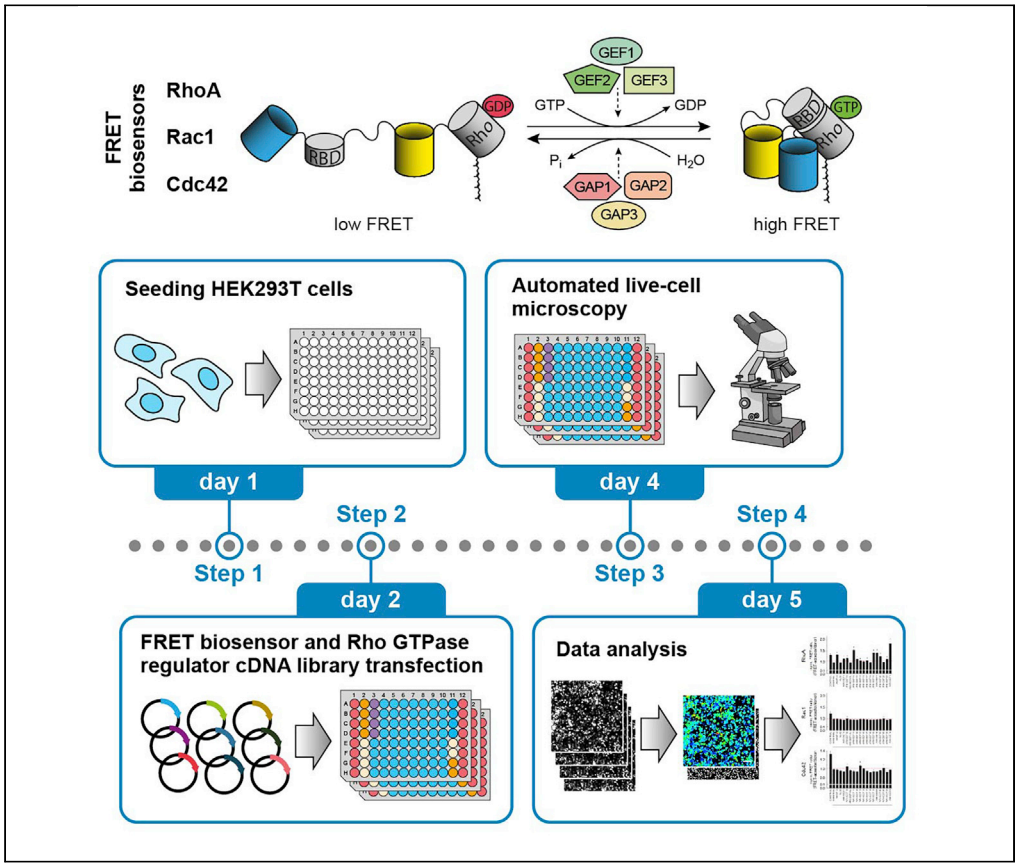
STAR Protocols

CellPress
OPEN ACCESS

Protocol

A screening-compatible live cell fluorescence resonance energy transfer-based assay for modulation of Rho GTPase activity



Rho family GTPases are central regulators of cytoskeletal dynamics controlled by guanine nucleotide exchange factors (RhoGEFs) and GTPase-activating proteins (RhoGAPs). This protocol presents a workflow for a robust high-throughput compatible biosensor assay to analyze changes in Rho GTPase activity by these proteins in the native cellular environment. The procedure can be used for semi-quantitative comparison of GEF/GAP function and extended for analysis of additional modulators. The experimental design is applicable also to other monomolecular ratiometric FRET sensors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Paul Markus Müller, Oliver Rocks

paulmarkus.mueller@fu-berlin.de (P.M.M.)
oliver.rocks@charite.de (O.R.)

Highlights

High-throughput biosensor assay to analyze changes in Rho GTPase activity in live cells

Semi-quantitative comparison of RhoGEF or RhoGAP function

Established for RHOA, RAC1, and CDC42 biosensors, applicable to others

Suitable for screening of modulators of RhoGEF or RhoGAP activity

Müller & Rocks, STAR Protocols 3, 101705 December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101705

STAR Protocols

CellPress
OPEN ACCESS

Protocol

A screening-compatible live cell fluorescence resonance energy transfer-based assay for modulation of Rho GTPase activity

Paul Markus Müller[1,3,*] and Oliver Rocks[2,4,*]

[1]Institute of Chemistry and Biochemistry, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany

[2]Institute of Biochemistry, Charité - Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

[3]Technical contact

[4]Lead contact

*Correspondence: paulmarkus.mueller@fu-berlin.de (P.M.M.), oliver.rocks@charite.de (O.R.)
https://doi.org/10.1016/j.xpro.2022.101705

SUMMARY

Rho family GTPases are central regulators of cytoskeletal dynamics controlled by guanine nucleotide exchange factors (RhoGEFs) and GTPase-activating proteins (RhoGAPs). This protocol presents a workflow for a robust high-throughput compatible biosensor assay to analyze changes in Rho GTPase activity by these proteins in the native cellular environment. The procedure can be used for semi-quantitative comparison of GEF/GAP function and extended for analysis of additional modulators. The experimental design is applicable also to other monomolecular ratiometric FRET sensors.

For complete details on the use and execution of this protocol, please refer to Müller et al. (2020).

BEFORE YOU BEGIN

The protocol below describes a screening-compatible live-cell imaging assay for the modulation of Rho GTPase activities using second generation fluorescence resonance energy transfer (FRET)-based biosensors for the prototype GTPases RAC1, CDC42 and RHOA (Fritz et al., 2013, 2015; Martin et al., 2016). These are genetically encoded single-chain reporters with optimized dynamic range, containing a circularly permutated (cp) mTFP1 as a FRET donor, a GTPase binding domain to detect GTP loading, a cp-Venus as an acceptor fluorophore (exception: the RHOA biosensor contains wild-type Venus (Fritz et al., 2013)) and a Rho GTPase at the C-terminus. In cp fluorescent proteins, the original N- and C-termini are fused by a peptide linker, while new termini are formed near the chromophore. This allows the optimization of the spatial arrangement of donor and acceptor chromophores to yield high FRET efficiency (Nagai et al., 2004). We have also successfully used RAC1 and CDC42 biosensors carrying tandem effector domains (Hanna et al., 2014; Moshfegh et al., 2014), and it is conceivable that other recently developed Rho GTPase biosensors with enhanced dynamic range will also work using this protocol (Van Unen et al., 2015). Moreover, with minor modifications, the workflow can also be adapted for drug screening (Becker et al., 2016) or for the use of other single-chain FRET-based biosensors of similar design (Eccles et al., 2016).

A few considerations should be made to understand the output and performance of this assay compared to the two conventional approaches available, that are *in vitro* and cell-based pulldown assays. *In vitro* assays typically involve recombinant GTPases and purified truncated regulators comprising only their catalytic domains. The high accuracy of this method thus comes at the cost of no information about potential regulation of the catalytic activity or specificity of the RhoGEFs or RhoGAPs tested. Pulldown assays rely on the extraction of GTP-bound active GTPases from

cell lysate by their cognate effector proteins. Since in cells only a small fraction of total Rho protein is activated at any given time (<5% (Ren et al., 1999)), the signal available in cell-based assays is inherently weak. Pulldown assays still can yield an arbitrarily strong signal because their output is the result of enzymatic signal amplification. However, this method is susceptible to variations in extracted GTP-bound protein, to signal variance due to the fast nucleotide hydrolysis of GTP-Rho in the lysate and to error propagation during the multistep preparation protocol.

Single-chain FRET-based biosensors yield a ratiometric signal of the active over inactive Rho GTPase in intact cells and are thus immune to the above errors. The design of these sensors simplifies FRET imaging by incorporating donor and acceptor fluorophores as well as GTPase and sensing domain in a 1:1 ratio into a single polypeptide (Spiering et al., 2013). Their microscopic readout allows the simultaneous integration of hundreds to thousands of cells. On the downside, the ratiometric changes in GTP/GDP levels can be small so that weak GEF/GAP activities may fall below the detection threshold. However, careful execution of an optimized assay protocol can largely compensate for this drawback, resulting in very high reproducibility, particularly in an automated routine.

Experimental design

The protocol is designed to detect global changes in RHOA, RAC1 or CDC42 activity in live cells by means of FRET biosensors for these GTPases upon transient co-expression of RhoGEFs or RhoGAPs. The analysis of either GEF or GAP activities involves separate protocols that were designed to yield maximum changes in FRET ratios. It is therefore necessary to anticipate whether in the planned experimental setup either an activation or inactivation of Rho GTPases will occur.

The GEF assay involves coexpression of RhoGDI (Rho GDP-dissociation inhibitor) to maximize the dynamic range of the reporter for activation. This is necessary because ectopically expressed Rho biosensors exhibit an elevated basal activity due to the saturation of endogenous RhoGDI, resulting in the enrichment of the reporter at membranes where abundant activation by endogenous RhoGEFs occurs (Pertz et al., 2006; Boulter et al., 2010). Conversely, for the GAP assay we recommend generating a RhoGDI knockdown cell line (see below "generation of a RhoGDI knockdown cell line"). This even further increases the basal activity of the transfected sensors and maximizes the dynamic range for inactivation by coexpressed RhoGAPs.

In addition, we show how the protocol can be adapted for further applications. Firstly, we exemplify how to approximate the preferred substrate GTPase of promiscuous RhoGEFs and RhoGAPs. FRET ratios cannot be directly compared between the RHOA, RAC1 and CDC42 biosensors as they differ in design, fluorophore photophysics and dipole orientation. However, by experimental benchmarking of the dynamic range of each biosensor within each assay such a correlation is possible, as outlined in the section 'Approximation of the preferred substrate GTPase'. Secondly, besides the sole detection of RhoGEF and RhoGAP substrate GTPases, this assay can also be modified to semi-quantitatively compare relative activities of RhoGEFs or RhoGAPs. This can be useful for example to investigate the regulation of RhoGEF and RhoGAP activity or to compare the activities of different splice or truncation variants of these proteins. In our previous study, this approach allowed us to postulate that most RhoGEFs and RhoGAPs are subject to autoinhibition (Müller et al., 2020). Thirdly, this assay can be modified to monitor changes in Rho GTPase activity in the presence and absence of potential further modulators of RhoGEF or RhoGAP activity. We have used this strategy to investigate the mutual regulation of catalytic activity in the multi-RhoGEF complex of ARHGEF11, ARHGEF12 and PLEKHG4B (Müller et al., 2020). Wherever modifications of the basic protocol are required for these additional applications these will be specifically highlighted below.

Generation of a RhoGDI knockdown cell line

Timing: 3 weeks

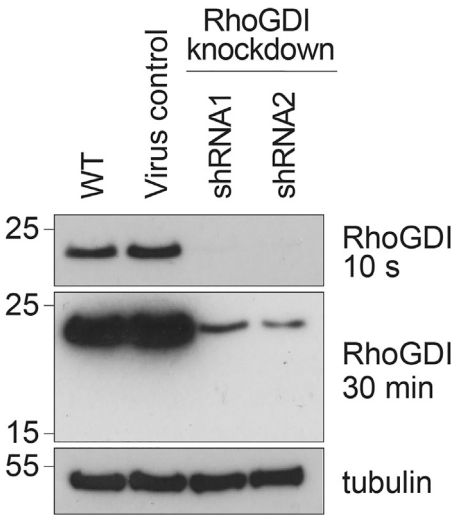


Figure 1. Western blot showing efficiency of shRNA-mediated RhoGDI knockdown

HEK293T cells were stably transduced with virus control or shRNA targeting RhoGDI (mission shRNA, Sigma-Aldrich, clone TRCN0000008004 (shRNA1) and clone TRCN0000008003 (shRNA2)), or were not infected (WT), and lysate was immunoblotted with RhoGDI antibody.

Figure taken from Extended Data Figure 2 (Müller et al., 2020).

Stable depletion of RhoGDI expression in HEK293T cells can be achieved by lentiviral delivery of short-hairpin RNA (shRNA) targeting this gene and subsequent selection of virus transduced cells. We recommend following the Addgene protocol using the pLKO.1-TRC cloning vector (available at: www.addgene.org/tools/protocols/plko/, chapters E-G).

1. Purchase shRNA against RhoGDI targeting the coding strand sequence 5′-CAAGATTGACAAGA CTGACTA-3′ cloned into the lentiviral vector pLKO.1-puro (mission shRNA, Sigma-Aldrich, clone TRCN0000008004). This sequence yielded a strong knockdown in HEK293T cells in our hands (Figure 1). Alternatively, the Addgene protocol describes the generation of such shRNA plasmid by cloning respective oligonucleotides into the pLKO.1 vector. Obtain the psPAX2 packaging plasmid (#12260) and the pMD2.G envelope plasmid (#12259) from Addgene (www.addgene.org).
2. Generation of lentivirus particles.
   a. Grow HEK293T cells in DMEM + 10% FBS without penicillin/streptomycin in a 6 cm dish to 50%–70% density.

   Note: Cells should at this step be kept without antibiotics.

   b. For cell transfection, add 1 µg pLKO.1 anti-RhoGDI shRNA plasmid, 750 ng psPAX2 packaging plasmid and 250 ng pMD2.G envelope plasmid to 100 µL Opti-MEM in a microcentrifuge tube and mix well with 6 µL PEI reagent (ratio of 1:3 (DNA [µg] : PEI [µL]). Incubate transfection mix for 20 min at 20°C–25°C and add dropwise to cells.

   Note: Other transfection reagents such as Fugene may suffice as well at this step.

   c. Incubate cells at 37°C and 5% CO_2 for 18–24 h.
   d. The next day, replace culture medium containing the transfection reagent with fresh complete DMEM medium (containing penicillin/streptomycin). Incubate cells at 37°C and 5% CO_2 for 24–30 h.
   e. At 48 h after transfection, harvest the medium containing lentiviral particles and store at 4°C. Add fresh complete DMEM medium to cells. Incubate cells at 37°C and 5% CO_2 for 24 h.
   f. The next day, again harvest medium from cells and pool with medium from previous day. Centrifuge medium at 200 × *g* for 5 min to pellet any cells or debris that were collected during harvesting and transfer supernatant to new tube.

Note: Virus may be stored at 4°C for a week. Freeze at −80°C for long-term storage. Avoid freeze/thaw cycles.

3. Lentiviral transduction.
   a. Prior to lentiviral infection of cells, determine optimal puromycin concentration for selection of virus transduced cells. The final puromycin concentration in your culture medium should range from 1-10 μg/mL. The minimum concentration resulting in complete cell death after 3–5 days should be used for selection in the following experiments. See Addgene protocol (http://www.addgene.org/protocols/plko/), chapter F.2 for details.
   b. Grow HEK293T cells in complete DMEM medium in six 6 cm dishes to 50%–70% density. Replace medium with fresh complete medium containing 8 μg/mL polybrene for increased efficiency of viral infection. Polybrene stocks can be made at a 1000× concentration in water (8 mg/mL) and stored for up to one year at −20°C.
   c. Infect HEK293T cells in the six dishes with 25 μL, 50 μL, 100 μL, 250 μL, 500 μL and 1,000 μL lentivirus-containing medium. Incubate cells at 37°C, 5% CO_2 for 12–24 h.

Note: Different volumes of virus containing medium are tested to determine the optimal amount of virus particles for transduction and gene silencing. To avoid an excessive MOI (multiplicity of infection), it is important to use an amount of virus sufficient to only transduce a fraction of the target cells.

4. Selection of virus transduced cells.
   a. Replace virus-containing medium with fresh complete medium. Incubate cells at 37°C and 5% CO_2 for 24 h to allow for sufficient expression of the puromycin resistance gene that is encoded on the transduced pLKO.1 vector.
   b. The next day (36–48 h after infection), add puromycin to the medium at the optimal concentration determined above (step 3a). Maintain one untransduced plate of cells in parallel. This plate will serve as a positive control for the puromycin selection.

Note: This is the beginning of the selection process leading to the selection of a stable cell pool.

   c. Observe the dishes every day to ensure that the cells in the untransduced dish are dying. Change to fresh puromycin-containing medium as needed every following two days.
   d. After completion of selection (4–6 days), choose the dish with stably transduced cells in which a small fraction of cells have died during selection. The cells in this dish will have an optimal MOI.
5. Assaying RhoGDI knockdown efficiency of stably selected cells.
   a. Determine the RhoGDI knockdown efficiency of stably transduced cells by Western blot analysis. Expected results are shown in Figure 1.

⚠ CRITICAL: All lentiviral procedures should be carried out in accordance with biosafety requirements of the host institution.

Custom-made humidity chamber

Timing: 1 day

When 96-well plates are kept for a longer time in a tissue culture incubator, edge wells are more susceptible to evaporation than center wells. This causes reduced cell growth in the edge wells due to hyperosmotic stress and also affects the apparent FRET ratio of the biosensors. We therefore strongly recommend to use a custom-made humidity chamber as described by (Walzl et al., 2012) to reduce this edge effect and preserve well-to-well consistency (Figure 2).

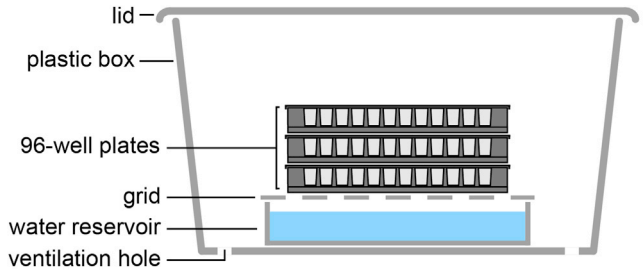


Figure 2. Schematic illustration of the custom-made humidity chamber
Schematic illustration of a custom-made humidity chamber, adapted from (Walzl et al., 2012).

6. Purchase a transparent ethanol-resistant plastic storage box with a non-airtight lid with the approximate dimensions LxWxH = 35 × 20 × 15 cm (similar to IKEA SAMLA, https://www.ikea.com/gb/en/p/samla-box-with-lid-transparent-s69440836/).
7. Drill holes in the bottom corners of the box to ensure proper CO_2 ventilation of the chamber.
8. Place two open 15 cm cell culture dishes on the bottom of the box and fill them with sterile water. These serve as water reservoirs and ensure a humid atmosphere inside the chamber. Place a custom made perforated acrylic glass grid on top of the tray to carry the stack of 96-well plates on top of the reservoir (5 mm thick acrylic glass perforated with drilled holes of 2 cm diameter at an area ratio of hole vs. acryl glass 1:1).

We suggest routinely controlling the consistency of obtained data across wells, as well as variations between wells that were recorded at the beginning and at the end of the measurement of a 96-well plate (time difference 45–90 min, dependent on total duration of image acquisition).

Preparation of expression plasmids

Timing: 1 week

9. Obtain the expression plasmids encoding pTriExRhoA-2G (#40176), pTriEx4-Cdc42-2G (#68814), pTriEx4-Rac1-2G (#66110) and miRFP670 (#79987) from Addgene (https://www.addgene.org) and mCherry-C1 from Takara Bio (#631972). The RhoGDI plasmid as well as mCherry-tagged RhoGEF or RhoGAP plasmids can be obtained from the author's laboratory upon reasonable request, or from other sources. For construction and sequences of RhoGEF/RhoGAP plasmids we refer to the Methods section and Table S1 in (Müller et al., 2020).

Note: Custom made constructs encoding regulators of Rho GTPase activity should be fused to a fluorescent protein to allow for quantification of expression levels. The emission spectrum of the fluorophore should not interfere with the cp-mTFP1 and cp-Venus fluorophores of the FRET sensors. We recommend cloning into the mCherry-C1 expression vector.

Note: If the assay is used to test the modulation of RhoGEF or RhoGAP activity by an additional interacting protein, we suggest fusing that protein to miRFP670 (Shcherbakova et al., 2016).

Arraying of plasmids

Timing: 1 day

If the assay is performed repeatedly with the same experimental conditions we recommend pre-diluting the RhoGEF and RhoGAP plasmids to a final concentration of 0.1 µg/µL and arraying them into a V-bottom 96-well plate in an order that mirrors the layout of the 96-well plate of the

experiment. This facilitates liquid handling, saves time and reduces pipetting errors. After usage, the plates should be secured with a microplate aluminum sealing tape to prevent evaporation and stored at −20°C. Before usage, thaw plates, spin down at 1,000 × *g* for 1 min and carefully remove the aluminum tape to prevent contamination across wells.

Note: A low plasmid concentration is required for precise pipetting. However, when subjected to frequent freeze-thaw cycles diluted plasmids are susceptible to degradation. While we observed no such effect for RhoGEFs and RhoGAPs arrayed in 96-well plates at 0.1 μg/μL, we strongly recommend freshly diluting the biosensor and RhoGDI plasmids as they are transfected at even lower concentrations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-RhoGDI (1:2000)	Santa Cruz Biotechnology	#sc360
Mouse anti-Tubulin (1:10000)	Sigma	#T6199
Chemicals, peptides, and recombinant proteins		
DMEM	Fisher Scientific	#10569010
FluoroBrite DMEM	Fisher Scientific	#A1896701
Opti-MEM I Reduced Serum Medium	Fisher Scientific	#31985047
Trypsin/EDTA solution	Merck	# 59430C
Penicillin/Streptomycin	Fisher Scientific	#15140163
Fetal Bovine Serum (FBS)	Biochrom	#S0115
Poly-L-lysine	Sigma-Aldrich	#P1399
PEI (Polyethylenimine, Linear, MW 25K)	Polysciences	#23966
Polybrene (hexadimethrine bromide)	Sigma-Aldrich	#H9268
Puromycin	Sigma-Aldrich	#P8833
Phosphate buffered saline (PBS), pH 7.2, without Ca++ or Mg++	Fisher Scientific	#20012019
Deposited data		
RhoGEF/RhoGAP substrate specificity data	(Müller et al., 2020)	http://the-rhome.com & Biostudies:S-BSST160
Experimental models: Cell lines		
HEK293T	ATCC	#CRL-3216
HEK293T-shRhoGDI	(Müller et al., 2020)	N/A
Recombinant DNA		
anti-RhoGDI shRNA pLKO.1-puro plasmid	Sigma-Aldrich	#TRCN0000008004
psPAX2 packaging plasmid	Addgene	#12260
pMD2.G envelope plasmid	Addgene	#12259
pTriEx-RhoA-2G WT	(Fritz et al., 2013)	#40176
pTriEx4-Rac1-2G WT	(Fritz et al., 2015)	#66110
pTriEx4-Cdc42-2G WT	(Martin et al., 2016)	#68814
miRFP670	Addgene	#79987
ARHGAP31 truncation (aa 1–820)	(Lamarche-Vane and Hall, 1998)	N/A
ARHGAP1	(Müller et al., 2020)	N/A
ARHGAP22	(Müller et al., 2020)	N/A
MCF2	(Müller et al., 2020)	N/A
RhoGDI	(Müller et al., 2020)	N/A
mCherry-C1	Takara Bio	#631972
miRFP670	Addgene	#79987
Software and algorithms		
ImageJ/Fiji	https://fiji.sc/	RRID: SCR_002285
Excel	Microsoft	RRID: SCR_016137
R	https://www.r-project.org	RRID: SCR_001905

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Olympus IX-81 microscope	Olympus	RRID: SCR_020341
Objective UPLSAPO ×10/0.4 numerical aperture (NA) air objective	Olympus	#N2249100
Excitation Filter TFP donor, FRET acceptor 430/25	Chroma	#S430/25× (discontinued, we recommend ET430/24× instead)
Excitation Filter cp-Venus acceptor 500/20	Chroma	#ET500/20×
Excitation Filter mCherry 572/23	Chroma	#ET572/35×
Excitation Filter miRFP670 640/30	Chroma	#ET640/30×
Dichroic Mirror TFP donor, FRET acceptor ZT442rdc	Chroma	#ZT442rdc
Dichroic Mirror cp-Venus zt514RDC	Chroma	#ZT514rdc
Dichroic Mirror mCherry HC BS 593	Semrock	# FF593-Di03-25 × 36
Dichroic Mirror miRFP670 R405/488/561/635	Semrock	# Di03-R405/488/561/635-t1-25 × 36
Emission Filter TFP donor 483/32	Semrock	#FF01-483/32-25
Emission Filter FRET acceptor, cp-Venus 542/27	Semrock	#FF01-542/27-25
Emission Filter mCherry 623/24	Semrock	#FF01-623/24-25
Emission Filter miRFP670 692/40	Semrock	#FF01-692/40-25
MT20 150 W xenon arc burner light source	Olympus	N/A
Motorized microscope stage	Märzhäuser	N/A
Autofocus system (laser based Z-drift compensator, ZDC)	Olympus	N/A
Temperature-controlled environmental chamber (Okolab or iXPlore Live)	Okolab or Olympus	N/A
ImagEM X2 water-cooled EMCCD camera	Hamamatsu	#C9100-23B
96-well μ-plate, no. 1.5 polymer coverslip, ibiTreat	ibidi	#89626
96-well plate, V-bottom clear wells	Greiner Bio-One	#651180
Microplate aluminum sealing tape	Corning	#6570
Hemocytometer	Carl Roth	#T735.1
Cell culture incubator with temperature and CO_2 control	BINDER	#CB-S260
8-channel pipette 0.5–10 μL	Eppendorf	#3125000010
8-channel pipette 30–300 μL	Eppendorf	#3125000052
300 μL multichannel pipette tips	Sarstedt	#70.3040
50 mL multichannel pipette reservoirs	Corning	#4870

MATERIALS AND EQUIPMENT

Polyethyleneimine (PEI) transfection solution		
Reagent	Final concentration	Amount
Polyethyleneimine (PEI) 25k	1 mg/mL	100 mg
HCl (1 M stock solution)	N/A	apr
NaOH (1 M stock solution)	N/A	apr
ddH_2O	N/A	fill to 100 mL
Total	**N/A**	**100 mL**

apr: as per requirement.

Add 100 mg of PEI to 90 mL sterile, deionized water (ddH_2O) in a glass beaker. Stir the solution and measure the pH with a pH meter. Slowly add hydrochloric acid (from a 1 M HCl stock solution) with a pipette to adjust the pH to < 2.0. Stir until the PEI is fully dissolved (~2–3 h), maintaining the pH < 2 throughout. Slowly add sodium hydroxide (from a 1 M NaOH stock solution) to adjust the pH to 7.0.

Pour the PEI solution into a graduated glass cylinder and fill with ddH_2O to a total volume of 100 mL. Sterile filtrate the solution through a filter membrane with 0.2 mm pore size to remove undissolved PEI particles. Prepare aliquots in 1.5 mL microcentrifuge tubes under sterile conditions.

Note: Store aliquots at −80°C. Frozen aliquots can be kept for at least one year. Thawed aliquots can be stored at 4°C for one month. Avoid frequent freeze-thaw cycles.

DMEM with 10% fetal bovine serum and penicillin/streptomycin (complete)		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	N/A	500 mL
Fetal bovine serum (FBS)	10%	56 mL
Penicillin-Streptomycin 10,000 U/mL (100×)	1×	5,6 mL
Total	**N/A**	**561,6 mL**

Note: Complete DMEM medium can be stored at 4°C for up to one month.

FluoroBrite DMEM with 10% fetal bovine serum and penicillin/streptomycin (complete)		
Reagent	Final concentration	Amount
FluoroBrite Dulbecco's Modified Eagle Medium (DMEM)	N/A	500 mL
Fetal bovine serum (FBS)	10%	56 mL
Penicillin-Streptomycin 10,000 U/mL (100×)	1×	5,6 mL
Total	**N/A**	**561,6 mL**

Note: Complete FluoroBrite DMEM medium can be stored at 4°C for up to one month.

FluoroBrite DMEM with 1% fetal bovine serum and penicillin/streptomycin (serum reduced)		
Reagent	Final concentration	Amount
FluoroBrite Dulbecco's Modified Eagle Medium (DMEM)	N/A	500 mL
Fetal bovine serum (FBS)	1%	5 mL
Penicillin-Streptomycin 10,000 U/mL (100×)	1×	5 mL
Total	**N/A**	**510 mL**

Note: Serum-reduced FluoroBrite DMEM medium can be stored at 4°C for up to one month.

Poly-L-lysine solution		
Reagent	Final concentration	Amount
Poly-L-lysine	0.1%	100 mg
ddH2O	N/A	100 mL
Total	**N/A**	**100 mL**
Dilute stock solution 1:40 for 0.0025% working solution.		

Note: Poly-L-lysine solution is stable for up to one month at 4°C. Store at −20°C for up to six months.

Microscope setup requirements for ratiometric FRET imaging

The single-chain Rho GTPase biosensors used in this work are designed to detect activation of Rho GTPases as a result of changes in fluorescence resonance energy transfer (FRET) between a donor

Table 1. Excitation filter, dichroic mirror, and emission filter combinations for ratiometric FRET imaging

Channel	Excitation filter	Dichroic mirror	Emission filter
donor (cp-mTFP1)	430/25	ZT442rdc	483/32
FRET	430/25	ZT442rdc	542/27
acceptor (cp-Venus)	500/20	ZT514rdc	542/27
mCherry	572/23	HC BS 593	623/24
miRFP670	640/30	405/488/561/635	692/40

fluorophore (cp-mTFP1) and an acceptor fluorophore (cp-Venus). Activation of the Rho GTPase biosensor leads to an increase in the FRET ratio calculated as the ratio of FRET channel fluorescence divided by the donor channel fluorescence. Therefore, suitable filter settings are required for the excitation of cp-mTFP1 together with cp-mTFP1 emission (donor channel) and excitation of cp-mTFP1 together with cp-Venus emission (FRET channel). For the identification of successfully transfected cells further filters are needed for cp-Venus, mCherry and, optionally, miRFP670 imaging (Table 1).

For convenient use of the filter settings, we recommend placing them separately into an excitation filter wheel, a dichroic mirror turret and an emission filter wheel. As image acquisition of the 96-well plate is performed automatically, all components of the microscope setup are required to be computer-controlled, including the shutter of the light source, an autofocus system and a motorized stage. A dry objective must be used to image across the 96-well plate. We used a 10×/0.4 NA air objective corrected for spherical and chromatic aberrations, but we have also successfully worked with air objectives with other magnifications.

Finally, a programmable software is needed that allows automated image acquisition.

The key setup requirements for the microscope are listed below:

- motorized inverted widefield microscope.
- autofocus system.
- microscope life cell incubation system to maintain 37°C, 5% CO_2, and humidity.
- light source.
- excitation filter wheel with mounted excitation filters.
- motorized dichroic mirror turret.
- motorized stage.
- emission filter wheel with mounted emission filters.
- scientific camera (EMCCD, sCMOS).
- programmable microscope control software.

> ***Note:*** If you use a biosensor with different donor and acceptor fluorophores make sure to adjust the filter settings accordingly.

STEP-BY-STEP METHOD DETAILS

Assay setup and initial calculations

> Timing: 1 day

This section defines the set-up of the assay, including the layout of the wells used in the 96-well plate and the amount of plasmid DNA needed.

1. Determine the number of experimental conditions N to be tested, such as the number of RhoGEF or RhoGAP constructs, and the type of biosensor to be used (RHOA, RAC1 or CDC42).

2. Include controls. A RhoGEF assay should comprise the following conditions:
   a. biosensor only.
   b. biosensor + RhoGDI. This control defines the low activity level baseline and thereby serves to assess the dynamic range of the biosensor.
   c. biosensor + RhoGDI + the RhoGEF MCF2 (DBL). MCF2 exhibits strong GEF activity toward RHOA, RAC1 and CDC42 and therefore serves as a positive control for all three biosensors.
3. A RhoGAP assay should comprise the following conditions:
   a. biosensor only.
   b. biosensor + positive control RhoGAP. We have successfully used ARHGAP1 as a RHOA-specific GAP, ARHGAP22 as a RAC1-specific GAP and ARHGAP31 (aa 1–820) as a CDC42-specific GAP. The truncated form of ARHGAP31 exhibits an increased catalytic activity compared to its full-length form.
4. Include a non-transfected condition that is necessary to acquire background fluorescence images for each detection channel.

   Note: Background fluorescence is due to cellular autofluorescence, imaging medium, uneven illumination, and camera noise. Averaged background images will be subtracted from the fluorescence measurement obtained from transfected cells.

5. Measure replicates as follows:
   a. Measure each condition in triplicate to assess variation by statistical testing.

   Note: This can be done as technical replicates on the same plate (identical reagents, culture conditions and handling, but independent transfections) or on three different plates (identical reagents and culture conditions but independent handling and transfections) or, ideally, as independent and temporally separate experiments on three different plates (independent reagents, culture conditions, handling and transfections).

   b. Measure the control conditions as quadruplets covering all well positions from edge to center. This allows for the detection of a potential edge effect (see above "custom-made humidity chamber").
   c. Additionally measure the biosensor only condition and the biosensor + RhoGDI condition for the RhoGEF assay at the end of each plate to ensure consistency of the measurements across the whole plate.
   d. The total number of wells W to be seeded therefore sums up to
      $W = 3 \times N + 16 + 16$ for a GEF assay and
      $W = 3 \times N + 12 + 16$ for a GAP assay. This includes 16 or 12 control wells for the GEF and GAP assay, respectively, and 16 background wells with cells that will not be transfected.
   e. Therefore, the number of wells T that will be transfected is calculated as
      $T = 3 \times N + 16$ for a GEF assay and
      $T = 3 \times N + 12$ for a GAP assay.
      Figure 3 shows an exemplary design of a 96-well plate.

   Note: For convenience, we avoid scrambling the positions on the 96 well plate. This is acceptable due to several precautions in this protocol that keep well-to-well variations minimal (such as low autofluorescence medium, frequent medium changes, humidity chamber, equilibrations and rigorous controls). However, if the protocol is adapted for other applications and any of these factors are changed, we do recommend scrambling to assess variation due to positioning on the 96 well plate.

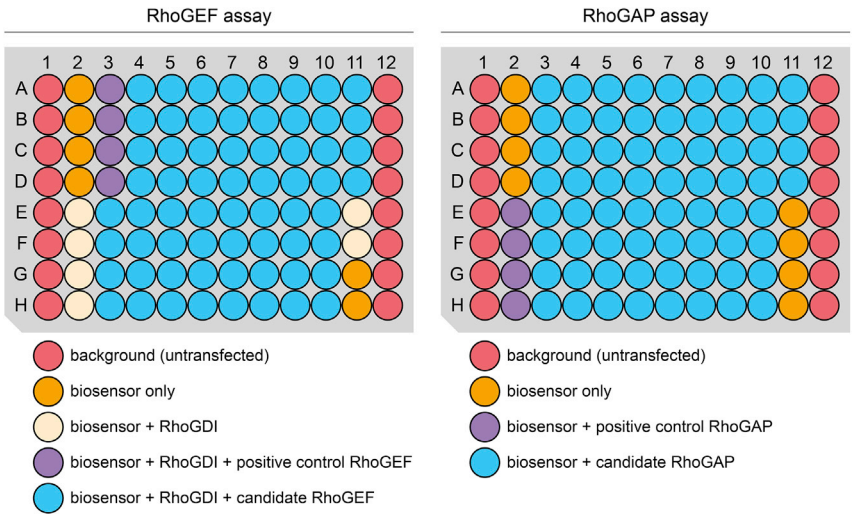


Figure 3. Layout of RhoGEF and RhoGAP activity assay screening in 96-well microplates

Exemplary layout of a 96-well plate for a RhoGEF assay (left) or a RhoGAP assay (right). All wells are seeded with HEK293T (GEF assay) or HEK293T RhoGDI knockdown cells (GAP assay). Cells in background wells are not transfected and are used for obtaining background fluorescence images. The layout is designed for column-wise recording with controls being recorded at the beginning and the end of each plate.

6. Calculate the total number of HEK293T cells (GEF assay) or HEK293T RhoGDI knockdown cells (GAP assay) for seeding. The seeding density is 55,000 cells/well. The total amount of cells therefore is W × 55,000 cells.
7. Predilute the RhoGEF and RhoGAP plasmids to be used to a final concentration of 0.1 μg/μL. We recommend arraying the plasmids in a V-bottom 96-well plate (see above "arraying of plasmids") to facilitate liquid handling and reduce pipetting errors.
8. Calculate the total amount of plasmid DNA needed for the transfection. The composition of the transfected plasmids for each condition including the required plasmid concentrations is shown in Table 2. The total amount of plasmid DNA per 96-well plate including a 10% pipetting error sums up as follows:

RhoGEF assay:

biosensor at 10 ng/μL: 40 ng × T × 1.1

RhoGDI at 40 ng/μL: 80 ng × (T - 6) × 1.1

mCherry at 40 ng/μL: 80 ng × 6 × 1.1

positive control RhoGEF at 100 ng/μL: 280 ng × 4 × 1.1

each candidate RhoGEF at 100 ng/μL: 280 ng × 3 × 1.1

mCherry at 100 ng/μL: 280 ng × 12 × 1.1

RhoGAP assay:

biosensor at 10 ng/μL: 30 ng × T × 1.1

positive control RhoGAP at 100 ng/μL: 270 ng × 4 × 1.1

Table 2. Composition of transfected plasmids per well for indicated conditions

	Condition	Biosensor 10 ng/µL	RhoGDI 40 ng/µL	mCherry 40 ng/µL	RhoGEF 100 ng/µL	RhoGAP 100 ng/µL	mCherry 100 ng/µL
RhoGEF assay	biosensor only	40 ng	-	80 ng	-	-	280 ng
	biosensor + RhoGDI	40 ng	80 ng	-	-	-	280 ng
	biosensor + RhoGDI + RhoGEF	40 ng	80 ng	-	280 ng	-	-
RhoGAP assay	biosensor only	30 ng	-	-	-	-	270 ng
	biosensor + RhoGAP	30 ng	-	-	-	270 ng	-

Controls with mCherry plasmid are pipetted at 2 different concentrations in order to keep plasmid DNA volumes and potential pipetting errors constant. For the RhoGEF activity screen, cells are transfected with a total amount of 400 ng DNA per well, composed of 40 ng FRET biosensor, 80 ng RhoGDI or mCherry control, and 280 ng RhoGEF or mCherry control. For the RhoGAP activity screen, cells are transfected with 300 ng DNA in total, composed of 30 ng FRET biosensor and 270 ng RhoGAP or mCherry control.

each candidate RhoGAPat 100 ng/µL: 270 ng × 3 × 1.1

mCherry at 100 ng/µL: 270 ng × 8 × 1.1

Note: While the observed FRET ratio is largely robust against variations in biosensor expression levels (see also "expected outcomes" and Extended Data Figure 2E (Müller et al., 2020)) the transfection efficiency and biosensor expression levels scale with the amounts of plasmid used. The amounts of plasmid described above were empirically balanced in order to maximize the effect size while keeping the biosensor expression at minimal but robust levels. Variations in amounts of plasmid might reduce the sensitivity of the assay.

Optional: Approximation of the preferred substrate GTPase. If a candidate RhoGEF or RhoGAP shows activity toward more than one of the target GTPases, the dynamic range of each biosensor can be benchmarked allowing for subsequent comparison of RHOA, RAC1 and CDC42 activities. To do so, first, the observed change in FRET ratio compared to the baseline FRET ratio (biosensor + RhoGDI for RhoGEFs or biosensor only for RhoGAPs) (ΔR) is measured. Subsequently, this value is normalized to the maximal observed change in FRET ratio (ΔR_{MAX}) for that specific biosensor. In our family-wide screen maximal RhoGEF-induced FRET ratio change was observed for MCF2 (RHOA), TIAM1 (RAC1) and PLEKGH4 (CDC42), while the maximal observed RhoGAP-induced FRET ratio change was observed for ARHGAP1 (RHOA), ARHGAP22 (RAC1) and ARAP2 (CDC42) (Müller et al., 2020). Alternatively, benchmarking can be achieved through the use of constitutively active point mutations of the biosensor representing maximal activation, or by dominant negative or effector binding deficient point mutations, representing maximal inactivation of the biosensors (Fritz et al., 2013; Hanna et al., 2014; Moshfegh et al., 2014; Martin et al., 2016). This way, the activity of any given RhoGEF or RhoGAP can be graded as % of the maximal observed activity, thereby allowing the identification of the preferred substrate Rho GTPase.

Optional: Direct semi-quantitative comparison of RhoGEF or RhoGAP variants. If the assay is used to semi-quantitatively compare the activity of two - or more - different variants of a RhoGEF or RhoGAP (e.g., splice isoforms or truncation constructs) their expression levels should not significantly differ to ensure that a difference in measured activity is not the result of different protein concentrations. In the latter case, the level of the stronger expressing variant can be empirically adjusted by diluting its plasmid concentration to reach equal expression, as can be determined by mCherry intensities (see also Methods section in (Müller et al., 2020)). The amount of plasmid in the transfection mixture of the diluted RhoGEF or RhoGAP is then filled up with miRFP670 to 280 ng or 270 ng, respectively.

Note: We observed that besides the amount of plasmid transfected (affecting the transfection efficiency), the expression strength of the plasmids in the mixture are interdependent (see also

Table 3. Composition of transfected plasmids for modulation of RhoGEF or RhoGAP activity by an additional modulator protein

	Condition	Biosensor 10 ng/µL	RhoGDI 40 ng/µL	mCherry 40 ng/µL	RhoGEF 100 ng/µL	RhoGAP 100 ng/µL	mCherry 100 ng/µL	Modulator 100 ng/µL	miRFP670 100 ng/µL
RhoGEF assay	biosensor only	40 ng	-	80 ng	-	-	140 ng	-	140 ng
	biosensor + RhoGDI	40 ng	80 ng	-	-	-	140 ng	-	140 ng
	biosensor + RhoGDI + RhoGEF	40 ng	80 ng	-	140 ng	-	-	-	140 ng
	biosensor + RhoGDI + modulator	40 ng	80 ng	-	-	-	140 ng	140 ng	-
	biosensor + RhoGDI + RhoGEF + modulator	40 ng	80 ng	-	140 ng	-	-	140 ng	-
RhoGAP assay	biosensor only	30 ng	-	-	-	-	135 ng	-	135 ng
	biosensor + RhoGAP	30 ng	-	-	-	135 ng	-	-	135 ng
	biosensor + modulator	30 ng	-	-	-	-	135 ng	135 ng	-
	biosensor + RhoGAP + modulator	30 ng	-	-	-	135 ng	-	135 ng	-

Controls with mCherry plasmid are pipetted at 2 different concentrations in order to keep plasmid DNA volumes and potential pipetting errors constant.

Figure 4 and "expected outcomes"). Therefore, rather than filling up the transfection mixture with an empty vector (no expression), we suggest using a mammalian expression vector expressing a protein that is inert to the assay.

Optional: Modulation of RhoGEF or RhoGAP activity by a further modulator. If the assay is used to test the modulation of a RhoGEF or RhoGAP activity by an additional protein, this protein should be expressed with an miRFP670 fluorescent tag. The amount of RhoGEF plasmid is then reduced to 140 ng or of RhoGAP plasmid to 135 ng and filled to 280 ng or 270 ng, respectively, with the additional miRFP670 tagged modulator protein or miRFP670 as control. In this case, the composition of the transfected plasmids for each condition including the required plasmid concentrations is shown in Table 3.

9. Passage HEK293T cells and grow them in complete FluoroBrite DMEM for next day seeding on 96-well plates and transfection the day after.

Cell seeding: Day 1

Timing: 3–4 h (including 2 h coating)

This section describes how poly-L-lysine coated 96-well plates are prepared and HEK293T cells are seeded.

10. Poly-L-lysine coating of 96-well plates to enhance cell attachment.
    a. Treat 96-well plates with 5 µg/well poly-L-lysine solution (200 µL per well of a 0.0025% working solution) for 20 min at 37°C.
    b. Wash twice with PBS and once with deionized water.
    c. Let plates dry with the lid open in a biosafety cabinet for at least 2 h prior to plating cells. Plates can be stored for at least one week upon drying.
11. Seeding of HEK293T cells or HEK293T RhoGDI knockdown cells.
    a. Prewarm Trypsin/EDTA solution, PBS and complete FluoroBrite DMEM at 37°C.
    b. Aspirate medium from 15 cm plate containing HEK293T cells that is 70%–80% confluent and was passaged the day before, wash plate with 20 mL sterile PBS and aspirate.
    c. Add 2 mL Trypsin/EDTA solution and incubate at 37°C, 5% CO_2 in humidified incubator for 3 min.
    d. Check under microscope: if cells start to detach, tap dish gently 5 times to mechanically dislodge cells.

e. Add 20 mL complete FluoroBrite DMEM to dish to remove any remaining adherent cells.
f. Spin cells down at 200 × *g* at 20°C–25°C and resuspend in 10 mL complete FluoroBrite DMEM. Carefully pipet up and down several times to ensure a single-cell suspension.
g. Determine cell number per volume using a hemocytometer or an automated cell counter. Adjust to 1.1 + 10^7 cells/50 mL in complete FluoroBrite DMEM.
h. Transfer cell suspension to a sterile single-well V-bottom plastic reservoir that fits an 8-channel multichannel pipette.
i. Use an 8-channel multichannel pipette to distribute 250 μL cell suspension (55,000 cells) into each well of a 96-well plate.

Note: a) Carefully resuspending cells before each pipetting step will ensure constant cell number. b) Make sure to place the ends of the pipet tips against the well walls near the bottom of wells to prevent cell solution from sticking to the wall or remaining in the pipet tip. c) Avoid the formation of air bubbles which may perturb even spreading of cells on the well bottom. Make sure air bubbles are expelled from pipet tips when resuspending cells in the source reservoir before transferring them to 96-well plate. Also, when dispensing cells into the 96-well plate, press the plunger only to the first stop. Changing pipette tips is not required between seeding different columns of the 96-well plate.

12. Place cells into custom made humidity chamber inside tissue culture incubator and allow cells to adhere at 37°C and 5% CO_2 for 18–22 h.

Cell transfection: Day 2

Timing: 3 h

This section describes the transfection of the cells that were seeded into 96-well plates one day before. It starts with the preparation and arraying of plasmid mixtures on 96-well plates for the transfection and continues with the preparation of the transfection mixture and transfection of HEK293T cells.

13. Preparation and arraying of plasmid mixtures on 96-well plates for transfection.
    a. Calculate the amount of required plasmids for all components as described above.
    b. Freshly dilute biosensor plasmid to 10 ng/μL and RhoGDI and mCherry controls to 40 ng/μL, if required, and array in 8 neighboring wells of a V-bottom 96-well plate in equal shares. This serves as a reservoir for the multichannel pipette.
    c. Thaw RhoGEF and RhoGAP plasmids arrayed on 96-well plate, spin down at 1,000 × *g* for 1 min to remove condensed water from the lid and carefully remove the aluminum tape to prevent contamination across wells.

Note: Plasmids encoding the biosensors, RhoGDI or mCherry should be diluted freshly on the day of transfection because storage of plasmid solution at very low concentrations impairs transfection efficiency.

    d. For transfection, array the required plasmids in a V-bottom 96-well plate according to the scheme in Figure 3 using a multichannel pipette. For the RhoGEF assay combine 4 μL of biosensor plasmid at 10 ng/μL, 2 μL of RhoGDI or mCherry control at 40 ng/μL and 2.8 μL of RhoGEF or mCherry control at 100 ng/μL. For the RhoGAP assay combine 3 μL of biosensor and 2.7 μL of RhoGAP or mCherry control at 100 ng/μL.

Note: To avoid pipetting errors due to loosely attached pipette tips visually inspect equal loading of the multichannel pipette tips for each pipetting step.

14. Change medium of HEK293T cells. Transfer approximately 22 mL complete FluoroBrite DMEM per 96-well plate, prewarmed to 37°C, into a multichannel pipette reservoir. Carefully remove medium from wells using a multichannel pipette without touching the cell layer and replace with 200 μL/well fresh medium using a multichannel pipette.
15. Check if cell attachment is unaffected after media change.

Note: To save plastic waste, pipette tips do not need to be changed between individual steps of the medium change. To do so, push the plunger to the second stop before removal of the old medium from the wells.

16. Preparation of the transfection mixture and transfection of HEK293T cells. Transfect cells with 3 μL of a 1 mg/mL PEI solution per 1 μg of plasmid DNA.
    a. Prepare PEI transfection solution with 1.2 μL PEI stock solution (1 mg/mL) and 28.8 μL FluoroBrite DMEM without additives per transfected well for the GEF assay and 0.9 μL PEI stock solution (1 mg/mL) and 29.1 μL FluoroBrite DMEM without additives per transfected well for the GAP assay. Prepare further 10% of the transfection mixture to account for potential pipetting errors and put in a multichannel pipette reservoir.
    b. Add 30 μL of the PEI transfection solution per well to the plasmid mixtures in the 96-well plate and pipette up and down for 5 times using a multichannel pipette.
    c. Incubate at 20°C–25°C for 20 min.
    d. Pipette up and down for 5 times and add transfection mixture to the cells.
    e. Place cells into custom made humidity chamber inside tissue culture incubator at 37°C and 5% CO_2.

⚠ CRITICAL: Cells should not exceed 60%–70% confluency on the day of transfection to assure an optimal transfection rate and cell attachment. If cells grow too confluent, transfection efficiency might be reduced and the cell layer might detach in the following days.

Medium change: Day 3

Timing: 15–30 min per 96 well plate

This section describes the medium change that is done 18–24 h after the transfection.

17. Prewarm serum-reduced FluoroBrite DMEM at 37°C.
18. Carefully remove medium from wells using a multichannel pipette without touching the cell layer and replace with 300 μL/well serum-reduced FluoroBrite DMEM using a multichannel pipette.
19. Place cells into custom made humidity chamber inside tissue culture incubator at 37°C and 5% CO_2.

Note: To save plastic waste, pipette tips that were used to add fresh medium to the previous column of the 96-well plate can still be used to remove the medium from the next column of wells.

Imaging: Day 4

Timing: 1.5 h

42–48 h after transfection the cells are used for data acquisition. This section describes the acquisition of the fluorescence microscopy images for later quantification of the FRET ratios.

20. Equilibrate the microscope live cell incubation system at 37°C, 5% CO_2 and humidity for 1 h to stabilize the system.

21. Take the transfected 96-well plate out of the humidity chamber in the tissue culture incubator and place the 96-well plate on microscope with the lid open. Let cells rest for 30 min.

Note: This step equilibrates any differences in temperature and CO_2 between the tissue culture incubator and microscope environment or changes that occurred during the transport of the plate. Removing of the 96-well plate lid reduces lag in equilibration of center wells versus edge wells.

22. Visually control cell viability, transfection efficiency and expression levels in a random selection of wells. Be careful not to bleach the sample.
23. Scan the entire 96-well plate, including background wells, using the following image acquisition settings:
    a. Acquire five non-overlapping fields of view per well.
    b. Depending on your microscope setup apply continuous z-drift compensation or focus the sample at every field of view.
    c. Take Images at 16-bit depth in the following channels and order:
        i. donor channel.
        ii. FRET channel.
        iii. acceptor channel.
        iv. cotransfection channels (mCherry and miRFP if required).
        v. brightfield.

Note: Acquisition of shorter wavelength channels before longer wavelength can theoretically cause photobleaching in longer wavelength channels. However, this effect is negligible here and we prefer to acquire shorter wavelength channel images first because they are the most critical for quantification.

    d. Keep exposure times and excitation light intensity constant for donor and FRET channels.

Note: Exposure time will be different for each channel and depend on the fluorophore, the expression level, the light source and especially the excitation and emission filters, as well as on the used objective and the camera sensitivity. We suggest that for the biosensor only conditions the mean intensity of the intensity histogram is located at approximately 5% of the dynamic range of the camera and the maximum intensity value does not exceed 50%. For EMCCD cameras, as used in this work, exposure times below 50 ms are not recommended as the signal-to-noise ratio might be compromised by the camera read noise (though true in general, the exact timing might be different for sCMOS cameras or other microscopic setups). We therefore recommend reducing the excitation light intensity in case exposure times reach this short-end limit. On the other hand, exposure times should ideally not exceed 100–150 ms to ensure minimal time lag between the donor channel and FRET channel acquisition to avoid motion artifacts in the FRET analysis. If time is critical, binning can be used to keep noise low. However, this is at the cost of image resolution.

Pause point: Data can be analyzed at any time.

Data analysis: Day 4 and day 5

Timing: 3–8 h, depending on size of data set and degree of automation of the analysis

This section describes the analysis of the acquired fluorescence microscopy images for the calculation of the FRET ratios and the quantification of fluorescence intensities. The calculation of a FRET ratio requires the generation of averaged background images for each channel, a background correction of all images, elimination of oversaturated pixels, segmentation of transfected cells

and the calculation of the FRET ratio image by division of the FRET channel image by the donor channel image. We recommend using ImageJ/Fiji for image processing. The workflow described below from step 25 onwards and in Figure 4 refers to a custom Fiji macro deposited together with the publication (Müller et al., 2020) at https://github.com/paulmarkusmueller/Mueller_et_al_2020 or at Zenodo: https://doi.org/10.5281/zenodo.7014704.

24. Visually inspect a random selection of the acquired images for each channel to control if image quality is appropriate over the entire 96-well plate (e.g., no z focus drift, sufficient transfection efficiency and cell viability).
25. Visually inspect all background images of each channel and discard images with fluorescent particles in the background. Create an average background image for each channel.

Note: These images will be used later for background correction.

26. In the FRET and the donor channel images exclude pixels that are oversaturated from further analysis. This is achieved by defining a threshold that is set to one value below the maximum intensity value the camera can record (e.g., 65534 for 16 bit images, 4094 for 12 bit images).
27. Convert the thresholded pixels into a saturation region of interest (ROI). These saturation ROIs will later be combined with further segmentations to a final ROI (step 29c).

Note: Exclusion of pixels needs be done before the background correction (step 28).

Note: This step serves as a precautionary measure. If saturated pixels appear on a regular basis or if large regions in the images are saturated the exposure time should be reduced (see also "imaging: day 4").

28. Optional: If raw images and averaged background images appear noisy, a Gaussian blur filter can be applied (we suggest using a radius of 1 pixel).
29. For background correction in each channel, subtract the respective average background image from the raw image.
30. Threshold the images to specifically select transfected cells for analysis.

Note: Thresholding can be done by fixed values or by thresholding algorithms. However, we recommend applying fixed thresholding values across the whole plate that will likely be less prone to variations in transfection efficiency. We suggest empirically and iteratively testing different threshold values to maximize the effect size of the positive controls while keeping the area of the ROI large to ensure low expression conditions are included in the analysis.

a. Generate ROIs representing transfected cells in the acceptor channel, the mCherry channel and, if required, the miRFP670 channel.
b. To reduce noise in the FRET ratio image, generate ROIs that exclude pixels with the value 0 in the donor and the FRET channel. If not sufficient, this threshold can be carefully increased.
c. Combine all ROIs from step 26, step 29a and step 29b for each field of view (FOV) using the "AND" logical operator.

Note: This step ensures that a pixel is only included in the final ROI if it is present in each of the individual ROIs. This way, analyzed regions only comprise cells that are successfully transfected with all designated constructs, while excluding saturated and optionally low intensity pixels.

Note: We recommend threshold values that define the final ROI primarily based on the acceptor channel, making this channel critical in limiting the area of the final ROI under normal

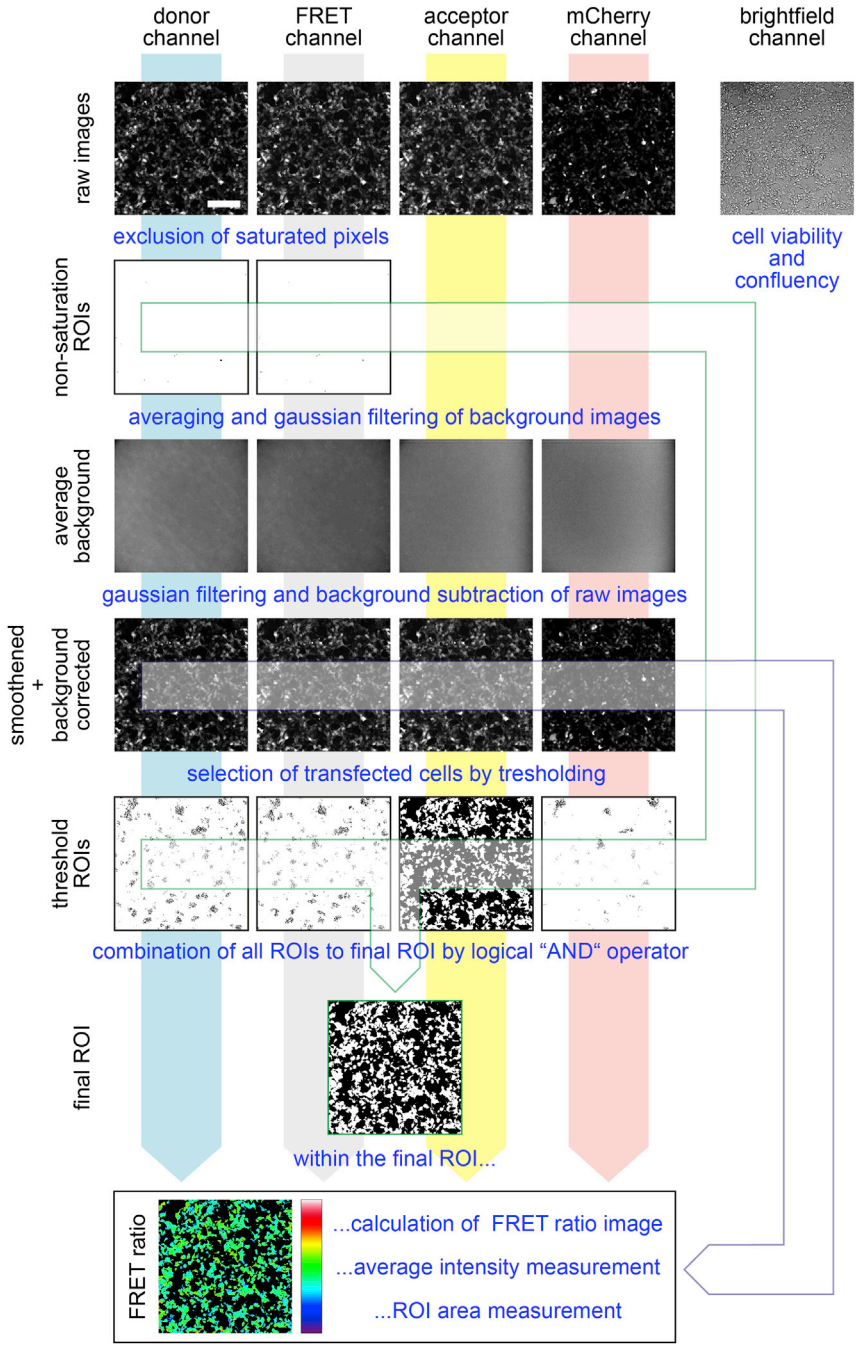


Figure 4. Image analysis workflow for FRET ratio calculation

From top to bottom: raw images of donor, FRET, acceptor and mCherry channel are processed and analyzed, brightfield images serve for visual inspection of cell viability and confluency. Non-saturation masks are created in donor and FRET channels to exclude saturated pixels from calculation of the FRET ratio. Average background images are created by averaging all recorded background images and smoothening by a gaussian filter. Smoothened average background images are then subtracted from raw images. The smoothened and background-corrected images are thresholded to generate masks that only include transfected cells. All masks are combined by logical AND operation to generate a final mask. The FRET ratio image is calculated by dividing the FRET by the donor image within the final mask. The average intensities are measured in the FRET ratio image and in the smoothened and background corrected acceptor and mCherry channel images and the area of the final mask is determined. Image processing, calculation and analysis steps are highlighted in blue. Contrast settings are not uniform for the shown images for visualization purposes. All images show the same FOV in different channels or as binary masks (black pixels are excluded from ROIs). Scale bar: 200 μm.

conditions (Figure 4 "threshold ROIs"). Only in cases where the RhoGEF or RhoGAP is expressed at very low levels the mCherry channel will become limiting.

31. Calculate the FRET ratio image in 32 bit image format by setting the pixels of the FRET channel image that are not included in the final ROI to "NaN" (not a number) and divide the FRET channel image by the donor channel image.
32. Measure the FRET ratio over the entire FRET ratio image.
33. As a control, measure the average intensity within the final ROI in all channels as well as the area of the final ROI for every FOV.

Note: These values serve to compare transfection efficiency and expression levels and to identify outliers.

EXPECTED OUTCOMES

A good transfection with the Rho biosensors and RhoGEFs or RhoGAPs of interest can yield up to 90% transfection efficiency and should not be lower than 20%–30%. With HEK293T cells at 10× magnification and 90% confluency this approximately corresponds to a minimum of 50–100 and up to 500 transfected cells per field of view. The transfection efficiency can be estimated by the area of the final ROI which should range between 50,000 and 250,000 μm^2 (Figure 5). ROIs smaller or larger than that can also be used for analysis. However, we suggest inspecting those wells for cell viability, dirt, cell clumps or debris by means of the brightfield images and the acceptor and mCherry channel images.

FRET ratios of control wells at the beginning of the measurement should not vary from those measured at the end (see wells 2A-2H and wells 11E-11H Figure 5A). Furthermore, no edge effect should be observed as evident by constant FRET ratios from outer to inner wells in the biosensor only and/or the biosensor + RhoGDI control wells (see wells 2A-2D and 2E-2F Figure 5A).

For the RhoGEF assay the FRET ratio of biosensor only should be at least 1.15 fold the FRET ratio of biosensor + RhoGDI, ideally this effect reaches 1.3 fold for the RhoA-2G sensor and 1.2 fold for the Rac1-2G and Cdc42-2G sensor. The positive control with the RhoGEF MCF2 should induce a maximum FRET ratio in the range of 1.7–2 fold the FRET ratio of biosensor + RhoGDI for RHOA and 1.2 fold the FRET ratio for RAC1 and CDC42. Note that the RhoA-2G and Rac1-2G FRET sensors exhibit a larger dynamic range than the Cdc42-2G sensor. TIAM1, a strong RAC1-specific GEF, reaches up to 1.7–2 fold the FRET ratio of biosensor + GDI (Figure 5B), while PLEKHG4 or FGD1, two strong CDC42-specific GEFs, do not exceed about 1.2 fold the FRET ratio of biosensor + RhoGDI, similar to the positive control MCF2.

For the RhoGAP assay the FRET ratio of the positive controls should decrease to 0.7 times the FRET ratio of biosensor only for RhoA-2G (in presence of ARHGAP19), for Rac1-2G to 0.85 (ARHGAP22) and for Cdc42-2G to 0.9 (ARHGAP31(1–820)). It should be noted that the correct filter settings are critical for the values given above.

As can be seen by the average mCherry intensities, the expression levels of different RhoGEFs and RhoGAPs can vary substantially (Figure 5B). Importantly, the assay is largely robust to such variations. For example expression of a RhoGEF at levels even below 10% of the mCherry control can still effectively activate the biosensor (see TIAM2 and VAV2 Figure 5B; see also Extended Data Figures 2F and 2G (Müller et al., 2020)).

Differences in RhoGEF or RhoGAP expression can also lead to differences in biosensor levels as determined by mean cp-Venus intensities in the acceptor channel. Even though the assay is robust also to these variations, we recommend to carefully inspect conditions where the average intensity

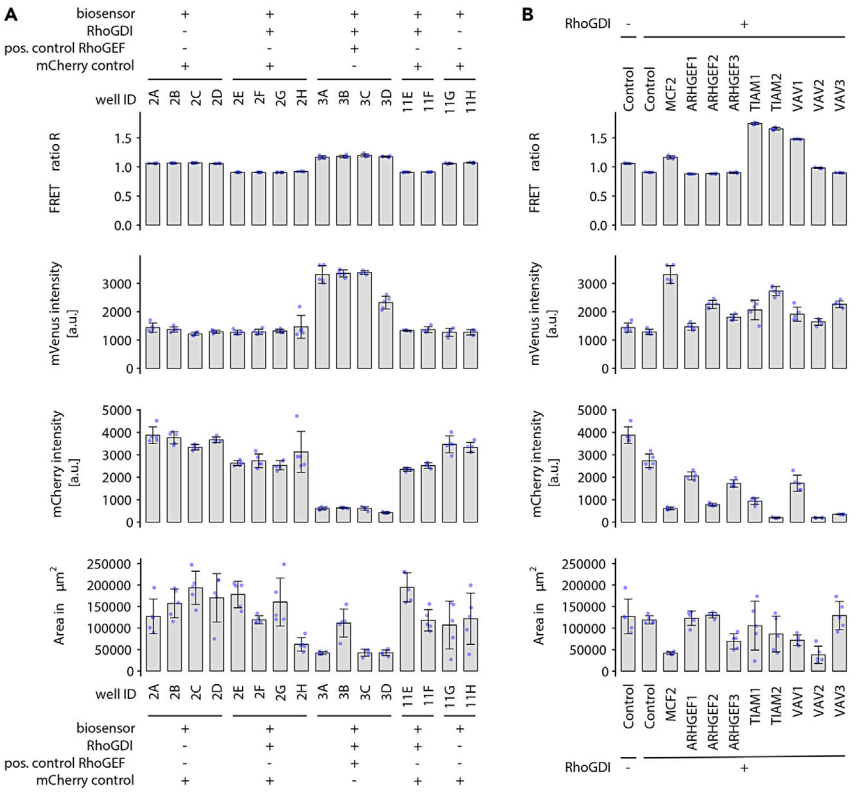


Figure 5. Example result of a RhoGEF assay using the Rac1-2G biosensor

(A) Analysis of all control conditions of the RhoGEF assay with the Rac1-2G biosensor according to the scheme in Figure 2. Wells 2A-2D and wells 11G,H show biosensor only, wells 2E-2H and wells 11E,F show biosensor + RhoGDI, wells 3A-3D show biosensor + RhoGDI + positive control RhoGEF (mCherry-MCF2). Wells 2A-3D are recorded at the beginning of the 96-well recording, while wells 11E-11F are recorded at the end.

(B) Example results of a small RhoGEF screen with the indicated transfected regulators (n=1 well per condition). (A+B) All values show the mean of 5 FOV per well +/- standard deviation, the data points of the individual FOVs are shown in blue. The FRET ratio *R*, the mean cp-Venus and mCherry intensity and the area of the final ROI are shown from top to bottom.

of the biosensors differs by more than 2–3 fold from intensity of the biosensor only or biosensor + RhoGDI condition, especially if the observed change in FRET ratio is relatively small (see Extended Data Figure 2E (Müller et al., 2020).

We suggest performing pilot experiments with positive and negative controls and a subset of regulators to test if the values described above are met.

QUANTIFICATION AND STATISTICAL ANALYSIS

The measured results from the technical replicates or the independent experiments (see section "assay setup and initial calculations") are averaged and their variation is assessed by statistical testing. In screening scale applications hits can be determined by applying thresholds, by statistical testing or by combining both. While thresholds define hits based on the effect size, statistical analysis is used to define significance levels. Because none of these approaches on their own allow valid conclusions about the biological relevance of an observed effect we use a combination of both in our protocol.

The threshold needs be carefully chosen. This can be facilitated by benchmarking based on known RhoGEF and RhoGAP activities. We reasoned that the effect size to identify biologically relevant hits in our experimental approach can be fairly small for two reasons. Firstly, changes in Rho GTPase

activity by full-length RhoGEFs and RhoGAPs are not necessarily actuated on a global level in the cell but rather in small localized pools and secondly, overexpressed full-length RhoGEFs and RhoGAPs do not always exert their full activity due to autoinhibitory regulation of their activity. Therefore, we suggest setting a threshold based on the standard deviation of the control condition, e.g., biosensor only +/- 2 to 4 fold the standard deviation of the biosensor only measurements (for details see (Müller et al., 2020).

Statistical validation of the identified hits requires a multiple comparison correction. We suggest applying the Benjamini-Hochberg procedure which controls the false discovery rate (FDR). First, p values are calculated by unpaired Student's t-test or a different suited statistical test. The individual p values are then sorted from lowest to highest and each p value is assigned a rank i with the smallest p value having the rank 1 and the largest p value the rank k. Next, the global significance level α_g is defined, commonly set to 0.05 but higher or lower levels can be considered. The local significance level α_i is thus defined as

$$\alpha_i = \alpha_g * (i / k)$$

with the rank $i = 1,\ldots, k$ and k = the total number of tests. Next, for each p value its local significance level α_i is assigned and starting from the smallest p value the null hypothesis is rejected for all $p \leq \alpha_i$ (''significant''). The first $p > \alpha_i$ marks the rank i from which on all null hypotheses are not rejected (''not significant'').

Optional: Approximation of the preferred substrate GTPase. As the calculated FRET ratios cannot be directly compared between the RHOA, RAC1 and CDC42 biosensors, these need to be normalized to their dynamic range. Thus, the observed change in FRET ratio (ΔR) for each candidate RhoGEF or RhoGAP as compared to biosensor + RhoGDI (for the RhoGEF screen) or biosensor only (for the RhoGAP screen), respectively, is divided by the maximal observed FRET ratio change (ΔR_{MAX}) that is observed in the screen. Alternatively, constitutively active point mutation variants of the biosensors can be used to estimate ΔR_{MAX} for the RhoGEF screen and, accordingly, dominant negative or effector binding deficient variants to benchmark ΔR_{MAX} for the RhoGAP assay. Each observed ΔR is then divided by the respective ΔR_{MAX} resulting in values between 0 and 1 for the RhoGEF assay and between 0 and -1 for the RhoGAP assay. Based on the assumption that the full dynamic range is benchmarked for each biosensor, the resulting values are comparable between the sensors for the different Rho GTPases.

Optional: Direct semi-quantitative comparison of RhoGEF or RhoGAP variants. A semi-quantitative comparison of activities of RhoGEF or RhoGAP variants (e.g., splice isoforms or truncation constructs) requires approximately equal expression levels of the regulators. If necessary, these can be empirically adjusted by plasmid dilution. To account for remaining minor differences in protein levels, the observed change in FRET ratio of a variant is normalized to its expression level by dividing ΔR by the corresponding mCherry intensity. Prior to this, the mCherry intensity can be normalized to the mCherry intensity of the respective control condition (biosensor + RhoGDI for the RhoGEF assay or biosensor only for the RhoGAP assay) to obtain values that are compatible across different 96-well plates and experiments. The main purpose of the mCherry-normalization is to generate values that can be used for statistical analysis. The size of the observed effect, however, has to be judged by means of the FRET ratios, rather than by the mCherry-normalized ΔR. It is therefore critical that the mCherry intensities are comparable. Furthermore, if ΔR of a supposedly more active variant of a regulator is not higher than the less active variant a conclusion cannot be drawn. This is because it cannot be ruled out that the system is over-saturated with regulators and the catalytically relevant fraction of regulator might indeed be smaller than the total population of expressed RhoGEF or RhoGAP. In such cases, the regulators have to be titrated in order to confirm that the observed effect also persists at lower expression levels.

Optional: Modulation of RhoGEF or RhoGAP activity by a further modulator. To analyze the modulation of RhoGEF or RhoGAP activity by an additional coexpressed modulator, the FRET ratios of the two conditions, with and without modulator, can be directly compared and used for statistical testing. However, a prerequisite is that the expression level of the RhoGEF or RhoGAP remains constant between the conditions. If required, the levels can be empirically adjusted by changing the RhoGEF/RhoGAP vs. modulator plasmid ratio.

LIMITATIONS

Our previous family-wide RhoGEF and RhoGAP activity screen has shown that the full-length forms of the regulators are often subject to autoregulation. Release of their autoinhibition may involve mechanisms such as phosphorylation, interactions with additional proteins or with lipid membrane domains. All these regulatory elements are present in the intact cellular environment and thus preserved in the assay setup. However, if for a particular GEF or GAP these elements are unknown and not actuated in the experiment, a modulation of Rho activity may not be detectable. Moreover, because the assay involves the overexpression of a GEF or GAP of interest, interacting endogenous cofactors (proteins or lipid domains) may become limiting. If these factors cannot be rebalanced in the experimental setup, regulatory interactions may not fully take effect. Likewise, an excess fraction of GEF or GAP may mislocalize and escape from regulation if their subcellular targeting depends on recruitment by endogenous factors. That being said, in those cases where the regulatory elements are known for a GEF or GAP or where a mechanistic hypothesis exists, the screening assay can be a very effective tool for further functional studies.

Furthermore, it is known that the overexpression of functional Rho GTPases, such as the biosensors, perturbs the stoichiometric balance between the GTPases and their solubilizing factor RhoGDI (Boulter et al., 2010) and thus the localization and the basal activity of the GTPases. While we rebalance the biosensor overexpression in the RhoGEF assay by reintroducing RhoGDI, we actually take advantage of this effect by hyperactivating the biosensors due to the stable knockdown of RhoGDI in the RhoGAP assay, thereby increasing the dynamic range of the assay. Theoretically, the higher accessibility of the biosensors in the RhoGAP assay could lead to non-physiological interactions between GTPase and GAP. Nevertheless, in contrast to other established Rho GTPase activity assays, our approach can routinely handle full-length RhoGEFs and RhoGAPs in their native regulatory environment.

As with any other experimental setup involving protein overexpression, the performance of this assay may be affected by non-physiological protein levels that may prevent correct protein folding or lead to proteolytic degradation. Furthermore, the fluorescent protein tags of the RhoGEFs and RhoGAPs may interfere with their structure, function or localization. However, out of 41 regulators tested in our previous study, the position of the fluorescent tag affected the localization of only three proteins (Müller et al., 2020).

Our protocol has been optimized for the use in HEK293T cells which exhibit an exceptionally high transfection efficiency and robustness to high ectopic protein expression levels, both being essential for this assay. The experimental workflow can be adapted to other cell lines as long as they meet these requirements.

The protocol will fail to yield robust data for GEFs or GAPs that cannot be expressed at sufficiently high levels (see section "troubleshooting"). For the same reason, GEFs or GAPs that are toxic to the cell at higher expression levels cannot be faithfully analyzed. However, in our hands, this was the case for only two out of 141 GEFs/GAPs.

Stable expression of the Rho biosensors could simplify the assay procedure. However, we found that such a shortcut in the protocol substantially reduces the dynamic range of the biosensor, presumably

due to compensatory mechanisms such as the adjustment of endogenous RhoGDI expression levels (Boulter et al., 2010).

In conclusion, the protocol presented here provides a reliable and robust method to measure RhoGEF or RhoGAP activity in the native cellular environment, offering a semi-quantitative analysis and statistical assessment. For in-depth studies of enzymatic activities or extended investigation of substrate specificities, including also other Rho GTPase family members, the results should be further validated *in vitro* using purified proteins.

TROUBLESHOOTING

Problem 1

Low overall transfection efficiency.

Potential solution

A high transfection efficiency is critical for the robustness and success of the assay. Before beginning with the screen, we recommend authenticating all plasmids, reagents and supplies for cell culture and transfection. Make sure that plasmids are freshly prepared and avoid freeze/thawing cycles at low concentrations (< 100 ng/μL).

The most common reason for weak expression is pipetting errors with the multichannel pipette. Make sure tips always fit tightly during pipetting and visually inspect equal pipetting volumes across all 8 tips during each pipetting step.

Make sure to split the cells one day before seeding and to use about 80%–90% confluent cells for seeding on day 1 so the cells are in continuous growth phase for 2 days before transfection. Cell confluency should be at about 90% on day 4, optimizing the seeding density of cells can help to reach this percentage.

We use a DNA:PEI reagent ratio of 1:3 (DNA [μg] : PEI [μL]). The ratio can be varied from 1:1-1:4.

We suggest waiting for 48 h after transfection until the cells are imaged. This time span can be increased to reach higher expression levels.

High background fluorescence masking the biosensor signal can be mistakenly interpreted as low transfection efficiency of the biosensor. A known source of background fluorescence is Riboflavin which is included in standard DMEM medium. This back fluorescence is especially high in epifluorescence applications in the blue to green light spectrum that is used for excitation of the donor and FRET channels. Therefore, normal DMEM medium reduces the signal-to-noise ratio. We highly recommend the use of specialized low fluorescence medium for live cell microscopy applications such as FluoroBrite DMEM.

Problem 2

Low expression level of one of the plasmids transfected.

Potential solution

The most common reason for weak expression is pipetting errors with the multichannel pipette. Make sure tips always fit tightly during pipetting and visually inspect equal pipetting volumes between all 8 tips at each pipetting step.

We recommend identifying outliers in protein expression by plotting the average intensity of the acceptor and the mCherry channels for each FOV and comparing it first to the FOVs in the same well and then to other conditions on the plate.

- If the expression levels differ from other regions in the same well, dirt or cell clumps might be located in that FOV. In this case the FOV is not considered for further analysis and the four remaining FOVs are used for averaging of that well.
- If the expression levels are similar over all 5 FOVs of that well, check if the expression is low for all transfected constructs or just for one of them. For the RhoGEFs and RhoGAPs it is known that some of them express only at low levels which is *per se* not problematic as changes in FRET ratio can be detected even at low expression levels of the regulators (see for example TIAM2 Figure 5B).

Problem 3

Toxicity of protein expression, cells round up, cells detach, small ROI.

Potential solution

Expression of some of the RhoGEFs and RhoGAPs leads to considerable cell rounding, often as a result of high RHOA activity. This effect is inherent to the system. In such cases caution has to be taken not to detach the cells when changing the medium on day 3. If cells detach or die, the RhoGEF or RhoGAP concentration can be further reduced and filled up with miRFP670 plasmid to the total 280 ng or 270 ng of plasmid DNA per well for the RhoGEF or the RhoGAP assay, respectively.

Cells can also detach as a result of an exceeding cell confluency leading to rolling up of the entire cell layer. Cell confluency should not be higher than 90% on the day of imaging.

Insufficient coating of wells with poly-L-lysine may have prevented a proper adhesion of the HEK293T cells. Make sure the entire surface of wells is covered with the coating solution and authenticate the reagent.

Problem 4

Edge effect as observed by a higher FRET ratio in the wells located at the edge of the plate as compared to the inner wells.

Potential solution

The edge effect mainly occurs as a result of two phenomena. Firstly, the evaporation in edge wells is higher than in center wells, leading to an increase in salt concentrations in the edge wells. Secondly, the edge wells are more prone to temperature changes, they cool down more rapidly when the plates are taken out of the incubator, while they heat up more rapidly when the plate is cooled down to 20°C–25°C and placed back into the incubator. This can result in differences in cell density and expression levels between outer and inner wells of the 96-well plate. It should be noted that to some extent such variations can be tolerated by the assay. If, however, the FRET ratio varies between outer to inner wells in the control conditions, the following precautionary measures included in the protocol should be checked again:

- The use of a humidity chamber as described above (Figure 2) prevents a decrease in humidity as a result of repeated opening of the incubator and accelerates the re-humidification of the samples.
- Daily changes of medium compensate for differences in evaporation between wells.
- Before imaging, the 96-well plate should be equilibrated with the lid open on the microscope.
- Additionally, 96-well plates can be placed on pads tempered to 37°C to maintain temperature while working with the plates in the biosafety cabinet.

The easiest way to avoid the edge effect, if affordable, is not to use the edge wells for analysis. The phenomenon is practically not observable between the second and third outermost wells of a 96-well plate.

Problem 5

Low dynamic range of the assay as determined by the positive control.

Potential solution

A low dynamic range of the assay can originate from suboptimal filter settings. To capture the highest dynamic range of the FRET effect, filters with the optimal excitation and emission wavelength and bandwidth have to be chosen (see Table 1).

Additionally, we observed that a low dynamic range can originate from the use of biosensor and regulator plasmids with different promoters. This can lead to poor protein expression levels and poor overall performance of the assay. It is therefore important to use the same promoters in the experiments that yield high protein levels (typically CMV).

As described above (problem 1), a high background fluorescence masking the biosensor signal can also result in a decreased dynamic range. We strongly recommend the use of specialized low fluorescence medium for live cell microscopy applications such as FluoroBrite DMEM.

Problem 6

Discrepancy between control conditions recorded at the beginning and at the end of the 96-well plate recording.

Potential solution

Such discrepancies likely arise from strong evaporation during image acquisition. This can be compensated by increasing the humidity in the microscope life cell incubation system or by increasing the speed of image acquisition. It can for example be helpful to focus only once in every well, if focusing is a time-consuming step.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Oliver Rocks (oliver.rocks@charite.de).

Materials availability

Materials used in this study can be obtained from Addgene or are available from the lead contact upon reasonable request.

Data and code availability

All data collected and analyzed in this study are available at http://the-rhome.com. The code for FRET analysis is available at https://github.com/paulmarkusmueller/Mueller_et_al_2020 or at Zenodo: https://doi.org/10.5281/zenodo.7014704. Any additional information required to reanalyze the data reported in this paper is available from the technical contact, Paul Markus Müller (paulmarkus.mueller@fu-berlin.de).

ACKNOWLEDGMENTS

We thank Olivier Pertz and Luis Hodgson for kindly providing the biosensor constructs used in this work. We thank Rafael D. Fritz for helpful discussions and the MDC Advanced Light Microscopy Facility for technical assistance. We acknowledge financial support from the Open Access Publication Fund of Charité – Universitätsmedizin Berlin and the German Research Foundation (DFG).

AUTHOR CONTRIBUTIONS

Conceptualization, P.M.M. and O.R. P.M.M. designed the work and executed experiments and analyses with overall scientific guidance by O.R.; Writing, O.R. and P.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Becker, M.S., Müller, P.M., Bajorat, J., Schroeder, A., Giaisi, M., Amin, E., Ahmadian, M.R., Rocks, O., Köhler, R., Krammer, P.H., and Li-Weber, M. (2016). The anticancer phytochemical rocaglamide inhibits Rho GTPase activity and cancer cell migration. Oncotarget *7*, 51908–51921. https://doi.org/10.18632/oncotarget.10188.

Boulter, E., Garcia-Mata, R., Guilluy, C., Dubash, A., Rossi, G., Brennwald, P.J., and Burridge, K. (2010). Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. Nat. Cell Biol. *12*, 477–483. https://doi.org/10.1038/ncb2049.

Eccles, R.L., Czajkowski, M.T., Barth, C., Müller, P.M., McShane, E., Grunwald, S., Beaudette, P., Mecklenburg, N., Volkmer, R., Zühlke, K., et al. (2016). Bimodal antagonism of PKA signalling by ARHGAP36. Nat. Commun. *7*, 12963. https://doi.org/10.1038/NCOMMS12963.

Fritz, R.D., Letzelter, M., Reimann, A., Martin, K., Fusco, L., Ritsma, L., Ponsioen, B., Fluri, E., Schulte-Merker, S., van Rheenen, J., and Pertz, O. (2013). A versatile toolkit to produce sensitive FRET biosensors to visualize signaling in time and space. Sci. Signal. *6*, rs12. https://doi.org/10.1126/SCISIGNAL.2004135.

Fritz, R.D., Menshykau, D., Martin, K., Reimann, A., Pontelli, V., and Pertz, O. (2015). SrGAP2-Dependent integration of membrane geometry and slit-robo-repulsive cues regulates fibroblast contact inhibition of locomotion. Dev. Cell *35*, 78–92. https://doi.org/10.1016/J.DEVCEL.2015.09.002.

Hanna, S., Miskolci, V., Cox, D., and Hodgson, L. (2014). A new genetically encoded single-chain biosensor for Cdc42 based on FRET, useful for live-cell imaging. PLoS One *9*, e96469. https://doi.org/10.1371/JOURNAL.PONE.0096469.

Lamarche-Vane, N., and Hall, A. (1998). CdGAP, a novel proline-rich GTPase-activating protein for Cdc42 and Rac. J. Biol. Chem. *273*, 29172–29177. https://doi.org/10.1074/jbc.273.44.29172.

Martin, K., Reimann, A., Fritz, R.D., Ryu, H., Jeon, N.L., and Pertz, O. (2016). Spatio-temporal co-ordination of RhoA, Rac1 and Cdc42 activation during prototypical edge protrusion and retraction dynamics. Sci. Rep. *6*, 21901–21914. https://doi.org/10.1038/srep21901.

Moshfegh, Y., Bravo-Cordero, J.J., Miskolci, V., Condeelis, J., and Hodgson, L. (2014). A Trio-Rac1-Pak1 signalling axis drives invadopodia disassembly. Nat. Cell Biol. *16*, 574–586. https://doi.org/10.1038/NCB2972.

Müller, P.M., Rademacher, J., Bagshaw, R.D., Wortmann, C., Barth, C., van Unen, J., Alp, K.M., Giudice, G., Eccles, R.L., Heinrich, L.E., et al. (2020). Systems analysis of RhoGEF and RhoGAP regulatory proteins reveals spatially organized RAC1 signalling from integrin adhesions. Nat. Cell Biol. *22*, 498–511. https://doi.org/10.1038/s41556-020-0488-x.

Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M., and Miyawaki, A. (2004). Expanded dynamic range of fluorescent indicators for Ca(2+) by circularly permuted yellow fluorescent proteins. Proc. Natl. Acad. Sci. USA *101*, 10554–10559. https://doi.org/10.1073/PNAS.0400417101.

Pertz, O., Hodgson, L., Klemke, R.L., and Hahn, K.M. (2006). Spatiotemporal dynamics of RhoA activity in migrating cells. Nature *440*, 1069–1072. https://doi.org/10.1038/NATURE04665.

Ren, X.D., Kiosses, W.B., and Schwartz, M.A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. *18*, 578–585. https://doi.org/10.1093/emboj/18.3.578.

Shcherbakova, D.M., Baloban, M., Emelyanov, A.V., Brenowitz, M., Guo, P., and Verkhusha, V.V. (2016). Bright monomeric near-infrared fluorescent proteins as tags and biosensors for multiscale imaging. Nat. Commun. *7*, 12405. https://doi.org/10.1038/NCOMMS12405.

Spiering, D., Bravo-Cordero, J.J., Moshfegh, Y., Miskolci, V., and Hodgson, L. (2013). Quantitative ratiometric imaging of FRET-biosensors in living cells. Methods Cell Biol. *114*, 593–609. https://doi.org/10.1016/B978-0-12-407761-4.00025-7.

Van Unen, J., Reinhard, N.R., Yin, T., Wu, Y.I., Postma, M., Gadella, T.W.J., and Goedhart, J. (2015). Plasma membrane restricted RhoGEF activity is sufficient for RhoA-mediated actin polymerization. Sci. Rep. *5*, 14693. https://doi.org/10.1038/SREP14693.

Walzl, A., Kramer, N., Mazza, G., Rosner, M., Falkenhagen, D., Hengstschläger, M., Schwanzer-pfeiffer, D., and Dolznig, H. (2012). A simple and cost efficient method to avoid unequal evaporation in cellular screening assays , which restores cellular metabolic activity. Int. J. Appl. Sci. Technol. *2*, 17–22.