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Biosensors for Characterizing the Dynamics of Rho Family GTPases in Living Cells

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Introduction and Overview

The biosensors developed in our laboratory have been based on different designs, each imparting specific strengths and weaknesses. Here we describe detailed protocols for the application of three biosensors exemplifying different designs -- first a design in which an environmentally sensitive dye is used to report the activation of endogenous Cdc42, followed by two biosensors based on FRET, one using intramolecular and the other intermolecular FRET. The design differences lead to the need for different approaches in imaging and image analysis.

Figure 1 shows the design of the three biosensors. MeroCBD (Fig 1a) is a sensor for Cdc42 activation in which a fragment of Wiskott Aldrich Syndrome protein that binds only to active Cdc42 is derivatized with an environmentally sensitive dye¹. In living cells, the dye undergoes a fluorescence change when the biosensor binds to endogenous, untagged Cdc42. This design is advantageous for several reasons: Cdc42 is not tagged with a fluorophore or other biosensor component, expression of exogenous Cdc42 is not needed, and the signal is substantially brighter than FRET because a bright dye is directly excited, rather than indirectly as in FRET. The major disadvantage is that the biosensor is not genetically encoded so must be loaded in the cells via microinjection, electroporation etc.

The FRET biosensor for RhoA is shown in figure 1B. A fragment of Rhotekin is attached directly to RhoA, and fluorescent proteins undergoing FRET are built into the chain connecting the RhoA and Rhotekin fragment. The C terminus of the RhoA is purposefully left free of fluorescent protein or other modifications, to maintain intact the regulation of RhoA by GDI. Unlike the Cdc42 biosensor, this biosensor is completely genetically encoded, greatly simplifying loading into cells.

Finally, the biosensor for Rac1 is shown in Fig.1C. Named Rac1 FLAIR, it is a modification of a design originally published using a covalently attached fluorescent dye for FRET. In the modification described here, the dye has been replaced with a fluorescent protein, rendering the biosensor fully genetically encoded. A fragment of p21 activated kinase (PAK) is used to bind specifically to activated Rac1. The main difference between this design and that of the RhoA biosensor is that the PAK fragment is not attached to Rac1. Rac1 FLAIR is an intermolecular FRET biosensor, frequently referred to as a dual chain sensor. The use of a dual chain design significantly enhances the sensitivity of the biosensor in comparison with

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single chain FRET biosensors. The latter usually show substantial FRET even when the biosensor is in the 'off' state; the two fluorophores are never fully separated. With the Rac1 sensor and other single chain sensors the dynamic range, the difference between the on and off states, is enhanced. There is a downside to the dual chain approach. The two components of the biosensor distribute differently in the cell, necessitating additional steps in image processing. Both single chain and dual chain biosensors are potentially perturbed by competition from native protein ligands; the dual chain sensor may be more likely to produce 'false negatives' as it may be more sensitive to ligands that compete for the GTPase binding fragment, while the steric bulk of the single chain sensor may prevent it from reaching all sites normally accessed by the GTPases. The issue of competition and its effect on biosensor readout is complex and beyond the scope of this article. We recently completed a careful study of the effects of varying the concentration of each component of the dual chain Rac1 biosensor. This did not appreciably alter the results of our cell protrusion studies (manuscript submitted).

MeroCBD, dye-based biosensor for Cdc42

As discussed above, the meroCBD biosensor for Cdc42 activation is based on a fragment of WASP (the Cdc42 binding domain, CBD) that binds only to activated Cdc42. This fragment is covalently coupled to a solvent-sensitive dye that undergoes a large change in fluorescence intensity when the CBD interacts with endogenous, activated Cdc42²⁻⁴. Because the dye responds to Cdc42 binding with an intensity change, rather than with a shift in λ_{max} , it is useful to attach a second fluorophore to the biosensor for ratio imaging. In this section we describe first production of CBD fused to EGFP, then conjugation of the CBD-EGFP with fluorescent dye, and finally image acquisition in living cells. Dyes are available from the Hahn lab and should be commercially available soon. Solvent sensitive dyes other than those that have been used to date should also respond to Cdc42 binding.

CBD-EGFP expression and purification

CBD-EGFP is expressed in the form of a C-terminal 6xHis fusion from the prokaryotic expression vector pET23. This vector has a strong T7 promoter, and is designed to work with BL21(DE3) strains of *E. coli* (Stratagene). It was determined experimentally that the highest levels of expression are observed when a plain T7 promoter (not T7lac) is used in combination with a BL21(DE3) strain, and not BL21(DE3)pLysS. The BL21(DE3) strain is more 'leaky' but this is not of great consequence as CBD-EGFP shows no appreciable toxicity. The protein is best induced and expressed at room temperature (26°C), which increases the proportion of correctly folded, soluble CBD-EGFP.

Key points include:

- 1) The bacteria strain is critical. BL21(DE3) are recommended. Do not use BL21(DE3)pLysS.
- 2) Use Talon resin (Co²⁺ affinity, Clontech Inc.). Do not use Ni-NTA resin. Use approximately 2 ml Talon resin (dry volume) for 3g of cell pellet.
- 3) Use the buffers suggested in this article. Apparently small changes have proven to greatly reduce yield.
- 4) Use enough buffer during lysis; i.e., for 3g cell pellet use 35 ml total lysis buffer.

Day 1—1. Competent BL21(DE3) cells are transformed with pET23-CBD-EGFP according to standard protocols⁵, and plated on LB-carb (100 µg/ml carbenicillin) plates. The 200µl transformation volume should be split over 2 plates.

2. Plates are incubated at 37°C overnight.

Day 2—3. 500 ml of LB-carb (100 µg/ml carbenicillin) are inoculated with the colonies from the plates. 5 ml of media are added on each plate and cells are resuspended into the media. The cell suspension is transferred into the 500 ml LB-carb and grown in a shaker at 37°C, 225rpm to OD₆₀₀ = 0.8–0.9. The culture is briefly chilled on ice to 26°C and put back in the shaking incubator, with temperature reduced to 26°C.

4. IPTG (1 M stock in water, kept at –20°C) is added to a final concentration of 0.2 mM, and the cultures are allowed to grow for another 6 hours at 26°C at 225rpm. IPTG concentrations of 0.2~0.5mM has been used successfully.

5. Cells are collected by centrifugation (Beckman JA-10 rotor, 10 min, 6000 rcf), and stored as a pellet at –20°C until use. Approximately 2.5–3 g of cells are usually obtained from each liter of culture.

Day 3—6. Cells (~3 g) are resuspended in 35 ml of Lysis buffer [50 mM NaH₂PO₄, pH 7.6, 300 mM NaCl, 10% glycerol, 5 mM MgCl₂, 2 mM β-ME, 1 mM PMSF] and lysed by sonication (4 pulses, 30 sec each on ice with 1 min rests).

7. The lysates are centrifuged at 13,000 rpm for 30 min, and the supernatant containing CBD-EGFP is carefully transferred into a 50 ml Falcon tube.

8. While the lysates are being centrifuged, 2 ml of Talon resin (dry volume) are transferred into a 50 ml Falcon tube and centrifuged at 700 rcf in a swinging bucket centrifuge. Two ml resin per 3 g cell pellet are recommended.

9. Talon resin is washed twice with 10 volumes of the lysis buffer (no β-ME and PMSF) in a 50 ml Falcon tube. Again, pellet the resin by centrifuging at 700 rcf.

10. The cell lysate is added to the washed Talon resin in the 50 ml falcon tube and inverted gently using an orbit shaker at room temperature for 40 min to 1hr, then wrapped in foil to avoid unnecessary exposure of EGFP to light. The resin is then separated by centrifugation at 700g in a swinging bucket centrifuge.

11. The supernatant is removed and saved (unbound fraction). If a large portion of unbound material is present in this fraction, consider increasing the Talon resin volume by preparing 2 tubes of 2ml resin and splitting the lysates into 2 tubes during the binding reaction.

12. The resin is washed twice (5 min each at room temperature, orbit shaker) with 20 ml of fresh buffer, excluding PMSF or βME.

13. The final wash is performed with 10 volumes of buffer containing 5 mM imidazole. It is important to prepare a fresh 5ml of 1M imidazole stock solution in the same buffer. Always prepare the stock imidazole solution fresh.

14. The elution is performed by adding 5 ml buffer containing 150 mM imidazole to the resin and rotated using the orbit shaker at room temperature for 5 min. Pellet the resin again by centrifugation.

15. The supernatant is removed and saved (eluted fraction).

16. The resulting 5 ml eluate is concentrated with the Ultrafree – 4 Centrifugal Filtration Device (Millipore; 5000Da cut-off) by centrifugation at 4 °C following the manufacturer's

directions. Check the concentration process every 20 min to ensure proper filtration. Do not over-concentrate; optimal final concentration should be approximately 120 μM . The concentration of CBD-EGFP is measured by taking a small aliquot (5–10 μL) and diluting into 50 mM Tris HCl (pH 7.5–8.0). Protein concentration can be determined using the absorbance at 280 with an extinction coefficient of 28260 ($\text{cm}^{-1} \text{M}^{-1}$), and the following equation:

$$[\text{CBD-EGFP}] \text{ (in mol/L)} = (\text{OD}_{280} * \text{dilution factor}) / 28260 \quad (\text{eq. 1})$$

On average, 10–20 mg of CBD-EGFP is obtained per liter culture.

17. If dye-labeling is to be performed the following day, a part of the concentrated protein is dialyzed overnight against 2 L of 50 mM NaH_2PO_4 (monobasic sodium phosphate) buffer at pH 7.5. Slide-A-Lyzer cassettes (PIERCE) with a molecular weight cut-off of 3,500Da can be conveniently used.

18. For long term storage, the protein is dialyzed overnight against 2 L of storage buffer [50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl_2 , 10% Glycerol] and is frozen at -80°C . This appears to be better than flash freezing for this protein.

Labeling EGFP-CBD with reactive fluorophore

For attachment of the dyes to cysteine, CBD-EGFP needs to be in 50 mM NaH_2PO_4 buffer, pH 7.5 at a protein concentration of 200 μM . A fresh solution of dye is prepared in pure DMSO by adding approximately 1 mg of dye into 30–40 μL DMSO. Once dissolved in DMSO, the dye should be used within 12 hrs. The exact concentration of the dye is determined spectrophotometrically by diluting a small aliquot of the DMSO solution 1:5,000–10,000 in additional DMSO. The ISOs-IAA dye extinction coefficient in DMSO at maximum absorption (593 nm) is 135,000 ($\text{cm}^{-1} \text{M}^{-1}$).

$$[\text{ISOs-IAA}] \text{ (in mol/L)} = (\text{OD}_{593} * \text{dilution factor}) / 135,000 \quad (\text{eq. 2})$$

We routinely obtain concentrations of 30–40 mM as the DMSO stock solution.

A 300 μL aliquot of fresh CBD-EGFP protein is transferred into a 2 ml Eppendorf tube wrapped in foil to protect from light. The dye is added to the CBD-EGFP solution in 2–3 aliquots to bring the final dye to protein ratio in the reaction to 6:1. This ratio can be further optimized depending on the reactivity of the dye if different dyes are used. We suggest 6:1 as a useful starting point. The protein-dye mix is vortexed once briefly at highest setting immediately after addition of the dye stock solution. Avoid excess mixing as this may denature the protein. Using higher dye to CBD-EGFP ratios (e. g. 10:1 or 15:1) in the reaction mixture can produce excessive amounts of precipitated material and “over-labeling” (dye to protein ratio in the purified covalent adduct greater than 1.0 due labeling of the exposed cysteine in EGFP). The optimal dye to protein ratio to use in the reaction mixture depends on the reactivity of the dye, so it can vary for different dyes, or dye from different sources/batches. The tube containing the reaction mixture is wrapped in foil and gently rotated on a rotating wheel stirrer at room temperature for exactly 1 hour. Here, the time is critical; longer reaction times result in over-labeling of the protein. After the reaction is complete, 1–5 μL βME is added to stop the reaction and the tube is incubated for 5–10 min at room temperature on the rotating wheel. The tube is centrifuged at room temperature to pellet insoluble material (i.e, 2 min at 13,000 rcf in an Eppendorf benchtop microcentrifuge). The supernatant is then loaded on a small G15 gel filtration column (0.5

cm $\times$ 6–8 cm) to separate the conjugate from free dye. This column is equilibrated and run in 50 mM NaH₂PO₄ buffer at pH 7.5. The first colored band to elute contains the dye-labeled CBD-EGFP (MeroCBD). Fractions of approximately 200 μ L each are collected. An aliquot (3–5 μ L) of each fraction is analyzed by 12% SDS-PAGE to confirm the presence and purity of the MeroCBD. Fluorescence visualization of the gel assures labeling with the dye, and can reveal a band of free dye running at much lower molecular weight, often near marker dyes used in the electrophoresis sample.

To evaluate the success of your procedure, and generation of an optimal biosensor, it is valuable to determine the dye/protein ratio once the biosensor has been freed from unattached dye. It is also important to know your protein concentration to optimize later microinjection in living cells.

An approximate protein concentration can be determined rapidly by taking an absorbance spectrum of the conjugate solution. Dilute a small aliquot (5–10 μ L) in 50mM Tris HCl, pH 7.5–8.0 and use OD280 and Equation (1) above. Importantly, the protein absorbance at 280 overlaps dye absorbance. This overlapping absorbance can be corrected by subtracting, from the OD280, a fraction of the dye's absorbance at its maximum (approximately 593 nm). However, this is variable due to the solvent sensitivity of the dye. For a more accurate measure it is best to compare a sample of the meroCBD (dye-labeled CBD-EGFP) to unlabeled CBD-EGFP by running them on the same gel, using different known concentrations of the unlabeled protein. Colorimetric assays, limited to those whose readouts do not overlap dye absorbance, have proven less reliable in our hands. The dye concentration is determined by taking an absorbance spectrum of meroCBD. 5–10 μ L of meroCBD is diluted in DMSO. DMSO as a solvent will denature and overwhelm the effect of the protein on the dye absorbance, resulting in a consistent dye extinction coefficient. OD at the dye absorbance maximum of 593 nm is used to calculate the dye concentration using Equation (2) above.

Only 40–60% of the CBD-EGFP is recovered as purified meroCBD, due to some denaturation/precipitation during labeling and loss during gel filtration. The final eluate concentration is usually 120–140 μ M. Labeling efficiency under these conditions varies between 0.7–0.9 dye/protein ratio. The meroCBD solution is divided into 15–20 μ L aliquots and stored at –80°C. Flash-freezing using liquid N₂ or dry ice appears to increase precipitation during microinjection. Alternatively, meroCBD may be kept at 4°C for up to one week.

Imaging meroCBD in living cells

We routinely microinject cells on coverslips and image meroCBD following 30 minutes of recovery time in a tissue culture incubator. MeroCBD can produce puncta (possibly autophagy) after 2–3 hrs in some cells, in which case the timing between microinjection and observation must be carefully controlled.

Cells are observed in a live-cell chamber atop an inverted epifluorescence microscope outfitted with an Hg arc-lamp light source. When imaging with a single camera, excitation and emission filterwheels can be used to alternately illuminate the dye and EGFP to obtain a ratiometric image set at every time point during the time-lapse experiment. If two cameras are available, two-camera modules such as those available from Olympus (U-DPCAD module), Technical Video (Technical Video Inc.), or Optical Insights (Dual-Cam) can be used to acquire two images simultaneously using a dual band-pass excitation filter. Since the dye and EGFP will have different brightnesses, band pass filters can be designed to pass different amounts of excitation light at each wavelengths through the dual band-pass filter.

When MeroCBD is made using I-SO type fluorophores^{2–4}, including the new dyes we currently employ (Dyes 53 and 87, manuscript in preparation) EGFP bleed-through into the dye channel is practically zero. This is made possible by using relatively narrow excitation and emission band-pass filters. We use a customized multiband-pass dichroic mirror (Fig.2) and the following excitation and emission filters: HQ470/40 and HQ525/50 for EGFP; HQ580/30 and HQ630/40 for dye (Chroma Technology, Rockingham, VT).

The interval between images will vary depending on the images. We routinely acquire images at 10s ~ 1 min time intervals using 2×2 binning on a full-field 1.3k by 1.0k cooled CCD camera. Importantly, the excitation light from the 100W Hg arc lamp source is too bright, so it is routinely reduced using neutral density filters for 22~36% transmittance (ND 1.4 ~ 1.0). The integration (exposure) time on CCD camera depends on the quantum efficiency of the CCD and the noise characteristics of the camera. The dye exposure is usually $0.5 - 0.3\times$ as long as the EGFP exposure. Dye exposure times may be reduced with some cameras; many current cameras, including those we use, are less efficient at the red wavelengths of the dyes. Users should fine-tune the exposure times depending on their optics and camera setup. Ideally, the integrated average intensity from both fluorescence channels should be similar to maximize the dynamic range of the data. Cameras with 12 bit depth have an intensity range from 0 ~ 4096; we routinely fill ~ 3000 of this dynamic range in a single field of view. We carefully monitor the pixel intensities near the cell periphery where the signals are usually low, aiming for approximately 100 ~ 200 over the background values. This translates to 2~3 in signal to noise ratio at the darkest regions.

Because different wavelengths are used to image EGFP and dye, many objectives (even apochromatic objectives) will focus on slightly different planes within the specimen. The z axis difference between these two focal planes needs to be calibrated and the objective slightly offset between the two images. We use a z axis position control on our microscope to accomplish this. Typically, the z-distance offset is determined by focusing on the same small object at different wavelengths, using image-based autofocus^{6, 7} followed by manual fine adjustment. A script is written to execute the shifts in z for each color acquired during an automated time lapse sequence. Moving the objective turret back and forth in different directions to account for these z-offsets will not give reproducible results due to hysteresis in the focus drive mechanism. Therefore it is better to acquire different colors so that the objective always changes z position in one direction. Alternatively, a linear-encoder feedback or piezo-stage system can be installed for precise z positioning to eliminate the hysteresis problem. It is important to always set the search range for autofocus to be more than twice as large as the total motions programmed in for z focus offset.

Genetically encoded FRET biosensors

Factors affecting FRET efficiency

FRET is sensitive to both the distance and orientation of the two fluorophores in a biosensor. When fluorophores are sufficiently far apart or have orthogonal dipole orientations, excitation of the donor leads simply to donor emission, rather than to FRET. However, when the distance is sufficiently small, and orientation enables sufficient dipole coupling, excitation energy is transferred from the donor to the acceptor, leading to decreased donor emission and increased emission from the acceptor. This produces a characteristic FRET excitation/emission spectrum, different from that of the donor or the acceptor alone.

GFP mutants optimized for FRET in living cells have shown impressive improvements. Mutants incorporate different trade-offs between brightness, FRET efficiency, photostability and the pH dependence of fluorescence characteristics^{8, 9}. Enhanced brightness improves the overall signal to noise ratio in cells, but is not always an improvement if it comes at the

cost of FRET efficiency or photostability¹⁰. The Cyan and Yellow fluorescent proteins (CFP and YFP) and their brighter, pH stable versions Cerulean and Venus^{9, 11} are relatively fast maturing, bright GFP mutants that have proven useful in many FRET biosensors. More recent mutants with improved FRET efficiency¹⁰ include CyPet and YPet, which exhibit 6.7-fold greater FRET efficiency than the original CFP – YFP pair, though more recent findings indicated this particular pair may be more prone to dimerization^{10, 12}.

FRET efficiency is quantified by the Forster equation:

$$R_o = [8.8 \times 10^{23} K^2 n^{-4} Q_d J]^{1/6} \quad (\text{eq. 3})$$

where R_o is the Foerster distance (distance at which energy transfer is 50% efficient) and K^2 is the dipole orientation factor, a function of the donor emission transition moment and the acceptor absorption transition moment. $K^2 = 2/3$ is generally assumed when fluorophore rotation can occur about the bond attaching the fluorophore to the protein¹³. Q_d is the fluorescence quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, generally assumed to be 1.4 for proteins¹⁴, and J is the spectral overlap integral, indicating the extent of overlap between the donor fluorescence emission spectrum and the acceptor excitation spectrum¹³.

In FRET biosensors, activation of the targeted protein leads to a change in the distance and/or orientation of the fluorophores. FRET efficiency is exquisitely sensitive to distance between fluorophores (varies as the 6th power of the distance between the fluorophores):

$$E = R_o^6 / (R_o^6 + R^6) \quad (\text{eq. 4})$$

where E is FRET efficiency, R_o is the Forster distance and R is the actual distance¹³. Changes in the relative angular orientations of the dipoles produce a lesser but nonetheless important effect; the dipole orientation factor K^2 can be assumed to be 2/3 only when both fluorophores are free to rotate isotropically during the excited state lifetime. A change in the fixed angle of the fluorophores in different biosensor states affected FRET because K^2 can change between 0 ~ 4^{13, 14}. The effects of fluorophore separation (linear displacement) versus angular reorientation cannot be readily separated in live cell studies, so FRET changes are not used to precisely determine distances between proteins in cells. Rather, the extent of FRET produced by fully activated versus inactive target protein is determined, and these endpoints are used to interpret FRET signals *in vivo*.

Expression of the RhoA single-chain biosensor

The genetically encoded biosensor for RhoA consists of (N-terminus to the C-terminus) a small RhoA binding domain (RBD) derived from the RhoA effector Rhotekin, ECFP, an unstructured linker of optimized length, pH-insensitive Citrine-YFP, and a full-length RhoA (Fig. 1B). Three different expression constructs for this single-chain biosensor have been developed, all available from the nonprofit distributor Addgene (www.addgene.org):

- 1) pTriEX mammalian expression and cloning vector (pTriEX-RhoA/ amp resistance).
- 2) pBabe retroviral expression vector (pBabe-RhoA/ amp resistance).
- 3) pBabe-sin-tet-CMV-puro retroviral vector for Tet-On/Off system (pBabe-sin-tet-CMV-puro-RhoA/ amp resistance).

The pTriEX backbone is used for the cloning of the biosensor and the complete biosensor cDNA can be cut out as a cassette by digesting with NcoI/XhoI. NcoI encodes the integral start codon, and XhoI is placed in-frame immediately following the stop codon after the RhoA GTPase. The pTriEX version of the biosensor allows overexpression in mammalian cells driven by the CMV promoter.

One must beware of the potential toxicity of GTPases upon overexpression¹⁵ and the importance of the relative intracellular concentrations of GTPases and upstream regulators in cells^{16, 17}. We have addressed these issues by using retroviral transduction to stably incorporate low copy numbers of the biosensor DNA using the pBabe expression systems¹⁵. Transduced cells are FACS sorted to obtain low to medium expressors only, so that the biosensor expression level is comparable to endogenous GTPase. We have shown that competition with endogenous effectors of the GTPase is not a significant problem at appropriate biosensor concentrations¹⁵.

In order to address the toxicity of overexpressed biosensor, a tetracycline-inducible retroviral construct based on a pBabe-sin-tet-CMV-puro backbone is used. Using the tet-off stable MEF/3T3 cell system (Clontech), infected cells are repressed using 1 microg/ml Doxycyclin. Cells are selected with Puromycin up to 10 microg/ml, increased gradually in concentration following the infection (2microg/ml increase per each successive selection cycle). At the end of selection, cells are induced for biosensor expression by removal of Doxycyclin and replating at a sparse concentration (1×10^4 cells per 10cm TC dish) for 48 hours. These cells are then FACS sorted to produce nearly 100% biosensor-positive cells. These are then repressed again by application of Doxycyclin.

For the induction of the biosensor prior to experiments, it is important to detach the cells by brief trypsinization and use sparse plating following the removal of Doxycyclin from the culture media 48hrs prior to the experiments. We routinely maintain repressed cells in 1 microg/ml Doxycyclin under standard tissue culture conditions. For induction, cells are detached by a brief trypsinization and spun at 300 rcf for 5 minutes. Supernatant media is suctioned out carefully and completely, and cells are resuspended in fresh culture medium without Doxycyclin. Cells are replated at 1×10^4 cells per 10cm TC dish without Doxycyclin and checked for fluorescence 24 hrs after the induction. The cells are kept in this condition for an additional 24 hrs prior to the assay. The total of 48 hrs after removal of Doxycyclin ensures that overexpressors die and only cells with a sustainable expression level survive for the experiment. Cells are detached and replated on coverslips coated with fibronectin (10microg/ml; Sigma) on the morning of the experiment and allowed to adhere for 5 hrs prior to imaging.

Imaging the RhoA biosensor

Imaging is performed in Ham's F12-K medium without phenol red, supplemented with 2 % fetal bovine serum. For a single-chain design, it is sufficient to simply take a ratio of the FRET emission over the donor emission¹⁵. The two fluorophores will bleach at different rates, so bleaching corrections may be required to counter a bias in the signal over time. Such photobleach corrections are covered in detail elsewhere¹⁸. Correcting for bleedthrough (discussed below) is not required for single chain biosensors, but can be used to improve dynamic range when the biosensor is bright enough to permit this additional image processing.

This biosensor design preserves upstream regulatory interactions with molecules requiring a free C terminus, such as GDI. While this is an advantage in accurately reflecting cell physiology, it requires that one maintain low, near-endogenous levels of biosensor to minimize perturbation of normal cell physiology. At these low biosensor levels one must be

careful to maximize light collection. It is not possible to compensate for low biosensor concentrations simply by increasing irradiation, as this bleaches the biosensor and increases phototoxicity. We routinely use an oil immersion 40× Olympus UIS2 DIC objective lens with a numerical aperture of 1.3, together with 2×2 binning on our cooled CCD camera. We find that using 60× or higher magnification objectives cuts down greatly on light collection while increasing the photobleach rate. The signal intensity scales to the 4th power of the numerical aperture and to the inverse square of the magnification. Therefore, switching from a 1.3 NA 40× DIC objective lens to a 1.42 NA 60× DIC objective lens will result in an approximately 37% decrease in signal intensity. It is important to use DIC rather than phase contrast objective lenses, as the phase objective contains a phase annulus that substantially reduces light transmittance. Neutral density filters of 0.6 ~ 1.0 (54.9% transmittance ~ 36.8% transmittance) are used to cut the brightness of the excitation light. It is better to use longer exposure with dimmer excitation rather than shorter, more intense irradiation; this reduces both photobleaching and phototoxicity. We routinely use methods to remove oxygen from the medium to further decrease photobleaching and phototoxicity, including the oxygen scavenger OxyFluor (Oxyrase, Inc.), antioxidants including vitamin C at 1mM concentration, and purging the assay medium with Argon gas.

We currently use a Roper-Photometrics CoolSnapESII camera, a Sony ICX285-based inline transfer cooled CCD camera cooled to 0C. This camera can be obtained with <6 e- read noise, and 0.1e-/pixel/second dark current. The quantum efficiency of the chip is approximately 60% for 450 ~ 625 nm light, and it has a small pixel size (6.45×6.45microns) for good spatial resolution. We routinely use 2 by 2 binning and expose 800ms for CFP and 400ms for FRET with a 36% transmittance neutral density filter in the excitation light path. These conditions usually result in gray values filling ~75% of the full 12-bit range of camera digitization. We do not recommend the current on-chip amplification-gain CCD (EM-CCD) cameras for biosensor imaging. While these cameras can capture images under extremely dark illumination conditions, the gain-circuitry introduces large amount of stochastic noise, which is problematic for ratiometric imaging (Fig.5). When mathematical operations are performed on raw images, the stochastic noise adds greatly to the total noise levels in the resulting ratio image. While there are techniques available for signal restoration from acquisition with EM-CCD and other noisy approaches^{19, 20}, we have found conventional cooled CCD cameras to be sufficient. Other viable options may include back-thinned, back-illuminated cooled CCD cameras with high quantum efficiency. These cameras offer ultra-high quantum efficiency (over 90%) but the pixel size is usually large (16×16 microns), reducing spatial resolution.

Two identical cameras can be mounted via a beamsplitter to simultaneously acquire the FRET and CFP channels. In such an approach, image registration can be affected by camera orientation as well as the angle of the dichroic mirror (causing rotation, x-y translation, image shear, mismatched scale and mismatched focus). One must carefully mount the cameras to minimize rotational and translational effects, while accounting for the focus differences. Typically, beamsplitters are designed with an adjustable parfocal compensation device that can independently adjust the focusing distance of either camera head. This should be performed empirically to minimize focus difference between the two channels. Rotation, x-y- translation, shear, scaling difference and curvatures in the field of view will need to be corrected computationally based on a priori calibration, as described below.

The choice of imaging medium is an important consideration when imaging FRET biosensors. Background fluorescence from the media is a significant issue at the wavelengths used. We have performed a quantitative comparison of various media available commercially (data not shown), and concluded that Ham's F-12K medium without phenol

red^{21, 22} is a good choice. Unfortunately, this medium is no longer commercially available. The published formulation can be found in Appendix 1.

Rac1 FLAIR, dual chain FRET biosensor for Rac1

The Rac1 biosensor we are currently using is a modification of an older design, wherein the FRET donor was EGFP-Rac1 and the FRET acceptor was a small binding domain derived from p21-activated kinase 1 (PAK1), labeled with the dye Alexa-546²³. We modified this biosensor by replacing the EGFP with the fluorescent protein CyPet, and replaced the Alexa-546 with the fluorescent protein YPet (Fig.1C). The new Rac1-FLAIR biosensor is wholly genetically encoded. It has been extensively validated and compared with other designs²⁴. Unlike the RhoA biosensor above, the donor and acceptor fluorophores are not on the same chain. This improves the dynamic range and hence the sensitivity of the biosensor (in single chain designs, some residual FRET is usually present even in the 'off' state, so the change induced by activation is not as great as in dual chain designs). The disadvantage of the dual chain design is that the two chains do not distribute equally through the cell, necessitating careful bleedthrough correction (see below). The two designs are prone to different effects on cell physiology and artefacts affecting the activation signal. Controls for the Rac1 FLAIR biosensor indicate that results have not been compromised by the separation of the chains²⁴.

Expression of the Rac1 biosensor is essentially the same as described for the RhoA biosensor above. The Rac1 biosensor is available from Addgene (www.addgene.org) in two formats:

- 1) Mammalian expression and cloning vectors: pECFP-C1 (Clontech Inc.) with ECFP replaced by CyPet (pCyPet-Rac1 / kanamycin resistance), and pEYFP-C1 (Clontech Inc.) with EYFP replaced by YPet (pYPet-PBD / kanamycin resistance).
- 2) pBabe-sin-tet-CMV-puro retroviral vector for Tet-On/Off system incorporating the CyPet-Rac1 and YPet-PBD (amp. resistance).

Because the donor and acceptor chains are on separate expression constructs, co-transfection/transduction of both components is required. Mouse embryo fibroblast cells (MEF/3T3) are transfected using Fugene6 (Roche, Ltd.) following the manufacturer's protocols. A DNA ratio of 3 : 2 is used to transfect CyPet-Rac1 and YPet-PBD, respectively, for optimal results in MEF or COS-7 cells. It is important to pre-mix the DNA solutions in the correct ratio, then add them to the Fugene-containing medium as a single aliquot. This optimizes correct mixing of both DNA constructs and results in an evenly distributed expression profile. 24 hours post transfection, cells are plated on fibronectin-coated glass coverslips for 3~4 hours prior to imaging.

Stable cell lines are preferred to reduce variability in the fluorescence profile from experiment to experiment as well as attaining better control of expression levels. For viral transduction using the pBabe Tet-off system, viruses for the donor and acceptor portions of the biosensor are co-transduced, and cells are selected for stable incorporation by successively increasing the puromycin concentration, similar to the procedure described for stable RhoA biosensor cells above. Once the stable population is produced, we FACS sort for low-medium brightness, with equal CyPet and YPet fluorescence emission. The collected fraction is then replated with Doxycycline (1 microg/ml) to repress the expression until 48 hrs prior to experiments. Examination of varying ratios and levels of expression for each construct have not affected the results of motility studies, but can influence sensitivity and the ease of bleedthrough corrections.

Imaging Rac1-FLAIR

Imaging is performed in Ham's F12-K medium without phenol red, supplemented with 2 % fetal bovine serum. For emission ratio imaging, the following filter sets are used (excitation, emission, respectively): CyPet: HQ436/20, HQ470/40; FRET: HQ436/20, HQ535/30; YPet: HQ500/20, HQ535/30 (Chroma Technology). The dichroic mirror ("Quad-Custom" Lot# 511112038, spectra shown in Fig.3) was custom manufactured by Chroma Technology Corp. for compatibility with all of these filter sets. A more recent sputter-coated ET series of dichroic mirrors and band-pass filters from Chroma could be used. For CyPet/YPet imaging or any other ECFP/EYFP-FRET based imaging, either filter set 59217 (single band exciters with a dual band emitter) or set 89002 (all single band filters) can be used effectively (Chroma Technology). Cells are illuminated with a 100W Hg arc lamp through a neutral density filter of 36% transmittance. At each time point, three images are recorded with the following exposure times, typical for the low biosensor concentrations used to minimize cell perturbation: CyPet (900ms), FRET (excitation of donor, observation of acceptor emission) (900ms), YPet (300ms) at binning 2×2. The image sets have been taken at 10s ~ 1min intervals. Ideally, one fills approximately 75% of the total 12 bit dynamic range of the camera for each channel. This produces a good signal to noise ratio and does not saturate the dynamic range should any one channel dramatically increase brightness. Imaging is detailed further in the RhoA biosensor section above.

Bleedthrough correction for intermolecular biosensors is described in the following discussion of imaging procedures.

Image processing procedures

Shading correction

The first step in image analysis is to correct for uneven illumination in the field of view. This is present in almost all images, including those taken using flat-field ("Plan") corrected objective lenses. In order to correct for shading, one obtains images from fields of view that contain no samples. Images of each fluorescence channel are obtained using the same camera integration times and illumination conditions as are used for the real images that will be corrected. The images for shading correction are acquired following the acquisition of the real fluorescence image sets using either cell-free areas within the same coverslip, or mounting a fresh coverslip with identical media and mounting conditions. In the latter case, it is convenient to mark a spot in the middle of the fresh coverslip on the side where cells would be present on an actual sample coverslip, so that one can focus on the edge of the spot to find the correct plane of focus. Usually, 20~30 shading images are acquired at the appropriate camera integration times and illumination settings for each fluorescence channel. The resulting image set for each channel is averaged to produce a single shade corrected image for each fluorescence channel. The averaging process is important because each image frame contains stochastic camera noise. This noise can be reduced by averaging many frames of the same field of view. Once the shading images are acquired and averaged, one simply divides the sample field of view by the corresponding shading image. Here, care must be taken to prevent floating point errors from the image analysis software. Software including Metamorph (Universal Imaging Co., Downingtown, PA) and ISEE Inovision (ISec Imaging Systems, Raleigh, NC) do not process floating point data. Matlab (Mathworks, Natick, MA) codes can be written to process images using a multiple decimal-point precision. Metamorph offers a convenient plug-in module for shade correction where a scaling factor can be specified to increase the relative pixel values so that floating point operation is avoided. One can either scale to the maximum pixel value in the shading image or specify some fixed value. The latter is usually the better choice as one can specify the

same scaling factor (i.e., 1,000) for images from both channels of fluorescence, thereby maintaining the relative intensities of the image pairs.

Background Subtraction

Because of the flat-field correction, cell-free background areas within the field of view have the same intensities. One can use an area with minimal debris in the field of view as the background. The pixel intensities within such a region are averaged and subtracted from the whole image. Small variations in background can be introduced by debris etc in your field of view. It is important to choose the same background position in each fluorescence image to minimize artifacts. Metamorph offers a convenient plug-in module ("Use region as background") to expedite the process. Using this utility, one can select a background region in the first fluorescence image of a ratio pair, and copy/save the region and paste/load onto the second fluorescence image. When using this Metamorph utility, it is important to note that processing of stack images require additional considerations. If the region is selected on the top plane and the utility is run for all planes in the stack, the averaged background value from the first plane is subtracted from each of the subsequent planes. In order to work around this problem, the region on the top plane can be saved and loaded onto each of the subsequent planes and background subtracted. Similarly, the same region can be loaded onto each plane of the fluorescence ratio pair images and processed.

Image Masking

Ratio division can introduce noise and hot spots in regions outside of the cell. Furthermore, pixel intensities in the thin, peripheral regions of the cell can be near background levels, making reliable quantitative ratio calculations difficult. In order to limit the area within which ratio calculations are performed, a binary mask can be applied to the cell, setting areas outside of the cell uniformly to zero. The mask can be based on an image of a volume marker (i.e. fluorescent dextran) or a membrane marker in a fluorescence color different from that of the biosensor. This is used to unambiguously specify the true cell boundary in cases where intracellular distribution of the biosensor does not correspond to the cell shape. In the case of most GTPase biosensors, distribution of the biosensors sufficiently delineates the cell boundaries, so this is unnecessary.

To produce the binary mask, all fluorescence image stacks (dye and EGFP channels, or ECFP and FRET/Citrine-YFP channels) are intensity thresholded. If the shade correction and background subtraction have been performed correctly, this is a straightforward process (i.e. using the "Threshold Image" command in Metamorph). With properly processed images, the histogram distribution is such that there will be a large peak in the zero pixel intensity position, followed by a spread of non-zero pixels in the image histogram distribution (Fig.4). Inclusive thresholding is manually performed by trial and error, observing which setting appropriately includes the cell pixels and excludes surrounding (i.e. using the Metamorph "Threshold Image" function). For a 16 bit image, the upper bound needs to be set at 65,535, the maximum value for 16-bit dynamic range. When processing a series of images from a time lapse study, it is important to check the low-end threshold value selected using the first plane ($t = 0$ time point) against the last image plane ($t = \text{end}$). Photobleaching will shift the low-end intensity distribution downward, resulting in under-selection of cell area at later times. In order to work around this problem, the threshold values are usually determined for later time points, resulting in a relaxed thresholding for earlier time points. Once the threshold values are set, a binary mask can be produced based on the selected inclusive threshold (in Metamorph, the "Clip" tab within the "Threshold Image" module is used to produce a binary mask in 16-bit). This will produce a binary mask where regions outside the threshold selected area are uniformly zero while inside is 65,535. Divide the binary mask with a constant (65,535) to produce a true binary mask containing

the value one inside the masked region and zero outside (Arithmetic function in Metamorph). Binary masks produced for each fluorescence channel are multiplied into the corresponding fluorescence image stacks to produce the masked fluorescence image stacks prior to automated image registration.

Automated Image Registration

The ratio calculation requires a division of one image by another. It is essential that the fluorescence ratio image pair be perfectly aligned prior to division. There are a host of potential sources for misalignment: placement of optical filters, dichroic turret movement, x-y-z stage movement, temperature fluctuations and ambient vibration, and sub-pixel misalignment. Fig.5 indicates the result of misalignment on ratiometric analysis. The edge artifacts in the form of high ratio values on one side and low ratio values on the other, as well as similar symmetrical artifacts within the cell, provide clues that image misregistration has occurred. Subpixel registration routines are available in most image processing software. For some experiments only manual registration will suffice, but in many cases, especially when there are subcellular structures with clearly defined edges, automated registration is possible. We developed an automated method for image registration based on normalized cross-correlation technique²⁵. A modified version of this routine, applicable for registration of up to 3 channels of fluorescence image sets, is provided in Appendices 2 and 3.

The routine for processing the 2-channel data set is “regAny2.m” and for the 3-channel data set is “regFCY2.m”. The regAny2.m will take individual tiff files with the running number index and align the FRET channel with respect to the CFP channel to an accuracy of 1/20th of a pixel (maximum pixel shift allowed = 10 whole pixels). Similarly, the regFCY2.m will align the FRET and YFP channels against the CFP channel. In both cases, the relative displacements in x and y are recorded on a frame by frame basis for an entire time series, and the median values of the distance displacements in x and y are applied to the whole time series at the end of the routine. This mode of registration is based on the assumption that the majority of the channel misalignment will stay constant for the duration of a single time lapse experiment. If individual x-y displacements are corrected on a frame by frame basis, we have observed an unacceptable level of “micro-jitter” in the final movie playback. Detailed requirements for the naming convention, file formats etc., are listed within the headers of the programs. One tip to making this program run faster and smoother is to crop the cell image as tight as possible (observe to make sure for all timepoints that the cell stays within the region of interest selected and cropped), making sure both channels to be aligned are cropped using the same cropping factor. This will minimize the computational load required to translationally align the images as there will be fewer number of total pixels to compute the cross-correlation coefficients compared to processing the entire field of view.

Image Shear, Rotation and Scaling Correction

Two separate cameras can be used to simultaneously acquire the two images required for ratio imaging (CFP and FRET, or EGFP and dye). This can be valuable when rapid image sequences are required, or when rapid changes in cell shape or biosensor distribution can produce motion artifact. For simultaneous imaging with two cameras, one uses a beam splitter and appropriate filters/dichroic to direct different wavelengths to each camera. Differences in positioning of the two cameras can produce image misalignment other than the straightforward X-Y translation encountered with one camera. Such additional misalignments include shear, rotation and scaling between the two images. These problems primarily arise from imperfect mounting of the cameras and the dichroic mirrors in the beam splitter. Alignment should first be optimized manually, but an automated solution based on *a priori* calibration is required to sufficiently correct these effects. We present here a polynomial-based method for *a priori* calibration. Fig.6 shows calibration grid images and

ratiometric images of RhoA activity before and after the correction. It is clear that manually aligning one corner of the grid leads to misalignment of the far corner. The software consists of two parts (Appendices 4 and 5): 1) a priori calibration software “morphPrep.m”, used to determine the coordinate transformation control points; and 2) the transformation program “morph.m”, which applies a 3rd-order polynomial-based coordinate transformation using the calibrated control points. We find it best to apply these corrections prior to image masking and X-Y translational alignment. For calibration, one can use a grid-type stage micrometer imaged in brightfield, or microbeads coated with fluorophores to simulate fluorescence imaging conditions. Imaging of the calibration specimen should be performed at the end of an experiment (be careful not to move the two cameras during the imaging experiment!). This correction method is not applicable to a majority of cases where a single camera and filterwheel are used to acquire multiple images in succession. However, in some cases fluorophores of very different wavelengths can scale differently due to the behavior of chromatically corrected optics (i.e. ECFP versus Cy-7). This method can be used effectively to correct for such issues if *a priori* calibration is performed.

Ratio Calculation

The masked and registered images are divided to produce a ratio image. For meroCBD, the dye image is divided by the EGFP image. For the FRET sensors, the FRET channel is divided by the donor image. The floating point error consideration is important in this step; for software that does not carry out floating point calculations, all numbers must be multiplied by a constant. This converts decimal portions of intensity values to larger numbers, so that they are not truncated or otherwise modified prior to division. In the Metamorph “Arithmetic” module, a scaling factor of 1,000 is specified as a multiplication factor during the ratio calculation. A smaller factor (10~100) can be used. However, this is not recommended since it tends to produce a non-smooth histogram distribution (Fig.7). The same scaling factor should be used when comparing ratio calculations from different experiments with the same biosensor.

Ratio images are scaled and often displayed as a pseudocolor map to reflect the range of ratio values within the image. Many methods are used for this, and the range of values within an image cannot readily be used to determine the dynamic range of the biosensor. For example, the lowest and highest pixel intensities are often not included in the scaling, as these can be due to spurious artifacts or noise. If one chooses to eliminate the lowest and highest 1% of pixel intensities a 99-fold change is shown within the image. Eliminating the lowest and highest 5% reduces this to a 20 fold range.

Ratio calculations for intermolecular FRET biosensors

In the case of intermolecular FRET biosensors such as the biosensor for Rac1, a bleedthrough correction is required. Images for the bleedthrough correction should be obtained routinely as part of each experiment, so that variations in the microscope system over time do not come into play. Control cells expressing either CyPet alone or YPet alone are used to determine how much light ‘bleeds through’ into channels where it is not desirable. For example, CyPet excitation is used to generate FRET. However, when this is done, some light emitted from CyPet itself appears in the FRET emission channel. This ‘bleedthrough’ must be quantified so that it can be subtracted out in real experiments. For this correction, intensity is measured using the CyPet excitation and CyPet emission channels, then measured again under the same exposure conditions using the CyPet excitation and YPet channels that will be used to monitor FRET. This reveals what percentage of the intensity measured using the “direct” CyPet fluorescence channels would also appear in the FRET channels. Bleedthrough coefficients determined thusly are the coefficients α and β in the following equation:

$$R = \frac{FRET_t - \alpha \cdot CyPet - \beta \cdot YPet}{CyPet} \quad (\text{eq. 5})$$

where R is the Ratio, $FRET_t$ is the total FRET intensity as measured, α is the bleed-through of CyPet into FRET channel, β is the bleed-through of YPet into FRET channel and $CyPet$ is the total CyPet intensity as measured. Bleedthrough into the CyPet channel (denominator) is negligibly small. By calculating the linear slope of the relationship between FRET and CyPet intensities upon CyPet excitation of cells expressing only the CyPet, the bleed-through parameter α can be determined. Similarly, by relating bleed-through into the FRET channel upon CyPet excitation of cells expressing only the YPet, the bleed-through contribution of YPet excitation by CyPet excitation into the FRET channel β can be determined. For our microscope and exposure conditions, the α parameter was found to be routinely within 0.4~0.5 and the β parameter ~0.2. These are dependent on the optical configuration of the microscope used. We provide a convenient routine, “BT_AB.m” for calculating the α and β parameters from raw images and associated shading images (Appendix 6), utilizing a segmentation based on the K-means clusters method²⁵. The ratio of corrected FRET over CyPet can be calculated and used as a measure of Rac1 activation.

In time-lapse experiments, CyPet and YPet photobleach at different rates. The ratio can be corrected for photobleaching as described elsewhere¹⁸. Briefly, by algebraic manipulation, (5) can be rearranged to:

$$R = \frac{FRET_t}{CyPet} - \beta \frac{YPet}{CyPet} - \alpha = \Gamma - \beta \cdot \Psi - \alpha \quad (\text{eq. 6})$$

where Γ is the fraction representing total FRET intensity over total CyPet intensity, and Ψ is the fraction representing total YPet intensity over total CyPet intensity, and both α and β are bleed-through constants described before. By taking double exponential fits of the decays of both Γ and Ψ , the correction function, $\bar{R}^{-1}$, can be calculated as outlined previously¹⁸. We provide here a convenient routine “PB5.m” that takes the masked and registered image sets (CyPet, YPet and FRET) plus the *a priori* determined α and β parameters, and calculates the photobleach corrected ratio R and corrected FRET, which is represented by the numerator of equation (5) (Appendix 7). These codes are available for download from the Hahn lab web page. Appendix 8~11 are also require functions which are called from within the routines presented here.

Concluding Remarks

Biosensors have become valued tools in cell biology, with commercial software and specifically designed microscopes greatly enhancing their accessibility and ease of use. It is important to apply this software with an understanding of the variables that can affect biological conclusions. Final images are sensitive to subtle changes in image processing procedures, or the use of biosensors under conditions that affect cell biology. We hope the procedures outlined here, for three different biosensor designs, can be a guide for proper application of a host of new biosensors developed by other laboratories to shed light on previously invisible protein dynamics.

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Fig.1.

Fluorescent biosensor designs. **A. MeroCBD**, biosensor of endogenous Cdc42 activation. Here a fragment of Wiskott Aldrich syndrome protein (WASP) that binds only to activated Cdc42 is covalently derivatized with an environmentally sensitive dye. When the WASP fragment encounters and binds to activated Cdc42, the solvation of the dye by water is reduced, leading to a fluorescence change. Advantages of this design include the ability to study endogenous protein, and enhanced sensitivity due to direct excitation of a bright, long wavelength dye (as opposed to indirect excitation via FRET). The disadvantage is the need for microinjection, electroporation or some other means to 'load' the covalently tagged protein biosensor into cells. **B. RhoA biosensor**. Here a fragment of Rhotekin that binds only to activated RhoA is attached to RhoA as part of the same protein chain. Two different fluorescent proteins undergoing FRET are in the chain between RhoA and the Rhotekin fragment, such that binding of the fragment to activated RhoA alters the distance and/or orientation between the fluorescent proteins, affecting FRET. This biosensor is fully genetically encoded, greatly simplifying loading into the cell. Because the Rhotekin fragment is attached to the RhoA, image processing is simplified relative to the dual chain sensor shown in C (see text). **C. Rac1 FLAIR, biosensor of Rac1 activation**. This design is similar to that of the RhoA biosensor, but here the PAK fragment used to bind activated Rac1 is not part of the same chain as the Rac1. The use of a dual chain, intermolecular FRET design enhances sensitivity because, unlike the single chain design, there is no FRET when the biosensor is in the 'off' state. Additional image processing (bleedthrough correction) is required because the biosensor components can distribute differently throughout the cell.

Fig.2.
Transmittance spectra for the Custom dichroic mirror (“Scripps Custom”) (Chroma Technology), Lot# 511111886.

Fig.3.

X–Y translational image registration artifacts generate features on ratio images. In panel A, a correctly registered ratio image of MeroCBD is shown. Panel B shows the ratio image from the same cell without registration. In the latter case, the dye image was misaligned by –5 pixels in both x and y directions. The white arrows in both figures point to regions where misalignment produces edge artifacts and other artifactual features. In panel B, a lower ratio rim can be seen on one side of the nucleus, and a higher ratio on the opposite side. Similarly, edge artifacts appear as higher ratio on predominantly one side of the cell, with an artefactually low ratio on the opposite side.

Fig.4.
Transmittance spectra for the Custom dichroic mirror (“Quad Custom”) (Chroma Technology), Lot# 511112038.

Fig.5.

A representative histogram from a shade corrected and background subtracted image, prior to masking. The prominent histogram peak at zero intensity is followed by a continuous distribution of pixels at a range of positive intensity values. Threshold masking to remove pixels of too low an intensity is performed using this histogram. One maximizes selection of pixels from within the cell while removing pixels outside the cell, including noise around the periphery.

Fig.6.

Image shear between two cameras creates artifacts in ratio images. In the upper panels, calibration grid images from two cameras are overlaid with and without shear correction (red arrow: two channels aligned relative to the corner indicated). In the lower panels, the ratio of RhoA biosensor readouts from the two camera channels are shown with and without shear correction. A peripheral ruffle shows a large artifactual feature when correction is not applied (white arrow).

Fig.7. Effect of the multiplication factor during radiometric calculation. In panel A, using a scaling factor of 1000 produced a smooth histogram distribution in a ratio image. Panel B shows the same ratio image with the scaling factor of 25. Though the general distribution pattern is similar, the histogram distribution in panel A retains higher bit-depth resolution.