nm between various conditions to see if the responses are as expected (*see Fig. 3*). Conditions where biosensor is active or being activated by upstream regulators should result in high FRET. Dominant negative mutant should yield low FRET (*see Note 12*), as should effector and GTPase-binding mutations or GAP/GDI co-expressions. Co-expression of activating GEF fragment in the absence of exogenous GDI should result in similar activation levels as the constitutively activated mutant biosensor expression (*see Note 13*).
5. Perform Western blot of the cell lysates from the conditions used in fluorometric analysis to confirm expression of full-length biosensor mutants. Confirm the expression of tagged GEF and GAP domains used in the fluorometry analysis using Western blot, additionally probing for β -actin as a loading control.

¹⁰-In both adherent and detached cell conditions, measure one well with “mock”-transfected condition, containing no fluorescence but otherwise transfected with empty pcDNA3.1 vector or equivalent. This will produce the emission spectra of cellular autofluorescence which will be subtracted from the raw data.

¹¹-Optimizations: Good transfection efficiency is critical. 24 h post-transfection, we find that individual cellular expression levels of fluorescence may be brighter but not as many cells are expressing fluorescence compared to 48 h post-transfection. Ultimately in our hands, 48 h post-transfection produces the best result. Different GEFs and GAPs could show differential strength of effects as well. Titration experiments should always be performed to find the best condition to achieve the maximum extent of response. To test biosensors other than Rho GTPases, this technique described here can be readily extended, by co-expressing various upstream activators and inhibitors and then measuring the appropriate FRET response. There has been a debate as to whether or not to normalize the fluorescence emission spectra to the donor peak emission. We do this because it makes the comparison of changes in FRET very intuitive. In addition, ratiometric FRET imaging in cells is such that FRET emission channel is normalized against the donor emission channel to calculate ratio, which is analogous to how we present this data in fluorometry. However if one wishes to see how the donor peak drops and the FRET peak rises and vice versa (as per the definition of a FRET response), then one must calibrate against the total expression levels of the biosensor by measuring the acceptor direct excitation/emission, and scale the donor excitation/emission scans according to it in order to overlay the concentration-corrected spectra.

¹²-The dominant negative mutant (T17N mutant version in the case of Rac/Cdc42 GTPases) is a very important condition to test in order to show that the biosensor is appropriately designed so that the binding domain does not spuriously bind to an otherwise inactive GTPase and produce high FRET. During design and validation of biosensors, the dominant negative condition could produce high FRET for the following reasons: (1) the difference in native affinity of the binding domain for the on versus off states of the GTPase is not high enough (not selective enough) and thus the proximity of the domains within a single-chain construct results in inappropriate interactions due to apparent high local concentration, yielding high FRET, or (2) cellular GEFs bind to the dominant negative mutant [32–34] and sterically impinge on the FRET pair of fluorescent proteins resulting in high FRET. To address the first issue, we have successfully incorporated an autoinhibitory regulation into the binding domain that reduces the binding affinity when GTPase is in the off state, but still allows efficient binding when the GTPase is activated [3]. This approach substantially reduced FRET in the dominant negative mutant, ensuring correct interaction of the domain to the active but not inactive GTPase. Additional approaches could include the use of point mutations to slightly reduce the binding affinity of the domain for the GTPase [4]. To determine if GEF binding is the cause of increased FRET in the T17N dominant negative mutant, other GTPase mutants that are known to cause strong binding to GEFs [35, 36], e.g., G15A and D118A, can be assessed for their impact on FRET. If they result in similarly high FRET levels as the T17N mutant, this issue can be typically corrected by modifying the linker structures within the biosensor (details of the structure design are beyond the scope of this discussion). In some GTPase sensor designs, only GTPase-binding defective mutants and not dominant negative mutants were used for the validation of the biosensor. This is inadequate and could produce biosensors with moderate-to-low off rates, which would result in a bias toward activation and high FRET when used in cells. Thus, a proper functional validation of biosensor must include the dominant negative mutant version.

¹³-Biosensors containing non-monomeric versions of fluorescent proteins can result in spuriously high FRET upon overexpression of the constitutively active version of the sensor. This is due to the tendency of these fluorescent proteins to dimerize and/or oligomerize coupled with the confinement of the active versions of the sensor to the plasma membrane, which reduces the degrees of freedom of its components compared to overexpression in cytoplasmic/non-membrane-targeted locations. This situation results in an aggregation-driven FRET, both inter- and intramolecularly. The extent of this effect can be determined by performing a careful titration of expression levels and measuring the FRET/donor response as a function of the concentration of the expressed biosensor, similar to Fig. 2, to best characterize the concentration cutoff to eliminate intramolecular FRET effects due to aggregation. Importantly, the monomeric versions of the biosensor should be used to sidestep this problem in order to obtain the most reliable readouts in live-cell imaging experiments where the membrane loading-associated aggregation could skew the data toward high activation.

3.3 Biosensor Effector Competition

This analysis characterizes the extent of biosensor binding to endogenous cellular effector targets, which may result in overexpression/signaling artifacts (*see* Note 14). The proximity of the components within single-chain biosensors results in an apparent high local concentration of each domain of the biosensor; thus the chance of the biosensor interacting with endogenous cellular binding targets, as opposed to the built-in affinity domain, is low. However it is important to verify that this is indeed the case for each new sensor. Here we assess the binding characteristics of the biosensor by providing exogenous binding domain in excess in a competitive binding assay.

1. Transfect the following conditions into LinXE cells as in Subheading 3.1: (a) untransfected; (b) fluorescent protein (FP)-labeled constitutively active Rac GTPase; (c) FP-labeled dominant negative Rac GTPase; (d) constitutively active version of the Rac GTPase biosensor; and (e) constitutively active version of the Rac GTPase biosensor that contains the GTPase-binding deficient (H83D-H86D) mutations in the built-in affinity domains (in this case the PAK-binding domain—PBD).
2. 24 h following transfection, collect cells by brief trypsinization, resuspend in cold DPBS, and then pellet by centrifuging at $300 \times g$ for 3 min. Keep on ice at all times.
3. Lyse the cells by adding 500 μ L of cold cell lysis buffer for pulldown (protease inhibitor cocktail and PMSF added just prior to this step), and pipette vigorously to disperse all cells. Let the cell lysis proceed on ice for 30 min.
4. Centrifuge at $20,000 \times g$ at 4 °C for 15 min to clear the lysate.
5. Transfer the supernatant to clean 1.5 mL microcentrifuge tubes. Remove and reserve 50 μ L each of the cell lysates as “10% input” fractions. Mix the 10% input fractions with 5 $\times$ loading buffer with DTT, boil at 95 °C for 5 min, and set aside for Western blotting.
6. Add 10–15 μ L equivalent of PAK-PBD agarose bead slurry to each sample tube and incubate at 4 °C with rotation for 1–2 h (*see* Note 15).

¹⁴If the GTPase built into the biosensor binds to endogenous effector targets and can signal downstream when activated (as opposed to only binding to the built-in binding domain within the biosensor), this would result in overexpression artifacts. This is suboptimal because it would directly amplify the signaling pathway of interest, thus making the biological interpretation of results difficult. Our biosensors do not bind to exogenously supplied excess binding domain when activated; thus, the overexpression artifacts are minimized. However, this does not mitigate the dominant negative effect stemming from overexpression of these biosensors. This is due to the fact that our sensors respond to all upstream regulators with minimal signaling to downstream targets so they could diminish the upstream signaling fidelity by acting as “signaling sinks” when overexpressed. Thus, we try to minimize this issue by achieving low expression levels of the biosensor in target cells that is just above the signal-to-noise ratio sufficient for data acquisition. This typically translates to a signal-to-noise ratio of 2–3:1 comparing the dimmest part of the cell in the foreground to the background fluorescence levels on the microscope [37, 38]. In addition, control analysis should be performed to determine if expression of biosensors in cells of interest impacts the major phenotype that one wishes to study. We perform this type of analysis routinely by quantifying the rate of protrusion and other cell motility parameters. There are various methods previously described to achieve these imaging conditions and expression levels of the biosensor, including the stable expression of biosensors using viral transduction and inducible expression strategies [27, 37, 39, 40].

¹⁵Increasing the amount of PAK1-PBD-agarose slurry added could push toward more competitive binding. This parameter should be optimized based on how the control lanes in the resulting blot look. We normally use 10–20 μ L slurry volume per tube under these conditions.

7. Wash three times with 1 mL of lysis buffer; the first wash should be done with the lysis buffer containing protease inhibitors (*see* Note 16).
8. After the final wash, leave approximately 80 μ L of the solution at the bottom of the tube with the slurry, add appropriate amount of the 5 $\times$ loading buffer with DTT, boil at 95 $^{\circ}$ C for 5 min, and set aside for Western blotting.
9. Set up SDS-PAGE gel, and proceed with Western blotting. Example blots are shown in Fig. 4 (*see* Notes 17 and 18).

3.4 Biosensor Characterization in Cells Using Microscopy Imaging

Once the biosensor is characterized and validated *in vitro*, we normally further characterize it in target cells (fibroblasts, tumor cells, etc.). This involves expression of mutant versions of the sensor and measurement of the ratio of FRET/donor in cells using a microscope, along with live-cell exogenous stimulation experiments to ensure that the sensor responds to physiologically relevant stimuli.

1. Transfect constitutively active and dominant negative versions of the biosensor into target cells of interest.
2. 24 h after transfection detach and replat cells onto appropriately prepared 25 mm coverslips (*see* Note 19).
3. Time before fixation following replating depends on the cell type and how long they may take to attach. For fixation, treat cells on coverslips with 3.7% formaldehyde in DPBS at room temperature for 20 min, shielded from light.
4. Rinse three times with DPBS, mount the coverslips, and image them. Use ratiometric analysis [27] to process the data. Example results are shown in Fig. 5.

¹⁶For centrifugation of beads, we use 30 s at $500 \times g$ at room temperature in a bench top centrifuge. When removing the unbound lysate and/or the lysis buffer during wash steps, do not vacuum suction or otherwise try to remove every last bit of the solution. Best approach is to use a P1000 pipet and manually remove the solution so that approximately 80–100 μ L remains at the bottom of the microcentrifuge tube every time. This will ensure that the slurry is not disturbed.

¹⁷The Roche anti-GFP antibody will detect all GFP variants including mCerulean and mVenus; thus we normally use this antibody to detect for both the controls and the biosensor bands. In addition, GTPase-specific antibody can also be used but these could sometimes give background in the 10% lysate input blot because of nonspecific interactions that are common in direct GTPase detection. Ponceau S solution can be used to detect the PAK1-PBD in the bound fractions in order to control for equal loading. Incubate the freshly transferred membrane with Ponceau S for 5–10 min, and then wash with distilled water. Alternatively, an anti-GST antibody could be used.

¹⁸Expected results from the pull-down experiment: The pull-down experiment is designed to show that other cellular GTPase effectors would not compete against binding to activated GTPase within the biosensor. As such, a mutant version of the biosensor containing a constitutively activated GTPase and a functional binding domain should not be pulled down by supplying an excess, exogenous binding domain (in the case of Rac subfamily GTPases, PAK1-PBD is used) [3, 20]. In the case of Rho-subfamily GTPases, Rhotekin-RBD is used [7, 10]). This is because the active GTPase mutant within the biosensor would preferentially bind the integral binding domain due to the high local proximity and availability. When appropriate mutations are introduced into the integral binding domain that precludes it from binding to the constitutively activated GTPase within the biosensor, exogenously supplied excess binding domain should now be able to compete for the binding to the constitutively activated GTPase within the biosensor, resulting in this biosensor mutant being pulled down. The fluorescently tagged constitutively active or the dominant negative mutants of GTPase are used as controls for the pulldown by exogenous binding domain: the constitutively active GTPase should be pulled down, but the dominant negative GTPase should not be pulled down. The 10% input lysate blot is used to show the expression of mutants of these biosensors and the control GTPases in cells.

¹⁹For mouse embryonic fibroblasts (MEF/3T3), we routinely use fibronectin-coated coverslips. These are produced by treating the coverslips in 10 μ g/mL fibronectin in DPBS at room temperature for 1 h [10]. For breast tumor cells, we use gelatin-matrix-coated coverslips which are prepared as described previously or fibronectin coating depending on the assay [41]. For macrophage cell line RAW/LR5, we directly plate them onto cleaned coverslips. Transfection procedure for RAW/LR5 cells using the Fugene HD reagent was previously described in detail [39].

5. For live-cell characterizations, mount transfected cells with the wild-type version of the biosensor on coverslips onto live-cell imaging chamber such as the Attolflour chamber (Molecular Probes), or such as that shown previously [26], and stimulate using known stimuli (*see* Note 20). General considerations of live-cell ratiometric imaging technique are beyond the scope of this chapter, but more details can be found elsewhere [27]. An example of such an exogenous stimulation experiment is shown in Fig. 6 (*see* Note 21).

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²⁰.For imaging, we routinely use BWD buffer supplemented with appropriate amount of serum or bovine serum albumin. Ham's F-12K medium without phenol red or FluoroBrite DMEM without phenol red is also an excellent choice for live-cell imaging.

²¹.Cells can be starved following established protocols for the specific cell line of interest and stimulated using any number of methods, including serum stimulation, use of chemokine/cytokine, and growth factors such as PDGF and EGF. This approach is important to show that the biosensor properly responds to cellular signaling pathways initiated by well-known ligands under established protocols. It is ideal if biochemical pull-down results are available that match the observed kinetic of stimulated activation of the GTPase in question.

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Fig. 1.

Cartoon of the Rac2 biosensor design, based on the Rac1 biosensor [3], where it contains monomeric fluorescent proteins mCerulean1 and circularly permuted monomeric Venus as the FRET pair (a). The detection module PBD incorporates autoinhibitory mechanism to prevent spurious high FRET and to improve reversibility. Two red circles indicate H83/86D mutations [42] in the autoinhibitory PBD domain which prevents binding to other Rac GTPases when the autoinhibition is relieved through GTPase activation. (b) The biosensor depicted in (a) shown as a cDNA domain diagram

Fig. 2. Representative, normalized spectra of Cdc42-GDI-binding biosensor [1] showing titration of biosensor DNA during transfection. This biosensor detects binding of GDI to Cdc42. As such, titrating the biosensor in transfection reveals a saturation point against endogenous GDI in LinXE cells (800 ng of biosensor DNA in total 4 μ g of DNA transfected per a well of a 6-well plate). Reproduced from Hodgson et al. 2016 Nature Chemical Biology [1]

Fig. 3.

Typical validation and characterization fluorometry for a GTPase biosensor. In this example, a biosensor for Rac2 GTPase is shown [20]. (a) Representative, normalized spectra of Rac2 biosensor showing emission scans between 450 and 600 nm upon excitation at 433 nm. Active = G12V mutant; active + GDI = G12V mutant plus 2× excess GDI; inactive = T17N mutant. (b) Fluorometric validations of Rac2 biosensor, showing various mutants with or without 2× excess GDI co-expression. Mutants of Rac2 that are able to bind GDI (WT, G12V, T40C) show low FRET whereas the Q61L mutant which does not bind GDI shows

high FRET. (c) The wild type and mutants of Rac2 biosensor with or without H83/86D mutations (GTPase-binding deficient) [42] in PBD1 (2× PBD H/D = both PBD1 and PBD2 are GTPase-binding deficient), with or without 2× excess GDI. (d) The wild-type version of Rac2 biosensor co-expressed with Rac2-targeting (in green) or non-Rac2-targeting GEFs, with or (e) without 2× excess GDI. (f) The wild-type Rac2 biosensor co-expressed with 4× excess targeting (in red) or non-targeting GAPs. Fluorometry data: Average $\pm$ SEM of 3 independent experiments performed in triplicates. * $p < 0.00001$ versus WT alone; # $p < 0.00001$ versus G12V alone. Originally published in The Journal of Immunology. Veronika Miskolci, Bin Wu, Yasmin Moshfegh, Dianne Cox, and Louis Hodgson. 2016. Optical tools to study the isoform-specific roles of small GTPases in immune cells. J. Immunol. Vol: 196(8): 3479–93. Copyright © 2016 The American Association of Immunologists, Inc

Fig. 4.

Western blot of PAKI-PBD-agarose pulldown of fluorescent protein (FP)-tagged, constitutively active and dominant negative GTPase for assay control, and the constitutively active version of a GTPase biosensor with functional or GTPase-binding-deficient mutations in the built-in affinity-binding domain, overexpressed in LinXE cells. Total lysates and β -actin were used as loading controls. Ponceau S staining is also shown to indicate the presence of GST-PBD in the pull-down fractions. Lane 1: untransfected control. Lane 2: constitutively active mutant version of the biosensor with GTPase-binding-deficient mutations in the built-in affinity-binding domain. Lane 3: constitutively active mutant version of the biosensor with functional built-in affinity domain. Lane 4: dominant negative mutant version of the GTPase tagged with an FP. Lane 5: constitutively active mutant version of the GTPase tagged with an FP. Pulldown and 10% lysate input: detected using anti-GFP antibody

Fig. 5.

Ratiometric images of transiently overexpressed constitutively active (G12V) or dominant negative (T17N) Rac2 biosensor in RAW/LR5 cells [20]. Scale bar = 10 μ m. Quantification of whole-cell Rac2 activities are also shown, n = at least 15 cells/condition, mean $\pm$ SEM, p < 0.00001. Originally published in The Journal of Immunology. Veronika Miskolci, Bin Wu, Yasmin Moshfegh, Dianne Cox, and Louis Hodgson. 2016. Optical tools to study the isoform-specific roles of small GTPases in immune cells. J. Immunol. Vol: 196 (8): 3479–93. Copyright © 2016 The American Association of Immunologists, Inc

Fig. 6. Live-cell imaging of Rac1 and Rac2 activation dynamics during fMLP response by macrophages [20], shown as a proof-of-principle validation of exogenous stimulation. Time-lapse biosensor activity image (top panel) and DIC (bottom panel) of RAW/LR5 cells with (a) Rac1 and (b) Rac2 biosensor and fMLP-receptor stably expressed, stimulated with 100 nM fMLP; scale bar = 5 μ m. (c) Quantification of whole-cell average Rac1 and Rac2 activities, normalized to average whole-cell activity prior to stimulation (baseline levels during the first 4 min before stimulation). Data are the Ave. $\pm$ SEM of 11 cells for Rac1 and 10 cells for Rac2; black * p < 0.05, 10–270 s versus 0 s (Rac1); red * p < 0.05, 10–480 s

versus 0 s (Rac2), one-tailed, paired Student's *t*-test was used. Originally published in The Journal of Immunology. Veronika Miskolci, Bin Wu, Yasmin Moshfegh, Dianne Cox, and Louis Hodgson. 2016. Optical tools to study the isoform-specific roles of small GTPases in immune cells. J. Immunol. Vol: 196(8): 3479–93. Copyright © 2016 The American Association of Immunologists, Inc

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