

Optogenetic Control of RhoA to Probe Subcellular Mechanochemical Circuitry

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Spatiotemporal localization of protein function is essential for physiological processes from subcellular to tissue scales. Genetic and pharmacological approaches have played instrumental roles in isolating molecular components necessary for subcellular machinery. However, these approaches have limited capabilities to reveal the nature of the spatiotemporal regulation of subcellular machineries like those of cytoskeletal organelles. With the recent advancement of optogenetic probes, the field now has a powerful tool to localize cytoskeletal stimuli in both space and time. Here, we detail the use of tunable light-controlled interacting protein tags (TULIPs) to manipulate RhoA signaling in vivo. This is an optogenetic dimerization system that rapidly, reversibly, and efficiently directs a cytoplasmic RhoGEF to the plasma membrane for activation of RhoA using light. We first compare this probe to other available optogenetic systems and outline the engineering logic for the chosen recruitable RhoGEFs. We also describe how to generate the cell line, spatially control illumination, confirm optogenetic control of RhoA, and mechanically induce cell-cell junction deformation in cultured tissues. Together, these protocols detail how to probe the mechanochemical circuitry downstream of RhoA signaling. © 2020 by John Wiley & Sons, Inc.

Basic Protocol 1: Generation of a stable cell line expressing TULIP constructs

Basic Protocol 2: Preparation of collagen substrate for imaging

Basic Protocol 3: Transient transfection for visualization of downstream effectors

Basic Protocol 4: Calibration of spatial illumination

Basic Protocol 5: Optogenetic activation of a region of interest

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INTRODUCTION

To gain a better understanding of how signaling achieves spatiotemporally structured subcellular protein complexes and cytoskeletal organelles, we must be able to exert experimental control over these pathways. The use of light-sensitive moieties in combination with structured illumination provides a promising route. In the past, the availability of photo-responsive elements that allow for switching, binding, or uncaging has been limited. Recent advances in optogenetics have allowed for molecular dissection of

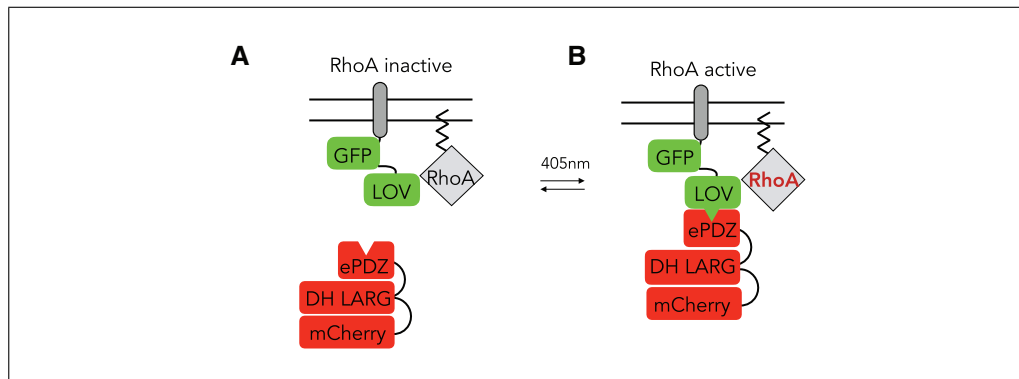


Figure 1 (A) Schematic of the TULIP system in the dark state. Stargazin-GFP-LOVpep sits in the closed conformation at the plasma membrane next to inactive RhoA. 2xPDZ-mCherry-LARG sits diffusely in the cytoplasm. (B) Schematic of the TULIP system in the activated state. Activation with 405-nm light causes a conformational change in Stargazin-GFP-LOVpep that increases its binding affinity to 2xPDZ-mCherry-LARG, recruiting it to the membrane, where it activates RhoA.

spatiotemporal signaling modules. Optogenetics utilizes photosensitive proteins that change conformation upon exposure to specific wavelengths, resulting in altered protein-protein interactions and modulation of downstream signals. This technique is appealing, as it can be used to study the effects of the location, intensity, periodicity, and duration of light pulses and subsequent signaling activity.

Nearly a decade ago, a seminal paper by Strickland and colleagues described how light-sensitive protein domains could be repurposed as optogenetic dimerization tools (Strickland et al., 2012). This initial study introduced the light-oxygen-voltage sensing (LOV) domain of *Avena sativa* phototropin 1 (AsLOV2). LOV domain conformations are light-sensitive, making them ideal for optogenetic use. This system was named TULIP for the tunable light-controlled interacting protein tags. In their original paper reporting the use of the TULIP system, the authors successfully documented the activation of two cellular signaling modules in yeast. Specifically, they dissected the yeast mating pathway induced by a canonical GPCR pathway responsible for a MAPK cascade associated with both growth arrest and polarized secretion. First, they documented light-dependent recruitment of a truncated Ste5 and full-length Ste11 to activate the MAPK pathway for subsequent cellular growth arrest. Then, they demonstrated that this system could successfully be used to control GTPase signaling using light-directed recruitment of Cdc42 to induce mating projections (or shmoos) in a polarized fashion. Together, these data showed the effectiveness of the TULIP system in regulating the activity of nucleotide-exchange factors, scaffold proteins, and kinases.

RhoA signaling is an ideal signaling pathway for optogenetic control. Rho-dependent signaling, regulated in space and time, drives a myriad of biological processes (e.g., development, homeostasis, and disease) (Lecuit, Lenne, & Munro, 2011). RhoA is a small, membrane-bound GTPase that largely controls the cellular basis of contractility through activation of its downstream effectors, actin and myosin (Lessey, Guilluy, & Burridge, 2012). RhoA activation is achieved by nucleotide exchange mediated by guanine nucleotide exchange factors (GEFs). The optogenetic strategy here is elegant: drive the localization of a RhoA-specific GEF to the plasma membrane for centralized activation of RhoA and its subsequent downstream effectors (Fig. 1). Recent optogenetic tools have subcellularly localized RhoA GEFs for RhoA activation in dividing (Wagner & Glotzer, 2016), nonadherent (Meshik, O'Neill, & Gautam, 2019; O'Neill et al., 2018), and adherent cells in culture (Oakes et al., 2017), and more recently in tissue both in culture (Cavanaugh, Staddon, Munro, Banerjee, & Gardel, 2020; Staddon, Cavanaugh, Munro, Gardel, & Banerjee, 2019; Valon, Marín-Llauradó, Wyatt, Charras, & Trepap,

2017) and in vivo (Izquierdo, Quinkler, & Renzis, 2018; Krueger, Quinkler, Mortensen, Sachse, & Renzis, 2019). These studies have successfully probed the complex nature of RhoA-mediated contractility on cell-cell and cell-matrix forces, in addition to deciphering mechanosensitive signaling pathways that regulate cellular morphology and tissue-scale morphogenesis.

Specifically, the TULIP system has proved to be a versatile tool in this analysis of mechanochemical signaling in driving the formation of subcellular cytoskeletal organelles. RhoA activation drives spatiotemporally structured subcellular organelles, like those of the cytokinetic ring, actin stress fibers, or the contractile actin belt anchored at adherens junctions, to name a few. Using the TULIP system, Wagner and Glotzer exogenously activated RhoA to find that it was sufficient to induce cytokinetic furrow formation in single anaphase cells (Wagner & Glotzer, 2016). Oakes and colleagues used the system to probe the molecular basis of actin stress fiber elasticity in single cells, showing a zyxin-dependent mechanism (Oakes et al., 2017). More recently, it was discovered that RhoA was sufficient to induce stable cell-cell junction deformations past a critical strain threshold to trigger mechanosensitive endocytosis in epithelial monolayers (Cavanaugh et al., 2020; Staddon et al., 2019). Altogether, these studies show the diverse applications of the TULIP system. We believe that, with the right engineering, optogenetic RhoA can be used for any application. Further studies using optogenetically activated RhoA will only continue to advance our understanding of cell and tissue mechanics.

In this article, we first describe the strategic planning associated with choosing and designing an optogenetic system. We then describe five protocols for optogenetic studies of RhoA in epithelial tissues: (1) generation of stable cell lines (Basic Protocol 1); (2) preparation the substrate for imaging (Basic Protocol 2); (3) transfection for visualizing downstream effectors (Basic Protocol 3); (4) spatial illumination calibration (Basic Protocol 4); and (5) optogenetic activation of a region of interest (ROI) (Basic Protocol 5).

STRATEGIC PLANNING

Choosing the Optogenetic System

A number of dimerization systems have been developed, each with diverse properties for different biological applications. The choice of optogenetic system will depend on factors such as wavelength compatibility, dynamic range, and requirements for activation speed, reversibility, and depth of tissue to be imaged. We recommend choosing the optogenetic system with the desired reversibility kinetics, as this is important for achieving local, spatially resolved subcellular control of signaling processes. Fast dimerization kinetics can be on the order of seconds, while slower kinetics is on the order of minutes to hours. Systems with slow reversal kinetics may be particularly useful if a more permanent phenotype is desired or if more persistent signaling is needed. However, since physiological signaling occurs within milliseconds to seconds, we recommend using faster dimerization kinetics to mimic in vivo signaling kinetics. The current optogenetic dimerization systems described in the literature are listed in Table 1.

Many systems have already been published for subcellular control of RhoA. If fast kinetics is desired, we recommend using the iLID/SspB or TULIP system, as these provide high temporal resolution of RhoA activation. The RhoGEF in TULIPs associates within less than 10 s and dissociates within 30–60 s (Cavanaugh et al., 2020; Oakes et al., 2017; Staddon et al., 2019; Strickland et al., 2012; Wagner & Glotzer, 2016); the iLID/SspB system shows similar association and dissociation kinetics (Meshik et al., 2019; O'Neill et al., 2018). Slower kinetic systems have been seen with the CRY2/CIBN light-gated dimerization system. This system was used to manipulate RhoGEF association within

Table 1 Optogenetic Dimerization Probes

System	Association wavelength	Dissociation wavelength	Tag sizes (amino acids)	Lifetime	Reference
PhyB/PIF6	660 nm	740 nm	908/100	Inducible	Levskaya, Weiner, Lim, & Voigt (2009)
Cry2-CIBN	450 nm	Dark	498/170	5-10 min	Kennedy et al. (2010)
iLID/SspB	450 nm	Dark	144/110	<50 s	Guntas et al. (2015); Zimmerman et al. (2016)
TULIP	450 nm	Dark	153/194	<50 s	Strickland et al. (2012)
nMag/pMag	450 nm	Dark	150/150	Tunable	Kawano, Suzuki, Furuya, & Sato (2015)
FKF1/GI	450 nm	Dark	619/1173	Hours	Yazawa, Sadaghiani, Hsueh, & Dolmetsch (2009)
LOVTRAP	Dark	450 nm	143/59	Tunable	Wang & Hahn (2016)
PixD/PixE	Dark	450 nm	150/380	Seconds to minutes	Dine, Gil, Uribe, Brangwynne, & Toettcher (2018)
BphP1/PpsR2	740 nm	650 nm	732/465	Inducible	Kaberniuk, Shemetov, & Verkhusha (2016)
UVR8/COP1	280 nm	N/A	440/340	Permanent	Crefcoeur, Yin, Ulm, & Halazonetis (2013)

minute timescales, but RhoGEF dissociation was on the order of 20 min (Valon et al., 2017). In this case, actin accumulated and dissipated with similar kinetics as the RhoGEF. As such, the CRY2/CIBN system provides for a more permanent phenotype associated with RhoA contractility. This slower system, while not typically physiological, can provide for persistent actomyosin recruitment and RhoA signaling that can be used to study the effects of traction forces on substrates, for example. With fast or slow recruitment kinetics, the major strength of using an optogenetic approach is the ability to image a baseline state prior to activation, the response during activation, and a recovery period following activation. These three periods give key insights into the behavior and response of junctions and/or effector proteins with respect to the activation of RhoA.

Engineering the Optogenetic Constructs

When engineering the photosensitive protein, it is necessary to consider the desired sub-cellular location for recruitment. Most studies to date have anchored the photosensitive protein to the plasma membrane, where RhoA sits inactive. Here, we describe the use of the TULIP system that utilizes the photosensitive LOVpep domain attached to the trans-membrane protein Stargazin (Strickland et al., 2012) (Fig. 1). Recent papers have also probed membrane-recruitable RhoGEFs using various photosensitive proteins attached to a CAAX motif, which triggers posttranslational modifications necessary to drive the protein's plasma membrane association and insertion (Meshik et al., 2019; O'Neill et al., 2018; Valon et al., 2017). It is also conceivable to drive RhoGEF localization to other more-specific areas within the cell, under the logic of sequestering the RhoGEF away from the plasma membrane. For instance, one study drove RhoGEF activity specifically to the outer mitochondrial membrane by fusion to the mitochondrial matrix targeting sequence from subunit VIII of cytochrome *c* oxidase (Valon et al., 2017). Other targetable proteins may be apicojunctional proteins like E-cadherin, ZO-1, or members of the PAR

polarity family, although nearly any protein can conceivably be targeted with the right design.

RhoA is activated when the recruitable GEF binds to the photosensitive protein at the plasma membrane upon light activation (Fig. 1). GEFs are multidomain proteins capable of catalyzing nucleotide exchange within Rho GTPases (Schmidt, 2002). The full-sequence RhoGEF houses the catalytic DH domain and additional protein and/or lipid interaction motifs, suggesting that these domains act as protein scaffolding complexes and/or localization signals (Bos, Rehmann, & Wittinghofer, 2007). In nearly all isoforms, the catalytic DH domain is found adjacent to a PH domain that commonly binds to phosphoinositide ligands (Cook, Rossman, & Der, 2014) and may even aid in nucleotide exchange (Cherfils & Zeghouf, 2013). Other common functional domains include the SH3 and PDZ protein-binding domains and the RGS autoinhibitory domain, to name a few (Cherfils & Zeghouf, 2013). It is therefore vital to consider which type of GEF is used as a dimerizer, because different GEFs can result in diverse subcellular behaviors depending on the sequence motifs used (Cook et al., 2014). As a result, designing this optogenetic piece will depend on the nature of the experiment and desired subcellular behaviors.

With TULIPs, the LOVpep's cognate binding partner is an engineered tandem PDZ domain attached to the catalytic DH domain of the RhoGEF LARG (Wagner & Glotzer, 2016) (Fig. 1). The DH domain of LARG is a potent RhoA-specific activator and exhibits the highest catalytic activity reported for its GEF family (Jaiswal et al., 2011). Other groups have used the DHPH domain of the *Drosophila*-specific RhoGEF2 (Izquierdo et al., 2018), the DHPH domain of LARG (Meshik et al., 2019; O'Neill et al., 2018), or the DHPH domain of ARHGEF11 (Valon et al., 2017). Although others have included the PH domain in their recruitable GEF complexes, we recommend engineering dimerization constructs that utilize only the GEF's catalytic DH domain to reduce basal GEF activity. Isolating the DH domain removes functional domain compositions and domain organizations that link GEF activity to specific downstream signaling modules. For example, the PH domain of PDZ-RhoGEF has been shown to bind to activated RhoA to drive a potential feedback loop that either attenuates or enhances RhoA function (Chen et al., 2010). This effect may not be desirable in an optogenetic system, as activation of a feedback loop may result in unwanted phenotypes resulting from altered RhoA function. Additionally, in some GEF proteins, the PH or RGS domain may act on the DH domain in an autoinhibitory fashion, preventing RhoA activation despite any GEF photorecruitment (Chen, Guo, Sprang, & Sternweis, 2011; Cherfils & Zeghouf, 2013).

Visualization and confirmation of these optogenetic proteins depends on their fluorescent tags. Tagging the anchored LOV domain to GFP aids in confirming the uptake of the probe in screening and sorting the cells. Additionally, we recommend tagging the desired RhoGEF with mCherry or another red protein variant to confirm its localization and recruitment. It is also possible to tag 2xPDZ-LARG with a far-red protein or Halo tag conjugated with the *Janelia* Fluor far-red protein. There is also a commercially available YFP-2xPDZ-LARG that frees up the red channel. However, confirming the presence of this probe in cells is more difficult and relies on visualization of downstream effectors or relocation from the cytosol to the membrane to confirm the presence of the recruitable GEF.

When cloning, it is important to be conscious of the linkers between the LOVpep or PDZ domains and their respective proteins of interest. Linkers, or the lack thereof, can affect the conformation of the desired protein. This is especially important if tagging a protein that houses specific signaling functions, such as apicojunctional proteins or RhoGEFs that localize to the cytoskeletal machinery. The design of a suitable linker to join protein domains can often be complicated. Careful attention needs to be paid when designing a

linker with the right length, hydrophobicity, amino acid residues, and secondary structure. Flexible linkers preferably have small non-polar (e.g., Gly) or polar (e.g., Ser or Thr) amino acids (Chen, Zaro, & Shen, 2013). The most commonly used flexible linkers have stretches of Gly and Ser residues, the length and copy number of which can be optimized to separate the functional domains. For LOVpep, we have successfully used the flexible linker GSGGSGGSPR, and for tandem PDZ we have used QSTVPRARDPPVAT (Cavanaugh et al., 2020; Oakes et al., 2017; Wagner & Glotzer, 2016). Other linkers for optogenetic tags include GSGGSGSGGT (Wang & Hahn, 2016) or GSTSGSGKPGS-GEGSTKG (Whitlow et al., 1993). These published linkers are sufficiently long and flexible that they do not affect the binding of the protein to its downstream effectors. For the anchor protein, when using a CAAX motif or another targeting sequence to a specific subcellular location, linkers are optional.

GENERATION OF A STABLE LINE EXPRESSING TULIP CONSTRUCTS

The nature of the experiment necessitates different protein expression systems. To probe the effects of RhoA localization on cell-cell junctions within a tissue in culture, for example, we recommend generating a stable cell line constitutively expressing both dimerization constructs, because the likelihood of both optogenetic probes being present in two adjacent cells is very low. Transient transfection of both optogenetic probes may be sufficient for analysis of RhoA activation in single cells, although generation of a stable cell line will greatly ease experimentation. Generation of stable lines necessitates the cloning of optogenetic constructs into a lentiviral vector (e.g., pWPT) or other vector (e.g., retroviral, adenoviral) depending on cell type. We recommend using a viral vector with a selectable marker such as puromycin resistance for cell selection.

This protocol uses the FuGENE 6 transfection reagent to produce lentiviral DNA, which is then used to create a stable cell line constitutively expressing both TULIP constructs. The protocol is designed to generate lentivirus of one optogenetic construct for infection of cells in culture; it must be repeated to obtain lentivirus of the second optogenetic construct. Following successful expression and sorting of one construct (steps 1-14), the cells can be transfected and sorted again with the second construct. Alternatively, you could infect WT cells simultaneously with both viruses and perform dual-channel fluorescence sorting via FACS.

Materials

- 293T cells (ATCC CRL-3216)
- 1 µg/µl lentiviral DNA vector containing desired constructs
- 1 µg/µl pHR1-8.2-delta-R packaging plasmid (dR8.2) (Addgene, cat. no. 12263)
- 1 µg/µl VSV-G pseudotyping plasmid (Addgene, cat. no. 8454)
- Opti-MEM (Gibco)
- FuGENE 6 Transfection Reagent (Promega)
- 10 mg/ml Polybrene (EMD Millipore)
- Phosphate-buffered saline (PBS)

- 15-ml conical tubes (Corning)
- 0.45-µm Millex syringe filter unit (Millipore)
- 30-ml Luer-Lock disposable syringe (ExellINT)
- 8-well chambers (Ibidi)

Additional reagents and equipment for FACS

Day 0: Prepare cells for infection

1. Plate 293T cells and grow to 80% confluence.

Day 1: Transfect cells

2. Assemble reaction as follows and let sit for 10 min.
 - 7.5 μ l lentiviral vector
 - 5 μ l dR8.2
 - 1.25 μ l VSV-G
 - 685 μ l Opti-MEM
 - 33.75 μ l FuGENE
3. Add complexes to 10 ml fresh medium on the plate of 293T cells.
4. Place cells in the incubator and let sit for 3 days.

Day 3: Isolate virus for infection

5. Collect supernatant in 15-ml conical tubes.

At this time, all items coming in contact with lentivirus should be bleached and placed into a biohazard bag. Wear double gloves and a lab coat to protect the skin.

6. Spin down the virus-infused medium to remove any remaining cells and carefully collect the supernatant.
7. Filter sterilize the supernatant with the 0.45- μ m filter and 30-ml syringe to remove any debris.
8. Add 2 ml lentivirus and 2.4 μ l of 10 mg/ml Polybrene to 6 ml fresh medium on the desired cell line.

The TULIP system has been successfully used in Caco-2, DLD1, HeLa, and NIH 3T3 fibroblast cells, although it is feasible to use it in other cell lines.

We recommend snap-freezing the rest of the virus for later use in case the infection did not work. Keep in a -80°C freezer.

9. Return cells to the incubator and let sit for 1 day.

Day 4: Clean optogenetic cells

10. Remove medium from the dish and wash with PBS.

CAUTION: The medium and wash should be bleached, as the virus is still a hazard. The cells can be cultured normally after the wash, as they are no longer hazardous.

11. Add fresh medium to the optogenetic cells, return to the incubator, and allow to grow for a few days.

This will increase the number of cells expressing the optogenetic constructs in the entire cell population.

Day 6+: Isolate optogenetic cell line

12. Once the cells have been expanded, sort them for the desired fluorescence via FACS. Place cells in the dark immediately upon sorting.

We find that relatively low levels of Stargazin-GFP-LOVpep are tolerated quite well, but high expression of mCherry-2xPDZ-LARG is needed to produce a marked cellular response. We recommend sorting the cells for highest 50% expression of Stargazin-GFP and highest 5%-10% expression of mCherry-2xPDZ-LARG.

Ambient light can activate the TULIP system. While it is okay to have some exposure to light in the room, prolonged exposure and recruitment of the GEF to the membrane may result in cell blebbing or death.

13. Expand each clonal population.

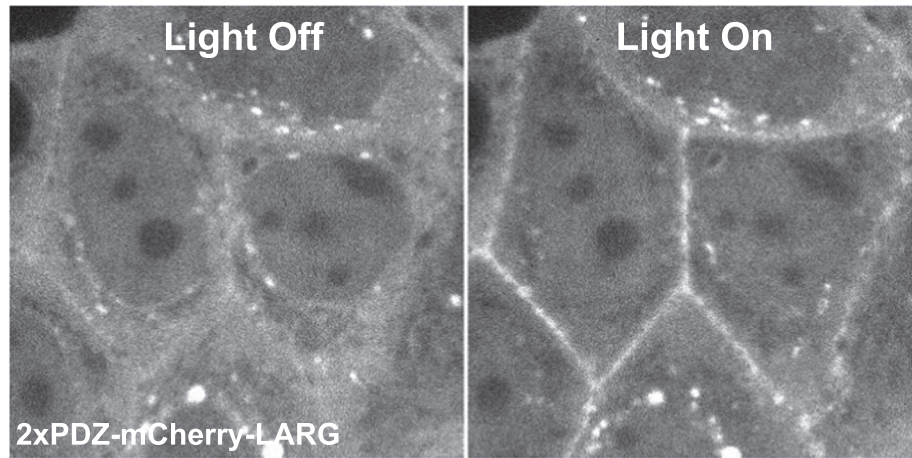


Figure 2 Representative images of a stable optogenetic cell line before and after light recruitment visualized by 2xPDZ-mCherry-LARG expression. With light off, cells have diffuse cytoplasmic localization of 2xPDZ-mCherry-LARG. With light on, cells show junctional recruitment of 2xPDZ-mCherry-LARG.

Screen clones for optimal construct expression

14. Split each clonal population, placing one portion in a cell culture dish and one in a single well of an 8-well Ibidi chamber. Make sure to keep a record of which clonal population is which.

The density of plating can be varied depending on the experiment.

15. To confirm expression of both constructs, take an image in the mCherry channel followed by an image in the GFP channel and then another image in the mCherry channel.

If the cytoplasmic RhoGEF shifts localization to the junctions, the clone is primed for optogenetic activation (similar to Fig. 2).

16. Expand the selected clone for use in subsequent protocols.

BASIC PROTOCOL 2

PREPARATION OF COLLAGEN SUBSTRATE FOR IMAGING

The extracellular matrix (ECM) composition can have drastic effects on cellular behavior and morphology. We also find that the substrate greatly affects the response of cell junctions to exogenous RhoA. Here, we describe how to plate optogenetic cells on polymerized collagen gels that allow tissues to be grown on a soft (<2 kPa) fibrillar network, which we have found optimal for constructing polarized epithelial monolayers. Alternative substrates include those generated using other ECM proteins such as laminin or fibronectin, Matrigel, or ECM-coated polyacrylamide gels required for applications like traction force microscopy. The substrate composition will depend on the specific experiment. For experiments examining cell-cell interactions using monolayers, we recommend making the substrate as soft as possible. Experiments examining interactions between cell-matrix adhesions may require different substrates. Successful completion of this protocol should result in a 2 mg/ml collagen gel that is less than ~300 μ m thick atop a glass chamber.

Materials

- 3.5 mg/ml rat tail collagen 1 (Col1; Corning)
- DMEM (Sigma-Aldrich) supplemented with 10% FBS (Hyclone; Thermo Fisher Scientific), 0.2 mM L-glutamine (Invitrogen), and 1% pen/strep (Invitrogen)

Table 2 Preparation of Substrate Solution

Reagent	1500 μ l total	1000 μ l total	800 μ l total	500 μ l total	300 μ l total
DMEM	593.9 μ l	395.9 μ l	316.7 μ l	198 μ l	118.8 μ l
HEPES	30 μ l	20 μ l	16 μ l	10 μ l	6 μ l
NaHCO ₃	35.8 μ l	23.8 μ l	19.1 μ l	11.9 μ l	7.2 μ l
Col1	840.3 μ l	560.2 μ l	448.2 μ l	280.1 μ l	168.1 μ l

1 M HEPES (Mediatech)
 7.5% NaHCO₃ (Thermo Fisher Scientific)
 Optogenetic cells (see Basic Protocol 1)

1.5-ml microcentrifuge tubes
 Chilled pipette tips
 4-well glass chambers (Ibidi)

1. Place collagen and a 1.5-ml microcentrifuge tube on ice to chill.
2. Using the volumes given in Table 2, add DMEM to the tube and let chill.
3. Add 1 M HEPES and let chill.
4. Add 7.5% NaHCO₃ and let chill.
5. Pipette 3.5 mg/ml collagen slowly into the mixture using a chilled pipette tip. Pipette up and down while stirring with the tip.

Ensure that the tip has equilibrated to the temperature of the ice. Do this while keeping the tube on ice.

6. Remove collagen mixture from ice and allow to polymerize.

Collagen will start polymerizing the instant it is removed from the ice. For a more meshy collagen, plate immediately onto chambers. For more bundled collagen, let sit as a liquid for >5 min.

7. Paint 80-100 μ l collagen mixture onto a glass chamber with the tip of a pipette. Make sure to spread it into all corners and be careful to avoid bubbles.

The volume used will affect the collagen gel thickness. If using a different chamber, the volume will need to be adjusted. The collagen gel thickness should be confirmed at the time of cell imaging. This can be done by using fluorescently labeled collagen and imaging a z-stack of the collagen proper.

8. Place chamber slides in an incubator for ≥ 5 min to allow the gel to solidify.
9. Apply ~ 150 μ l DMEM on top of the gel to keep it from drying before cells are plated.
10. Plate cells on collagen in a dark cell culture hood and grow in a dark incubator.

Cells can be plated sparsely and then grown for a few days in a dark incubator to give a confluent monolayer.

Some ambient light is okay, but the GEF may be recruited to the membrane upon significant light exposure.

TRANSIENT TRANSFECTION FOR VISUALIZATION OF DOWNSTREAM EFFECTORS

It is critical to confirm the localization of RhoA and any downstream effectors upon RhoA activation. Downstream effector analysis is accomplished by using reporters of RhoA

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activity, typically through transient transfection. These reporters can include the direct downstream effectors of RhoA, actin and myosin. RhoA activity can also be confirmed by using the RhoA biosensor, which houses the RhoA binding domain within the C-terminal portion of anillin. In the case of myosin and the RhoA biosensor, these constructs can be transiently transfected into cells for confirmation of RhoA. For visualization of actin structures, there is a commercially available cell-permeable far-red SiR-actin, although we do not detail its use here. It is also possible to use non-fluorescent outputs such as traction force microscopy to confirm RhoA activation (Oakes et al., 2017).

When performing multichannel imaging, it is important to take into account the spectral overlap between optogenetic photoexcitation and fluorescence imaging. The broad blue-light sensitivity of the LOV domains prevents imaging in the GFP channel, since LOVpep can be activated by blue light. Unfortunately, this limits the number of channels for imaging. If possible, the use of far-red fluorescent proteins and probes such as SiR-actin will allow for multispectral imaging. Since the number of far-red proteins is also limited, we commonly use proteins fused with Halo tags and conjugate them with Janelia fluor far-red proteins for visualization.

In our system, we have found that Caco-2 cells are extremely sensitive to electroporation and often have low transfection efficiencies (<20%), although this may be dependent on cell type. For monolayers, we do not recommend transfecting by electroporation, as it also results in marked cell death (>30%-40%), which can hinder the growth of a confluent and polarized monolayer. Instead, we recommend first forming the monolayer using Basic Protocol 2 and then performing transfection using cationic liposome-based reagents like Lipofectamine 3000. For single cells, this is less of an issue and any transfection method can be utilized, though efficiency will still vary. The following protocol uses Lipofectamine 3000 to produce an epithelial tissue with transient expression of downstream effectors like the RhoA biosensor or myosin. Generally, this protocol can be used to transfect any protein of interest for analysis of the effects of junctional RhoA localization.

Materials

Cells in 4-well Ibidi chambers (see Basic Protocol 2)
Dulbecco's phosphate-buffered saline (DPBS; Corning)
Opti-MEM (Gibco)
Lipofectamine 3000 Reagent (Thermo Fisher Scientific)
1-5 µg/µl reporter DNA
P3000 Reagent (2 µl/µg DNA; Thermo Fisher Scientific)
DMEM (Sigma-Aldrich) supplemented with 10% FBS (ThermoFisher scientific),
0.2 mM L-glutamine (Invitrogen), and 1% pen/strep (Invitrogen)

1. Plate optogenetic cells in 4-well Ibidi chambers in DMEM at 100% confluency or grow cells to a confluent monolayer.
2. Approximately 30-60 min before transfection, gently wash cells in the dark with 1 ml prewarmed DPBS.
3. Replace wash with 250 µl Opti-MEM and return cells to the incubator until the transfection reagents are ready.
4. Warm all reagents to room temperature.
5. For each transfection, prepare two 125-µl aliquots of Opti-MEM (A and B).
6. Add 6 µl Lipofectamine 3000 to tube A. Vortex briefly and spin down.
7. Add 2.5-5 µg DNA to tube B, followed by 5 µl P3000 reagent. Vortex briefly and spin down.

8. Add the entire contents of tube B to tube A and mix by pipetting gently. Incubate 10-15 min at room temperature.
9. To transfect cells, place them in a dark cell culture hood and gently add the entire mixture in small drops to one Ibidi well. Return to incubator for 12 hr.
The room light can be on, but the hood light should be turned off to prevent recruitment of GEF to the membrane.
10. Replace Opti-MEM with DMEM in the dark and return to incubator for another 12-36 hr.
11. Analyze transfected cells 24-48 hr after transfection.

CALIBRATION OF SPATIAL ILLUMINATION

Here, we describe our particular microscope setup and calibration protocol for optogenetic activation using MetaMorph software. While each microscope setup will be different, the most important piece of equipment is the digital micromirror device (DMD) coupled to a light source. The DMD is an intricate array of hundreds of thousands of hinge-mounted adjustable mirrors controlled via microscope acquisition software (e.g., MetaMorph). Mirrors that fall within ROIs that are drawn on the computer are rotated into the light path to reflect light from the source onto the sample. This setup has the advantage of illuminating all pixels in the ROI simultaneously. To ensure accuracy, the system should be calibrated before each experiment and for each objective used. Calibration is typically performed by clicking on a series of markers displayed in sequence by the DMD. This process registers the DMD to the camera pixels. DMD chips are typically smaller than the field of view (FOV) of the camera, and thus it is often useful to save a region of the fully illuminated chip as a reference for choosing regions during the actual experiment. Successful completion of this protocol will calibrate the DMD system for use in Basic Protocol 5.

Materials

Nikon Ti-E inverted microscope (Nikon)
 Yokogawa CSU-X confocal scanning head (Yokogawa Electric)
 Laser merge model with 491-, 561-, and 642-nm laser lines (Spectral Applied Research)
 Zyla 4.2 sCMOS camera (Andor)
 Mosaic digital micromirror device (DMD) coupled to a 405-nm laser (Andor)
 60× 1.49 NA ApoTIRF oil-immersion objective (Nikon)
 MetaMorph Automation and Image Analysis Software (Molecular Devices)
 Mirror slide

1. Turn on the microscope, Mosaic DMD, and light source.
2. Turn on the calibration flashlight perpendicular to the Mosaic light path.
3. Insert the 100% mirror in front of the calibration flashlight and ensure that the rest of the light path is clear.
4. Apply immersion medium to the objective.
5. Place the mirror slide in the slide holder with the mirrored surface closest to the objective.
6. Using transmitted light, focus on scratches in the mirror surface until they are crisp.
7. Rotate the Mosaic filter cube into position below the objective.

BASIC PROTOCOL 4

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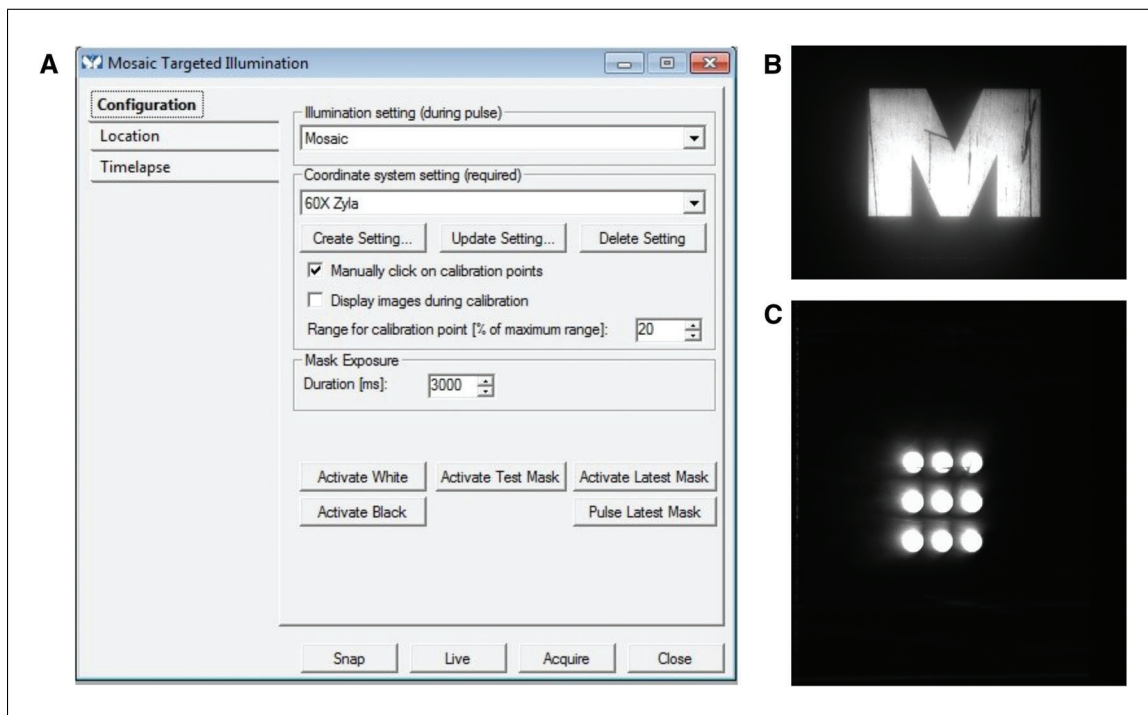


Figure 3 (A) Configuration toolbox within the Mosaic Targeted Illumination device box showing the illumination setting. Coordinate system is set to 60× Zyla; mask exposure is set to 3 s. The Activate Test Mask button is shown before the Mask Exposure setting. (B) Image of the activated test mask “M”. (C) Image of the completed, manually clicked calibration points for device calibration.

8. Under the “Devices” menu, select “Mosaic Targeted Illumination” to open the control panel.
9. Set the Illumination (during pulse) to “Mosaic” (Fig. 3A).
This ensures that the Mosaic filter cube is rotated into place below the objective if it is not already there.
10. Set the coordinate system to the desired objective (“60× Zyla” in Fig. 3A). Make sure the objective magnification (Mag) setting in MetaMorph matches the coordinate system setting.
11. Click “Activate Test Mask” (Fig. 3A).
12. In the Acquire box, click “Live” to show the test mask.
At this point you should see an M on the live screen (Fig. 3B).
13. Adjust the focus so the M becomes crisp and clear. Stop live imaging and click “Update setting”.
14. Click on the center of each white dot as it is displayed (Fig. 3C).
When all nine dots have been clicked, the system will be calibrated.
15. Confirm that the system is calibrated by drawing an arbitrary region on the image and testing the illumination.
16. Remove the 100% mirror from in front of the calibration flashlight and turn off the calibration flashlight.
17. Turn on the illumination light source and set to the desired intensity by clicking “Low” and “High” in the “Laser Controller” box (Fig. 4).

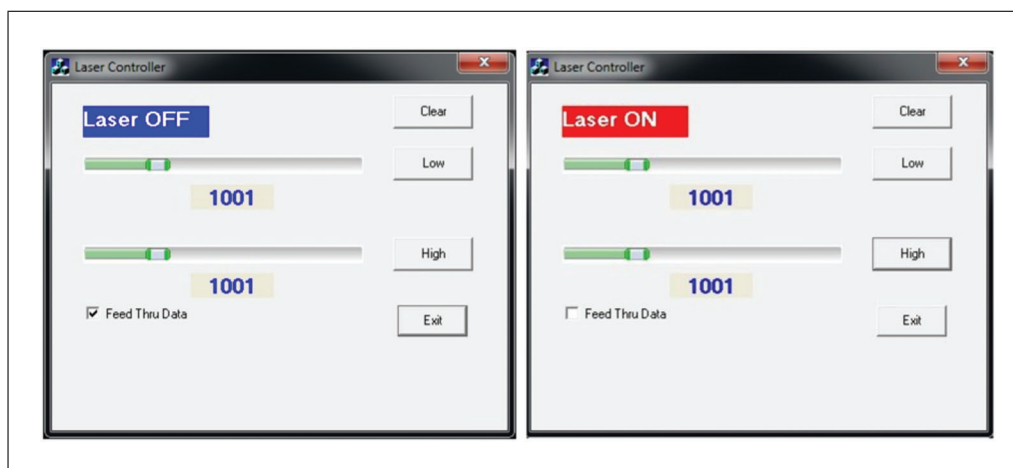


Figure 4 Images of laser controller with the laser in the OFF and ON states, with intensity set to 1000 AU.

IMPORTANT: Any change in the optical setup (e.g., objectives, filters) will require recalibration of the DMD. It is a good habit to simply perform the calibration before each experiment to ensure proper alignment and thus targeting of the activation.

OPTOGENETIC ACTIVATION OF A REGION OF INTEREST

This protocol describes how to use the Mosaic DMD to induce RhoA activation by light localization within an ROI. We generally recommend imaging three periods: the baseline state prior to activation, the response during activation, and the recovery period following activation. For example, we have imaged the junction steady state for 10 min before a 5-min activation and then documented junction recovery for 15 min post-activation (Cavanaugh et al., 2020; Staddon et al., 2019) (Fig. 5), allowing us to evaluate any cellular or protein response to exogenous RhoA activation. However, such protocols necessitate optimization. Within the activation period, there may be variations in pulse duration, pulse frequency, and interval spacing of RhoA activation that are dependent on the nature of the experiment. We recommend taking time to establish optimal activation schemes as necessary for each experiment. Successful completion of this protocol will produce timelapse images of cell junctions undergoing deformations resulting from exogenous RhoA.

BASIC PROTOCOL 5

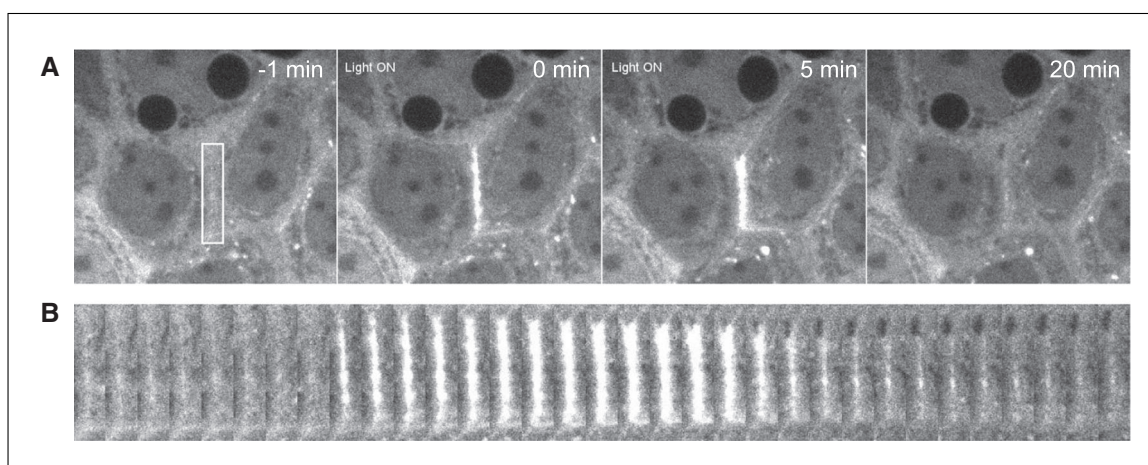


Figure 5 (A) Representative images of a stable optogenetic cell line expressing 2xPDZ-mCherry-LARG before, during, and after targeted junctional optogenetic activation. Activation of the ROI (white box) induces junctional localization of the GEF and shortening of the targeted junction over a 5-min activation period. A 15-min relaxation period (total time, 20 min) shows reversal of any junction contraction. (B) Representative kymograph showing expression of 2xPDZ-mCherry-LARG before, during, and after targeted junctional activation.

Additional Materials (also see Basic Protocol 4)

Stage incubator (Chamlide TC and FC-5N; Quorum Technologies)

Perform activation and acquire images

1. Make sure the light path from the Mosaic light source to the sample is free of any obstruction or mirrors.
2. Turn on the Mosaic laser using the “Laser Controller” (Fig. 4).

The laser power here needs to be determined empirically so that the optogenetic cells can be activated. This will be determined in part by the optics of the individual microscope. Due to the high microscope sensitivity, only a very small amount of light is needed to activate the protein. We, along with members of Tobin Sosnick's laboratory, have also found that too much light hinders activation of the LOV protein. We have had success generating contractile responses at junctions using light intensities between 6.7 and 10.5 μW (750-1000 AU) over regions that are $\sim 20 \mu\text{m}^2$, with a minimal junctional response at 4.3 μW (500 AU). For further information, see discussion of junctional responses as a function of light intensity in Cavanaugh et al. (2020) and Staddon et al. (2019).

3. Place the sample in the stage incubator, keeping the sample in the dark, and allow it to equilibrate.

The stage incubator will maintain the cells at 37°C. The humidified 5% CO₂ should be maintained at 50°C at its source to prevent condensation within the tubing.

4. Scan cells for optimal expression of 2xPDZ-mCherry-LARG. To confirm expression of both constructs, take an image in the mCherry channel followed by an image in the GFP channel and then another image in the mCherry channel.

If cytoplasmic RhoGEF shifts to the junctions, the cell is primed for optogenetic activation (similar to Fig. 2).

5. Wait >5 min until all 2xPDZ-mCherry-LARG has dissociated from the junctions and returned to the cytoplasm.

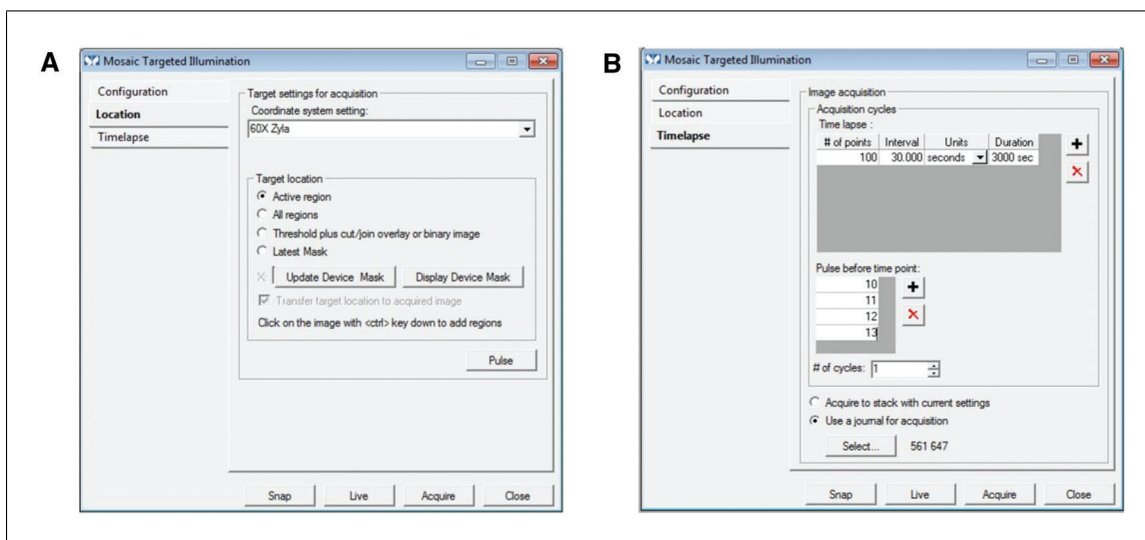


Figure 6 (A) Location toolbox within the Mosaic Targeted Illumination device setting. The coordinate system is set to 60 \times Zyla, with target location set to the active region. (B) Timelapse toolbox within the Mosaic Targeted Illumination device setting showing the number of time points set to 100 and the interval set to 30 s, with pulses before time points 10-13. A journal is used to image the 561- and 647-nm channels during each of these time points.

This can be confirmed by taking another image in the mCherry channel to visualize cytoplasmic RhoGEF.

Analyze results

6. Draw an ROI to be targeted on the previously acquired image (Fig. 5A).
7. In the “Mosaic Targeted Illumination” box, click “Location” (Fig. 6A). Make sure the coordinate system setting matches the Mag setting.
8. Select the region in the image and click the “Active region” button in the “Target Location” box (Fig. 6A).

This will specify the drawn ROI as the mask. It is also possible to click “All regions” if multiple ROIs are drawn.

9. In the “Mosaic Targeted Illumination” box, click Configuration (Fig. 3A). Specify the “Mask Exposure” duration in milliseconds.

We use 1000 ms, but this can be adjusted depending on the nature of the experiment.

10. In the “Mosaic Targeted Illumination” box, click “Timelapse” (Fig. 6B). Specify the acquisition cycles.

This setting depends on the nature of the experiment, as different activation schemes require pulses at different time points. During a period of activation, we typically illuminate the ROI with blue light prior to each time point. The program in Figure 6B has 100 time points at intervals of 30 s, with blue light pulsing before time points 10-13.

Here, you can also use a journal for acquisition like using different color channels for acquisition. In the example in Figure 6B, we have a journal specifying images in both 561 and 647 nm to image the recruitable GEF and any far-red effector protein or membrane stain. See Table 3 for an example protocol.

Table 3 Example Protocols (Journals) for Optogenetic Experiments

Goal	To confirm presence and functionality of optogenetic probes	Monolayer (imaging mCherry-LARG and Cell Mask Deep Red)	Single cell (imaging mApple-Myosin and Alexa 647 beads for traction force microscopy)
Journal	Acquire mCherry (GEF)	Timelapse loop (30-s intervals for 10 min)	Timelapse loop (20-s intervals for 15 min)
	Acquire GFP (Stargazin)	• Acquire mCherry	• Acquire mApple
	Acquire mCherry (GEF)	• Acquire Deep Red	• Acquire Alexa 647
	Confirm that GEF moves from cytosol to membrane	Timelapse loop (30-s intervals for 5 min)	Timelapse loop (20-s intervals for 15 min)
		• Mosaic Illumination in ROI (405 nm)	• Mosaic Illumination in ROI with (405 nm)
		• Acquire mCherry	• Acquire mApple
		• Acquire Deep Red	• Acquire Alexa 647
		Timelapse loop (30 s intervals for 15 min)	Timelapse loop (20-s intervals for 15 min)
		• Acquire mCherry	• Acquire mApple
		• Acquire Deep Red	• Acquire Alexa 647
			Remove cells from gel using 0.05% SDS
			Acquire Alexa 647 (relaxed gel image for TFM)

11. Click “Acquire” in the “Mosaic Targeted Illumination” box.

Cell-cell junctions should undergo contraction, similar to Figure 5.

12. Save Images under desired name.
13. Save ROI for use in image analysis.

IMPORTANT: *We detail here the use of the mosaic micromirror with the Targeted Illuminated system using MetaMorph, but other acquisition software and hardware setups exist. Acquisition can be greatly streamlined through the use of journals, but is beyond the scope of this protocol.*

COMMENTARY

Background Information

LOV domains of the phototropin blue light receptors contain a flavin-based blue-light-sensing chromophore and regulate light-mediated biological processes in microbes and plants (Möglich & Moffat, 2010). Specifically, the AsLOV2 protein is made up of a core per-arrnt-sim (PAS) fold with flanking alpha helices on both the N and C termini (Halavaty & Moffat, 2007). Upon blue light absorption, a conserved cysteine residue in the AsLOV2 core covalently binds to the flavin cofactor. This binding causes conformational changes that propagate along the PAS fold, leading to the uncaging of an ~20-aa amphipathic C-terminal alpha helix (Harper, Neil, & Gardner, 2003) known as the J α helix. The beauty of this system lies in its reversibility; J α is left intact in the dark state, but light stimulation can expose linear motifs in its amino acid sequence. Therefore, reversible caging of the J α can lead to masking or unmasking of signaling protein activity with light.

The nature of J α is such that it can block certain peptide epitopes in the AsLOV2 core. Upon photoexcitation, J α can undock and expose this specific epitope. This feature of the AsLOV2 protein is useful because it can be used to design a binding partner to create a dimerizable protein complex. For a AsLOV2 binding partner, the original Strickland paper used a high-affinity, high-specificity engineered variant of the Erbin PDZ domain, ePDZ-b1 (Huang, Koide, Makabe, & Koide, 2008). This domain is small, at ~194 amino acids. The ePDZ domain's affinity and lifetime in the photoexcited state are tunable by various mutations, effectively tuning downstream signaling events (Strickland et al., 2012). Recently, this system has been improved upon by substituting the ePDZ-b1 domain with a tandem PDZ tag (2xPDZ) that is functional in more diverse protein fusions (Wagner & Glotzer, 2016).

Troubleshooting

RhoGEF is not recruited to the membrane

The RhoGEF should be visibly recruited to the membrane upon photoactivation (Fig. 2). If it is not, there may be a problem with the expression levels of the optogenetic probes. It is possible that the ratio of Stargazin-GFP-LOVpep and 2xPDZ-mCherry-LARG is not optimal. In this case, we recommend choosing cells that have higher expression of either protein.

Cells are constitutively activated

It is possible that the light from bright computer monitors or ambient room light can activate the cells due to the presence of blue light. This can be easily solved by keeping the cells in the dark whenever possible and keeping them at a far distance from computer monitors or turning the computer away from the microscope.

Activation occurs outside the targeted ROI

Make sure that the “Active region” button is toggled in the “Targeted Illumination” control panel, and that the correct region is chosen.

Cells bleb or die

We find it a common occurrence that the cells may bleb in other regions of the cell upon photorecruitment of the GEF. If this is the case, or if the cells are dying, it is wise to ease off the 405 nm laser intensity. Too much laser power may result in an excess amount of RhoA activation that weakens the opposite membranes to cause cell blebbing or bursting.

RhoGEF aggregates

The RhoGEF often aggregates, showing up as clumps in fluorescence. This is natural and a feature of the 2xPDZ-mCherry-LARG.

Junctions do not contract

Although we expect robust junction contraction upon RhoA activation, there are

several reasons that the junctions may simply not contract. First, it is possible that the light intensity levels are not high enough to produce a marked cellular response. Light intensities would then need to be increased. Second, it is possible that the RhoGEF expression levels are not optimal for RhoA activation. In this case, we suggest choosing cells with higher RhoGEF expression. Third, although rare, we have found that clones may express a variant of the recruitable RhoGEF that renders it unrecruitable. This may be due to spontaneously occurring mutations in the DNA encoding the photosensitive dimerization domains, which are present in the DNA stock used to generate the stable cell line. We recommend sequencing the DNA used to make sure there are no mutations, and otherwise using another isolated cell clone.

Perfect Focus does not work

Painting 80–100 μ l of collagen on the chamber should be sufficient to produce an even layer of polymerized gel. This is advantageous because the gel thickness is conducive to using the Perfect Focus system on many microscopes. If Perfect Focus is not working, it is possible that the gel is too thick. If so, consider using less collagen for a thinner gel. It is also possible to coat the chamber with 80–100 μ l and then aspirate the excess collagen to produce a very thin gel layer.

Stable cell line loses expression

A common problem that we have is dilution of the optogenetic constructs over time. This can occur for a few reasons, although we believe that the cells expressing the optogenetic proteins are more prone to cellular extrusion because they are hypercontractile. To alleviate this, we recommend using cells at a low passage number. If the cells are still losing expression, we recommend repeating the fluorescent sorting of the cells to achieve better protein expression.

Understanding Results

Cells with good expression of both optogenetic constructs can be photoactivated such that there is a marked increase in junctional RhoGEF compared to cytoplasmic RhoGEF; the latter should result in reduced fluorescence (Fig. 2). The photoactivated junction will be visually distinguishable compared to other cells that have not been activated with 405-nm light. The unactivated cells should have diffuse cytoplasmic localization of the RhoGEF. Upon RhoGEF recruitment, the ac-

tivated junction will undergo contraction and shorten noticeably (Fig. 5), although the extent of this shortening may be dependent on the cell type and light intensity used. With this approach, junction lengths can be analyzed under wild-type and various inhibitor conditions. Additionally, the use of far-red protein labeling can allow visualization of the membrane, junctional components, or effector proteins as a result of RhoA localization and subsequent junction contraction.

Time Considerations

The largest time investment comes from obtaining the stable cell line. This may take a few weeks from infection to isolation and expansion of a clonal cell line. Careful attention needs to be paid when sorting and screening the cells for optimal fluorescence intensities, and multiple clones may need to be isolated to obtain the recruitable cell line with optimal expression levels of the constructs. Time is also spent on optimizing the microscopy parameters, including light intensity and pulse duration. It may take a few optogenetic experiments to determine optimal microscopy parameters. Imaging can take anywhere from minutes to hours, depending on the activation scheme needed to drive the desired cellular behavior. A typical experiment, from plating cells to the onset of imaging, should only take a few days. This is limited by how fast the cultured cells can grow into a confluent monolayer if testing cell mechanics at the tissue scale. At the cellular scale, cells can be plated the night before to ensure proper attachment to the desired substrate.

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