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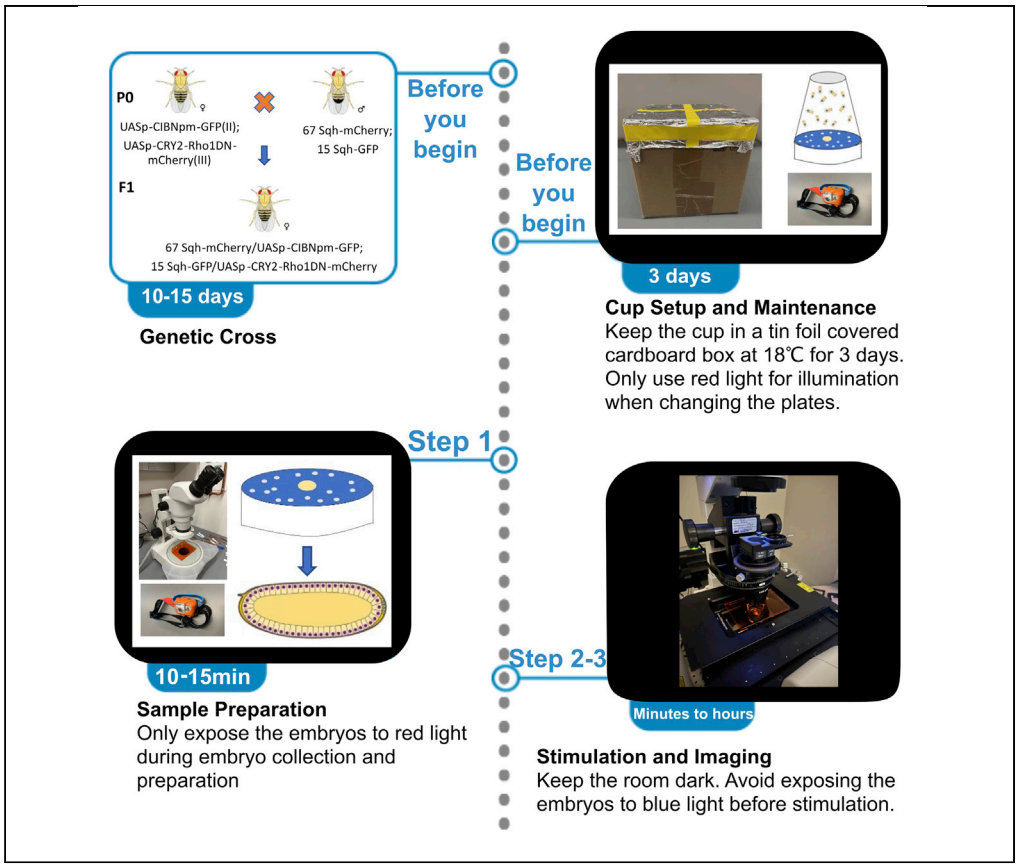
An optogenetic tool to inhibit RhoA in *Drosophila* embryos



We describe a protocol for optogenetic inhibition of the small GTPase Rho1 (RhoA) in *Drosophila* embryos, which allows rapid and spatially confined inactivation of Rho1 and Rho1-mediated actomyosin contractility. We provide step-by-step instruction for optogenetic manipulations of *Drosophila* embryos using confocal and multiphoton imaging systems. This tool is useful for determining the site- and stage-specific function of Rho1 in *Drosophila* embryos and for studying the immediate tissue response to acute elimination of cellular contractility.

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

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Highlights

Controlling RhoA activity in complex tissue contexts using the CRY2-CIB optogenetic system

Optogenetic-mediated acute inhibition of actomyosin contractility in *Drosophila* embryos

Detailed description of light-sensitive live embryo preparation for optogenetics

Step-by-step instruction for optogenetic manipulations using different imaging setups

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An optogenetic tool to inhibit RhoA in *Drosophila* embryos

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SUMMARY

We describe a protocol for optogenetic inhibition of the small GTPase Rho1 (RhoA) in *Drosophila* embryos, which allows rapid and spatially confined inactivation of Rho1 and Rho1-mediated actomyosin contractility. We provide step-by-step instruction for optogenetic manipulations of *Drosophila* embryos using confocal and multiphoton imaging systems. This tool is useful for determining the site- and stage-specific function of Rho1 in *Drosophila* embryos and for studying the immediate tissue response to acute elimination of cellular contractility. For complete details on the use and execution of this protocol, please refer to Guo et al. (2022).[1]

BEFORE YOU BEGIN

The optogenetic tool Opto-Rho1DN is constructed based on the CRY2-CIBNpm system, as illustrated in Figure 1.[1,2] This optogenetic tool can be activated by UVA light and blue light (400–500 nm), with maximal activation at 450–488 nm.[2–4] In addition, the tool can also be activated by 830–980 nm laser when performing two-photon stimulation.[2,5] Therefore, lights normally used for exciting GFP fluorescent protein (488 nm for single-photon laser or 920 nm for two-photon laser) can effectively stimulate the tool. Illuminating the sample with 514 nm (commonly used for exciting YFP) only activates the optogenetic system at high laser intensity (above 2 μW).[3] The optogenetic module remain unstimulated when the sample is illuminated with a wavelength of 561 nm (commonly used for exciting mCherry) or longer for single photon stimulation, or 1,040 nm for two-photon stimulation.[2,5] Thus, during sample preparation and pre-stimulation imaging, we only illuminate the sample using lights at the green-red range (> 532 nm), which in our practice do not cause unwanted light stimulation. We have successfully achieved desired Rho1 inhibition using the following combinations of optogenetic constructs, either with or without fluorescent tags on the optogenetic module.

Flies with transgenes encoding optogenetic components:

Opto-Rho1DN line 1: UASp-CIBNpm-GFP (II); UASp-CRY2-Rho1DN-mCherry (III).

Opto-Rho1DN line 2: UASp-CIBNpm (I); UASp-CRY2-Rho1DN-mCherry (III).

Opto-Rho1DN line 3: UASp-CIBNpm (I); UASp-CRY2-Rho1DN (III)

Note: We use line 1 as an example in steps of this protocol.

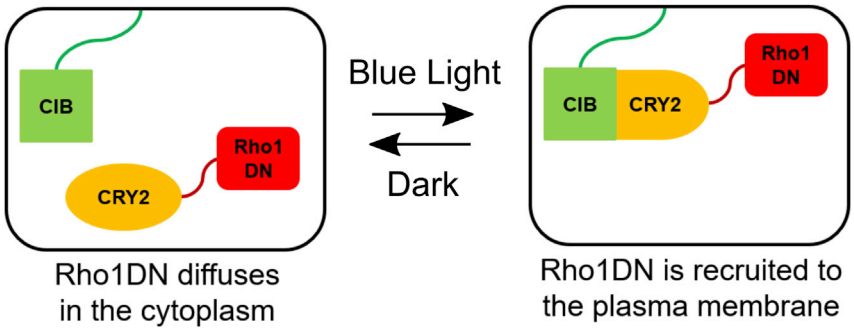

Figure 1. Design of Opto-Rho1DN

Dominant negative form of Rho1 (Rho1DN) is used to inhibit endogenous Rho1 by sequestering available Rho GEFs, which activate Rho1 by facilitating the exchange of GDP for GTP on Rho1. In the system, the endogenous membrane targeting signal in Rho1DN is mutated. As a result, CRY2-Rho1DN can only affect endogenous Rho1 activity when it is recruited to the plasma membrane, where Rho1 GEFs are localized to in many morphogenetic processes. After blue light stimulation, CRY2 changes its conformation and therefore binds to membrane tethered CIB1 (or the N-terminal CRY2-binding domain of CIB1, CIBN). This will relocate CRY2-Rho1DN from the cytoplasm to the plasma membrane, where it inhibits the activation of endogenous Rho1. Reproduced from Guo et al.[1]

Note: Flies containing the optogenetic constructs are crossed to flies expressing desired fluorescent markers and the maternal GAL4 driver. In this protocol, we use maternal-Tubulin-GAL4 67.15 ("67; 15") as the GAL4 drivers. 67 and 15 stand for maternal-Tubulin-GAL4 inserted to the second (II) and third (III) chromosomes, respectively.[6]

Note: We have successfully used maternal GAL4 combined with various fluorescent markers, but this protocol uses 67 Sqh-mCherry; 15 Sqh-GFP as an example. This line contains the maternal GAL4 67 and 15 and expresses mCherry and GFP tagged myosin regulatory light chain Sqh (Spaghetti squash) under the control of the *sqh* promoter.

Genetic cross

Timing: 10–15 days

1. Select virgin female flies from the optogenetic line UASp-CIBNpm-GFP (II); UASp-CRY2-Rho1DN-mCherry (III) and cross them to male flies with maternal GAL4 drivers and the desired fluorescent protein markers (in our case, the 67 Sqh-mCherry; 15 Sqh-GFP males).

   Note: It is not necessary to put this cross in dark if the female flies contain the UASp optogenetic constructs and the males contain the maternal GAL4 driver.

   Note: The reciprocal cross between 67 Sqh-mCherry; 15 Sqh-GFP females and UASp-CIBNpm-GFP (II); UASp-CRY2-Rho1DN-mCherry (III) males will in theory generates identical F1 flies. However, in this case, the F1 embryos will contain both maternally deposited GAL4 proteins and the UASp-CIBNpm-GFP and UASp-CRY2-Rho1DN-mCherry transgenes, which will cause expression of the optogenetic components during embryogenesis of the F1 flies. In this case, it is critical to put the cross in dark, because otherwise the activation of the optogenetic module may cause undesirable developmental defects in the F1 generation and impact the phenotypic analysis in the F2 embryos.

2. After ~10 days, select F1 female flies with the following genotype: 67 Sqh-mCherry/ UASp-CIBNpm-GFP;15 Sqh-GFP/UASp-CRY2-Rho1DN-mCherry, and set up a cup with male flies for embryo collecting.

Figure 2. Setting up and maintaining fly cups for embryo collecting

Left panel: A fly cup used for embryo collecting. To make fly cups, we remove the bottom of plastic beakers and replace the bottom with metal mesh for air ventilation. The opening of the plastic beaker is covered by an apple juice plate for female flies to lay eggs. A smear of fresh yeast paste is placed on the surface of the plate. Any commercially available fly cups that allow embryo collecting could serve the purpose. Middle panel: an aluminum foil covered cardboard box used for storing fly cups. Right panel: a headlamp used for providing red-light illumination in the dark room.

Note: The bottom of the cup is covered with an apple juice plate with a small smear of fresh yeast paste on the surface. The flies will lay eggs on the surface of the apple juice plate. These F2 embryos will be collected for the optogenetic experiments (Figure 2). The apple juice plate needs to be changed every day if the cup is kept at room temperature (~ 21°C–23°C), or every 2 days if the cup is kept at 18°C. To increase the expression of the UASp constructs, we routinely keep the cup at 18°C for at least 3 days before embryo collection. It also takes 2–3 days for the flies to adapt to the cup environment and be fed on the yeast paste to achieve best egg production.

Note: Since the optogenetic components are expressed maternally in F1 females, the female flies used to set up the cup do not need to be virgins, but they must have the correct genotype, and in this case, 67 Sqh-mCherry/UASp-CIBNpm-GFP; 15 Sqh-GFP/UASp-CRY2-Rho1DN-mCherry. For experiments involving early embryogenesis (up to stage 9), the genotype of the male flies in this cross does not noticeably influence the outcome. For our work, we routinely use the male flies from the same F1 population in the cross for convenience.

Note: The F1 flies are genotyped and collected on a CO_2 pad under a stereomicroscope (Nikon SMZ-745 stereoscope). This step can be performed in the presence of ambient light.

⚠ CRITICAL: After the cup is set up, keep the cup in a dark environment for the entire process to avoid exposing the cup to blue light. We put the cup in an aluminum foil covered cardboard box to avoid any possible exposure to the ambient light. We change the apple juice plate in a dark room, where we use a headlamp with red light as the only light source for illumination. Since the optogenetic components are only stimulated by blue light, exposure to green or red light will not cause premature stimulation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Clorox Ultra Germicadal Bleach (8.25% sodium hypochlorite)	VWR	10028-048
Halocarbon oil 27	Sigma-Aldrich	H8773-100ML
Methyl 4-hydroxybenzoate	Sigma-Aldrich	H5501-500G
Sucrose	VWR	0335-1KG
Bacto agar	BD	214010

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ddH_2O	NA	NA
Experimental models: Organisms/strains		
Drosophila: UAS-CIBNpm(I); UAS-CRY2-Rho1DN-mCherry (III)	Guo et al.[1]; Guglielmi et al.[2]	NA
Drosophila: UAS-CIBNpm-GFP(II); UAS-CRY2-Rho1DN-mCherry (III)	Guo et al.[1]; Guglielmi et al.[2]	NA
Drosophila: UAS-CIBNpm(I); UAS-CRY2-Rho1DN (III)	Guo et al.[1]; Guglielmi et al.[2]	NA
Drosophila: maternal-tubulin-GAL4 67 (II); maternal-tubulin-GAL4 15 (III) ("67; 15")	Hunter and Wieschaus[6]	NA
Drosophila: 67 Sqh-mCherry;15 Sqh-GFP	Guo et al.[1]	NA
Other		
VITCHELO V800 Headlamp with White and Red LED Lights	Amazon	NA
Black cloth for covering the microscope	Online	NA
UV Filter Shield for FM1403 Fluores (Orange-red plastic shield)	Bolioptics	FM14036151
SP Bel-Art 100-place polypropylene freezer storage box (Black, light-proof box for sample transfer)	VWR	30621-392
35 mm glass-bottom dish	MatTek	P35G-1.5-10-C
Apple juice	Grocery store	NA
60 mm × 15 mm petri dish with lid	Falcon	351007
Dumont Style 5 tweezers	VWR	102091-654
Eyelash tool (made from pure red sable round brush #2)	VWR	22940-834
CO_2 pad	Genesee Scientific	59-114
Nikon SMZ-745 stereoscope	Nikon	NA
Olympus FVMPE-RS multiphoton microscope	Olympus	NA
Nikon inverted spinning disk confocal microscope	Nikon	NA

MATERIALS AND EQUIPMENT

Apple Juice plate (modified from Müller[7]).		
Reagent	Final concentration	Amount
Methyl 4-hydroxybenzoate	1.2 g/L	1.2 g
Sucrose	20 g/L	20 g
Apple juice	NA	500 mL
Bacto agar	22 g/L	22 g
ddH_2O	NA	600 mL

Note: Prepare the apple juice solution by adding 1.2 g Methyl 4-hydroxybenzoate and 20 g Sucrose to 500 mL apple juice and boil the solution. Prepare the agar solution by adding 22 g Bacto agar to 600 mL water and boil. Then mix the apple juice solution and the agar solution. After boiling, the final volume of the mixed solution would be around 1L. Let the mixed solution cool to 65°C and pour the mixed solution to the 60 mm × 15 mm Falcon Petri dishes.

Note: Keep the apple juice plate in 4°C for future use. Maximum storage time for apple juice plate in 4°C is 1–1.5 month.

STEP-BY-STEP METHOD DETAILS

Sample preparation

Timing: 10–15 min

The purpose for this step is to collect the embryos at the desired stage and prepare them for stimulation and imaging without unwanted stimulation of the sample.

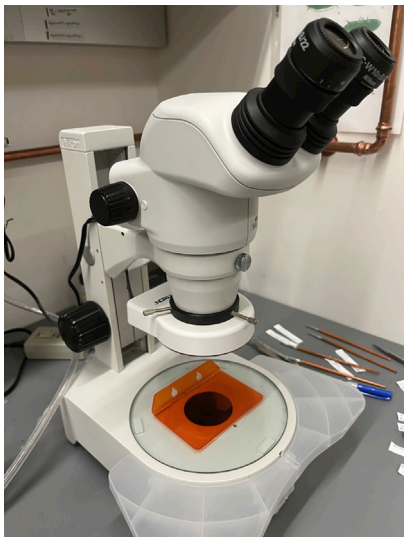

Figure 3. An upright Nikon stereoscope with orange-red plastic shield used for embryo collection

⚠ CRITICAL: Exposure of the specimen to ambient light should be strictly avoided in all steps in sample collection and preparation. In our experiments, these steps are performed in a dark room, with red light as the only light source for illumination. The optogenetic components are extremely sensitive to ambient light (more specifically, the UVA and blue light component (400–500 nm) of the ambient light). Even a brief exposure of the specimen to ambient light would cause premature stimulation.

1. Place an orange-red plastic shield (UV Filter Shield for FM1403 Fluorescence Microscope) on the stage of an upright Nikon SMZ-745 stereoscope in preparation for embryo collection.

   Note: When collecting the embryos, place the apple juice plate on top of the orange-red shield, which blocks the blue-green wavelengths from the transmitted light used for illuminating the specimen (Figure 3) and protects the sample from unwanted stimulation.

   ⚠ CRITICAL: All embryo collecting and preparation steps are performed in a dark room, where only red light is used for illumination.

2. Change the apple juice plate at the time of embryo collection.

   Note: For collecting embryos at the stage of early embryogenesis, change the apple juice plate 8–16 h (for cups kept at 18°C) before embryo collection.

3. Cover the surface of the plate taken off from the cup with a thin layer of Halocarbon oil 27.

   Note: In 30–60 s, the chorion of the embryo will become transparent, and the embryo will be visible under the stereomicroscope when illuminated with the transmitted light.

4. Collect 5–15 embryos at the desired stage from the apple juice plate using Dumont Style 5 tweezers. Avoid squeezing the embryos with the tweezers.

   Note: For our purpose, the embryos at late cellularization are selected in order to monitor mesoderm invagination (also known as ventral furrow formation). The bright field image of an embryo at the stage of late cellularization is shown in Figure 4.

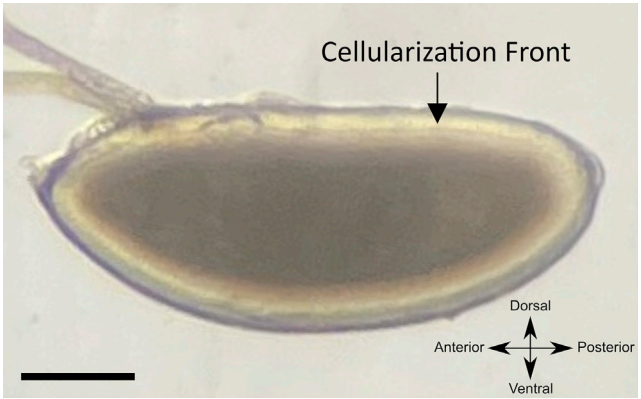

Figure 4. A typical *Drosophila* embryo at the mid-to-late cellularization stage

The black arrow labels the cellularization front that we use to determine the stage of the embryo in cellularization. The orientation of the embryo is indicated by the compass at the bottom of the figure. Scale bar = 100 μm.

5. Remove excess oil from the embryos by gently blotting the embryos on a small square piece of paper towel (~ 1.5 cm × 1.5 cm in size).
6. Dechorionate embryos using 40% bleach (~3% sodium hypochlorite) for about 2 min.

   Note: In our laboratory, this step is performed by transferring the embryos onto a small square piece of paper towel (~ 1.5 cm × 1.5 cm) that has been pre-soaked with 40% bleach.

   Note: 40% bleach should be made freshly every 3–5 days.

7. After ~2 min, pick up the paper towel with a tweezer and place it on a large piece of tissue paper to absorb excessive bleach.

   Note: The embryo side of the square paper towel should always be facing up.

   Note: Throughout the dechorionation and washing steps, make sure that the embryos remain stuck on the surface of the square paper towel.

8. Rinse the embryos using deionized water for 8 times.

   Note: For each wash step, gently soak the square paper towel into a drop of deionized water and then quickly dry the paper towel on a tissue paper. Repeat this process for 8 times to remove any residue bleach.

Figure 5. Light-proof box used for sample transfer

We use the SP Bel-Art 100-place polypropylene freezer storage box for this purpose.

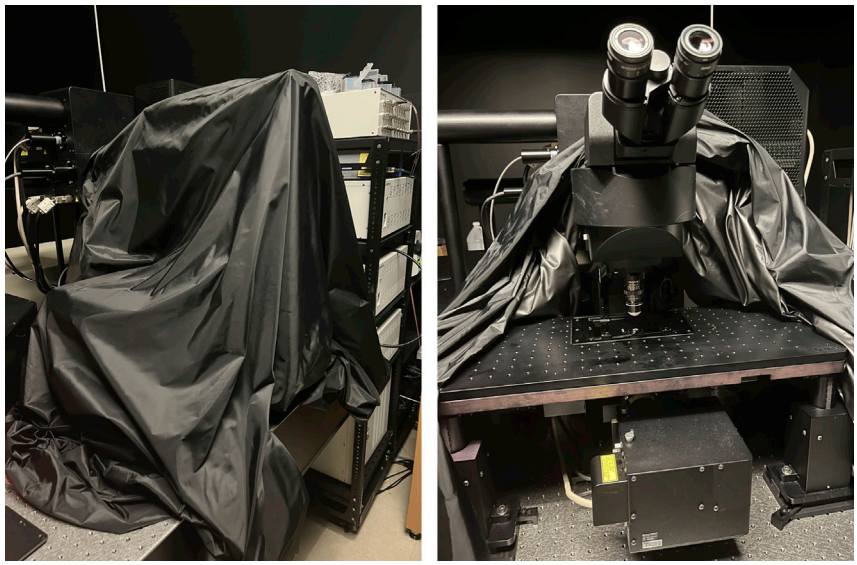

Figure 6. The setup for the Olympus FVMPE-RS multiphoton microscope with or without the black cloth cover
The light-proof black cloth cover is routinely used in regular imaging to protect the light-sensitive detectors. In the optogenetic experiments, it helps to avoid premature stimulation of the embryos.

9. Transfer the dechorionated embryo from the square paper towel to a 35-mm glass-bottom dish using an eyelash tool.
10. Add deionized water to the well in the middle of the dish to completely cover the well.

Note: the embryos should remain stuck on the surface of the glass-bottom of the dish after adding water to the well.

11. Use the eyelash tool to gently adjust the embryos to the desired position and orientation for imaging.
12. Transfer the 35-mm glass-bottom dish with the embryos to the confocal or multiphoton microscope.

⚠ CRITICAL: During the transfer process, avoid any unwanted stimulation from ambient light. In our experiments, we keep the sample in a light-proof box during the transfer (Figure 5).

Setting up the sample on the microscope stage

Timing: 5 min (for step 13)

The purpose for this step is to set up the embryo sample on the microscope in preparation for optogenetic stimulation and imaging. During this process, the key consideration is to avoid premature stimulation of the embryos from ambient light or other light sources associated with the microscope. Protection from premature stimulation can be achieved in different ways for different types of microscopes. In this protocol, we use the Olympus FVMPE-RS multiphoton microscope and the Nikon inverted spinning disk confocal microscope as examples. With some modifications, similar approaches can be employed when using other types of confocal or multiphoton microscopes.

13. Set up the sample for the Olympus FVMPE-RS multiphoton system:

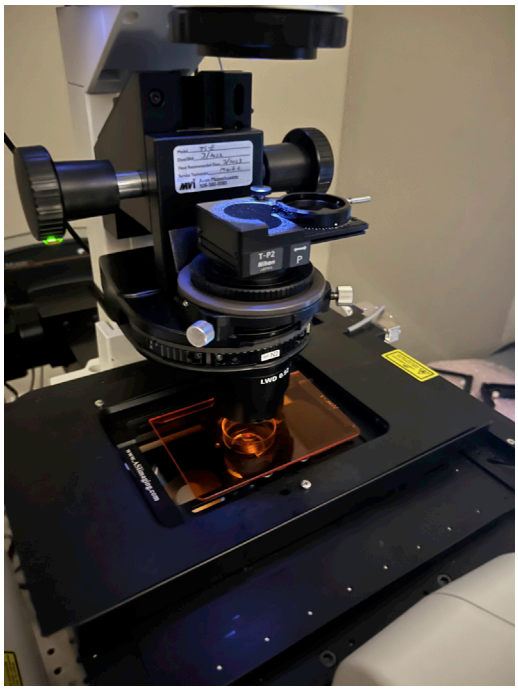

Figure 7. The setup for the inverted Nikon spinning disk confocal microscope

An orange-red plastic shield is placed between the light source for the transmitted light and the sample.

Note: In our regular imaging experiments, a light-proof black cloth is routinely used to cover the microscope to protect the light-sensitive detectors. For the optogenetic experiment, this black cloth also provides a superb protection of the samples from unwanted light stimulations (Figure 6).

a. Transfer the sample to the microscope stage. During this step, all light sources, including the room light and computer monitors, should be turned off to avoid unwanted sample stimulation.
b. Use the eyepiece to identify the embryo of interest and bring the embryo into focus. During this process, only use non-blue light for sample illumination.

Note: For the multiphoton system used in our experiments, this is achieved by taking advantage of the epifluorescence unit on the system. Specifically, the specimen is illuminated with green excitation light (532–554 nm) by using the standard TRITC filter set to filter out the unwanted wavelengths from a white light source. The green illumination light does not stimulate the optogenetic module while allowing adjustment of the position of the stage and the focus of the embryos. Illumination light with longer wavelength should also work, but we have not tested it on our system.

Note: The computer screen can be turned on once the sample is protected by the black cloth that covers the microscope.

14. Set up the sample for the inverted Nikon spinning disk confocal microscope:
    a. Transfer the sample to the microscope stage with all light sources turned off, including the room light and the light from computer screens.
    b. Place an orange-red plastic shield on top of the sample before turning on the transmitted light for sample illumination (Figure 7).

Note: The orange-red shield blocks the blue light component from transmitted light, thereby protecting the sample from unwanted stimulation.

Note: The computer screen can be turned on at this point, as long as the sample is protected by the orange-red plastic shield.

c. Use the transmitted light for sample illumination when adjusting the position of the stage and the focus of the embryo.

⚠ CRITICAL: Make sure there is no ambient light in the room before taking the sample out of the light-proof transfer box. The computer screen and touch panel controllers (if existing), as well as all other open light sources should be turned off or covered when the sample is not protected from ambient light.

⚠ CRITICAL: Keep the orange-red plastic shield on top of the sample during the entire stimulation and imaging process to avoid accidental stimulation of the sample by ambient light.

Sample stimulation and imaging protocol

Timing: minutes-hours depends on the purpose of the experiment (for step 15)

The purpose for this step is to perform optogenetic stimulation and observe desired phenotype upon Rho1 inhibition. We use the Olympus FVMPE-RS multiphoton microscope and the Nikon inverted spinning disk confocal microscope as examples to demonstrate the sample stimulation and imaging procedures. We will also discuss common considerations that are applicable to other confocal or multiphoton imaging systems.

15. For experiments performed using the multiphoton microscope, we use different lasers to achieve pre-stimulation imaging, stimulation and post-stimulation imaging. The general steps for our experiments on the multiphoton microscope are as follows:

Note: The Olympus FVMPE-RS multiphoton system used in our experiments is equipped with the InSight DS Dual-line multiphoton laser for simultaneous dual-wavelength excitation. The laser can generate two pulsed laser beams simultaneously. The wavelength of one laser beam is tunable between 680 nm and 1,300 nm, which is normally adjusted to 920 nm in our experiments for exciting green-yellow fluorescent proteins, such as GFP. The wavelength of the other laser beam is fixed at 1,040 nm, which is routinely used for exciting orange-red fluorescent proteins, such as mCherry. Imaging with these multiphoton lasers is achieved through a galvanometer scanner. The multiphoton system is also equipped with an IR/VIS stimulation unit for photo-activation/stimulation, which contains a 458 nm laser and an independent galvanometer scanner to allow photo-activation/stimulation within defined region of interest (ROI).

a. Turn off or cover all light sources in the room before taking the sample out of the transfer box. Make sure there is no other ambient light in the room.
b. Place the 35 mm glass-bottom dish on the stage and locate the embryos using the eyepiece.

Note: The embryos are illuminated with a beam of green light (532 nm–554 nm) normally used for epifluorescence with a standard TRITC filter set. In our case, the light source for epifluorescence is white light, and the TRITC filter is used to filter out the blue light component to avoid unwanted stimulation. In principle, any wavelength within the green-red range can be used at this step to illuminate the sample without the risk of premature stimulation.

c. Cover the microscope with a light-proof black cloth. Make sure that the sample is fully protected from light before turning on the computer screen and other necessary light sources.
d. Acquire pre-stimulation movies using the 1,040 nm laser. In this experiment, the 1,040 nm laser excites both CRY2-Rho1DN-mCherry and Sqh-mCherry.

Note: For the process we are interested in (ventral furrow formation), the Sqh-mCherry signal is enriched at the medioapical region of the ventral cells, whereas CRY2-Rho1DN-mCherry should remain in the cytoplasm before stimulation. When imaging these proteins in early embryos, the laser intensity we routinely use ranges from 4% to 10%, with a linear increase in laser intensity from embryo surface to a depth of 100 μm. The laser intensity used for pre- and post-stimulation imaging is determined empirically based on the balance between optimal signal-to-noise and prevention of photobleaching. Troubleshooting 1.

Note: In general, pre-stimulation images can be acquired by using excitation light that will not stimulate the optogenetic module, such as the 561-nm and 633-nm lasers commonly equipped on the confocal microscopes and the 1,040-nm laser on the multiphoton microscopes.

e. Stimulate the optogenetic module within defined ROI using the IR/VIS stimulation unit. Scan the ROI with the 458 nm laser continuously for ~ 12 s.

Note: For stimulating the entire embryo, we use 1× zoom and an ROI that is 512 × 300 pixel^2 in size (1 pixel = 1 μm).

Note: The laser intensity we normally use for stimulation is 0.1%–0.3%. A more rapid stimulation could be achieved using higher laser intensities. However, under those circumstances, the scattered laser light is sufficient to cause stimulation in regions outside of the ROI, which is less ideal if a spatially confined stimulation is desired. Troubleshooting 2.

Note: Stimulation of the optogenetic module can be achieved in multiple ways. For laser scanning confocal microscopes, sample can be stimulated by scanning a defined region of interest with a blue laser (400 nm–500 nm). For spinning disk confocal microscopes, stimulation will occur when a 488-nm blue laser is used for imaging GFP- or YFP-tagged proteins. For multiphoton microscopes, sample can be stimulated by scanning a defined region of interest with a 920-nm laser. Alternatively, stimulation of the sample can be achieved by using a separate photo-stimulation/photo-activation module equipped on the microscope, as described above. Of note, consistent with a previous report,[2,5] 920 nm two-photon laser results in slower activation of the optogenetic module compared to the 488 nm single-photon laser stimulation.

f. Acquire post-stimulation movies using both 1,040 nm and 920 nm lasers.

Note: In this experiment, the CRY2-Rho1DN-mCherry protein, which is excited by the 1,040 nm laser, should show a rapid translocation from the cytoplasm to the plasma membrane after stimulation. The Sqh-GFP protein, which is excited by the 920 nm laser, should show a rapid dissociation from the cell cortex. Troubleshooting 3 and 4.

Note: When imaging Sqh-GFP in early embryos, the laser intensity we routinely use is between 0.1% and 0.5%, depending on the imaging depth within the embryo. The laser intensity is determined empirically based on the balance between optimal signal-to-noise and prevention of photobleaching.

Note: The 920 nm laser will cause stimulation of the optogenetic module in all regions being imaged. Troubleshooting 5.

Note: For the experiment demonstrated in this protocol (acquiring mCherry signal for pre-stimulation imaging and acquiring both GFP and mCherry signals for post-stimulation imaging), one advantage of using the multiphoton microscope is that multiple embryos from the same glass-bottom dish can be used for sequential data collection. When acquiring the post-stimulation images for one embryo, the scattered light from the 920 nm beam (used for Sqh-GFP excitation) will not stimulate other embryos on the same dish. These embryos, if at appropriate stages, can be used in subsequent rounds of optogenetic stimulation and data collection. In contrast, when imaging Sqh-GFP using a single photon laser (such as the spinning disk confocal microscope as described below), the blue laser intensity required for exciting GFP is usually much higher than that required for stimulating the optogenetic module. Under such circumstance, the blue laser scattered from the embryo during post-stimulation imaging may cause stimulation of other embryos on the same dish, preventing the use of them for additional data collection.

Note: Laser intensity should be adjusted based on the expression levels of the fluorescent markers to achieve optimal signal-to-noise and meanwhile avoid photobleaching of the fluorophore.

Note: The activation of the optogenetic module by blue light is reversible, but the dissociation rate after turning off stimulation is much slower compared to the rate of light-induced dimerization. In our experiments, the membrane recruitment of CRY2-Rho1DN-mCherry resulted from a single round of stimulation (0.3% 458 nm laser for 12 s) is still clearly visible 10–15 min after the stimulation, which is consistent with the reported dissociation half-time of ~9 min.[2] However, in order to ensure a complete inhibition of Rho1, we repeat the stimulation step every 3–5 min.

16. For experiments performed using the inverted spinning disk confocal microscope, the general steps we use for our experiments are as follows:
    a. Turn off or cover the room light and the computer screens (including any touch panel controllers, if existing) and all other light sources in the room before taking the sample out of the transfer box. Make sure there is no other ambient light in the room.
    b. Place the 35-mm glass-bottom dish (sample) on the microscope stage.
    c. Place the orange-red plastic shield above the dish to protect sample from unwanted stimulation.
    d. Block any light from the computer screen that might stimulate the sample (the computer screen needs to be turned on at this point in order to carry out the next step).
    e. Turn on the transmitted light through the software control and locate the embryo with the eyepiece.

Note: The transmitted light illuminates the sample from above the stage. No blue light can pass through the orange-red shield, which prevents unwanted stimulation of the sample.

    f. Acquire the pre-stimulation movie with the 561 nm laser, which does not stimulate the optogenetic module.

Note: For imaging Sqh-mCherry in early embryos, we routinely use a laser intensity of 20%. For pre- and post-stimulation imaging, the laser intensity is determined empirically to achieve an optimal balance between adequate signal-to-noise and greatest protection of the sample from photobleaching. Troubleshooting 1.

    g. Stimulation of the optogenetic module and acquisition of the GFP signal is performed simultaneously using the 488 nm laser. The 561 nm laser is also used in parallel to acquire the signals in the mCherry channel.

Note: The intensity of the 488 nm laser we routinely use is 10%–20%. Note that this intensity is optimized for imaging Sqh-GFP (which is determined based on the trade-off between signal intensity and photobleaching) but is approximately two orders of magnitude higher than the laser intensity required for optogenetic stimulation. Robust membrane recruitment of CRY2-Rho1DN-mCherry is typically observed within the first few seconds of image acquisition. Troubleshooting 3, 4, and 5.

Note: Laser intensity should be adjusted based on the expression levels of the fluorescent markers to achieve optimal signal-to-noise ratio while minimizing photobleaching.

Note: For spinning disk confocal microscope, repeated stimulation is not necessary since the 488 nm laser is constantly stimulating the optogenetic module during the acquisition process. Troubleshooting 5.

⚠ CRITICAL: With the approach described above, due to the strong light scattering from the 488 nm laser, the entire embryo is stimulated upon exposure of the sample to the 488 nm laser, precluding the application of region-specific stimulation. In addition, other embryos from the same dish might also be stimulated by the scattered light, precluding the use of them for further data collection. If imaging the GFP channel is not necessary for post-stimulation acquisition, a much lower laser intensity for the 488 nm laser (e.g., 0.1%) can be used to stimulate the embryo. In this case, other embryos on the same dish will not be stimulated prematurely and can be used for additional data collection. Troubleshooting 2.

EXPECTED OUTCOMES

The membrane recruitment of CRY2-Rho1DN-mCherry should be noticed almost immediately after the stimulation step, and maximum recruitment should be achieved within 30 s using 10% 488 nm acquisition (Figure 8, Methods video S1). Stimulation of the optogenetic module in the apical constriction domain during ventral furrow formation will cause rapid disappearance (< 1 min) of apical myosin, the activation of which depends on the Rho1-Rok pathway. When the embryo is stimulated during the early phase of ventral furrow formation, obvious relaxation of the constricted tissue should be observed (Figure 9, Methods video S2).

LIMITATIONS

This tool is designed to inhibit Rho1 during early embryogenesis. The expression of the optogenetic module is driven by UASp, which is optimized for expression in the female germline but not in the somatic tissues. The effectiveness of the tool in somatic tissues beyond early embryogenesis remains to be determined. To apply this tool on later stages during *Drosophila* development, one possible approach is to replace UASp with UASt, which is optimized for expression in the somatic tissues. An important factor that will impact the Rho1-inhibition effect is the expression level of the optogenetic module, which depends on the specific GAL4 driver lines used in the cross. For example, in our experiments, when we drive the expression of Opto-Rho1DN using a driver line with a single copy of maternal GAL4 instead of two copies, the inhibitory effect on actomyosin contractility becomes substantially weaker. Therefore, further experiments are needed to evaluate the effectiveness of the tool when different GAL4 driver lines and/or UASt are used to drive the expression of the optogenetic module.

Using this tool, we have achieved a rapid inactivation of Rho1 during ventral furrow formation, as indicated by the rapid inactivation of apical myosin and disassembly of apical F-actin.[1] However, since the tool acts by sequestering the Rho1 GEFs, how quickly Rho1 and its downstream effectors will become inactivated may vary in different developmental and physiological conditions,

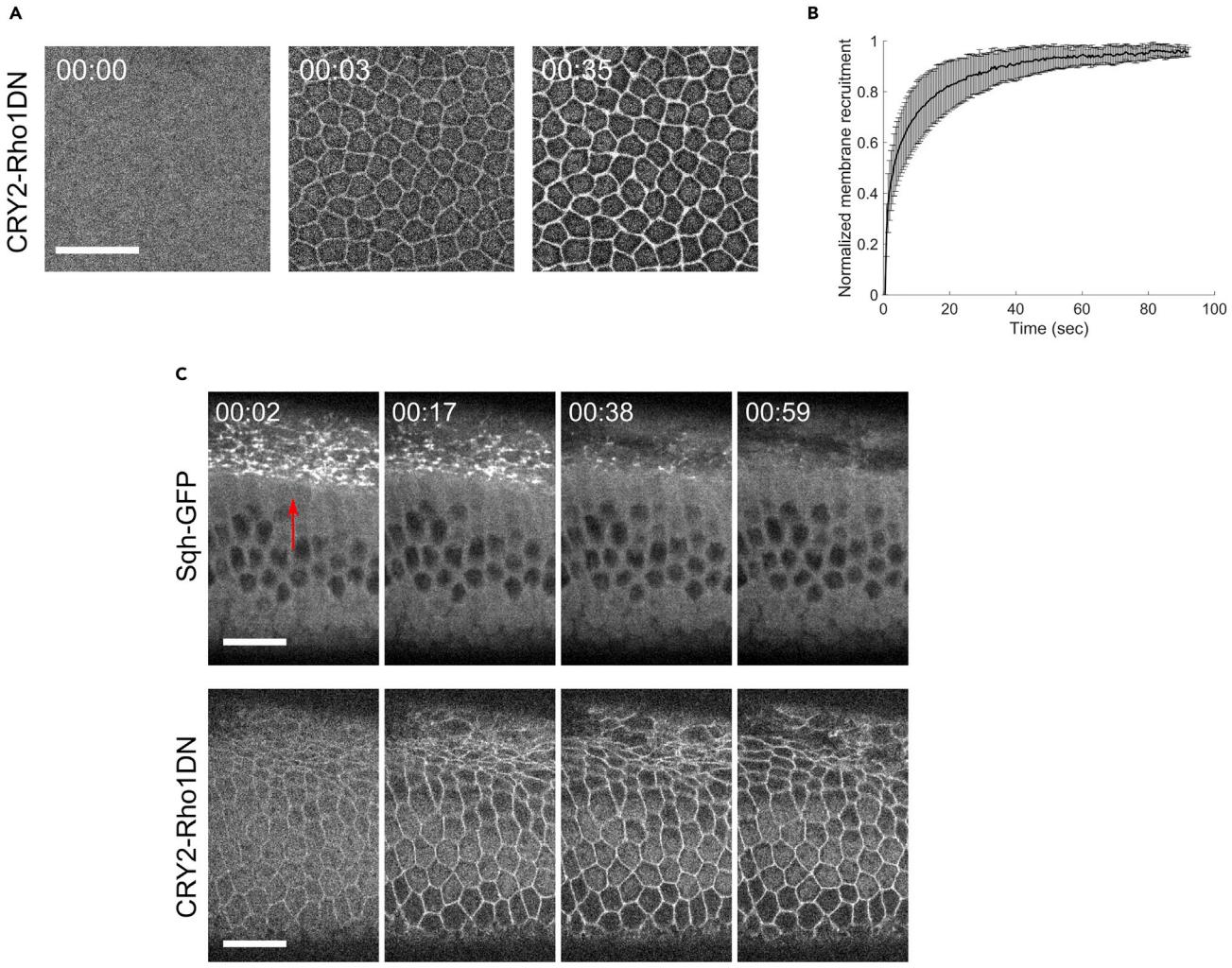

Figure 8. Tissue response after blue-light stimulated membrane recruitment of CRY2-Rho1DN-mCherry

(A) Confocal images showing the rapid membrane recruitment of CRY2-Rho1DN-mCherry upon blue light illumination.

(B) Relative abundance of membrane recruited CRY2-Rho1DN-mCherry over time after blue light stimulation. Error bar: s.d., N = 6 embryos.

(C) Activation of Opto-Rho1DN results in rapid dissociation of myosin from the ventral cell cortex (arrow) in a gastrulating embryo. N = 8 embryos. Note that in this experiment, F1 female genotype is UASp-CIBNpm/+; 67 Sqh-GFP/+; CRY2-Rho1DN-mcherry/15. Data acquired on the Nikon inverted spinning disk confocal microscope. All scale bar = 20 μm. This figure is reproduced from Guo et al.[1]

depending on the concentration and activity of proteins that function to inactivate Rho1 and its downstream effectors.

This tool is highly sensitive to blue light. When handling embryos for the optogenetic experiment, multiple additional procedures are used to avoid potential unwanted stimulation, such as the use of dark room with red light as the only light source for illumination and the use of the orange-red shield during embryo manipulation. While these procedures have been proved to be effective, they also complicate the protocol and require extra attention during the experiment.

For the experiments described in this protocol, we target activated CRY2-Rho1DN to the cell membrane by using a general plasma membrane localized CIBN anchor.[1,2] This approach might not be optimal if inhibiting Rho1 at a specific plasma membrane domain is desired. Since activated CRY2-Rho1DN proteins can diffuse in the cytoplasm before binding to the CIBN anchor, even if we confine

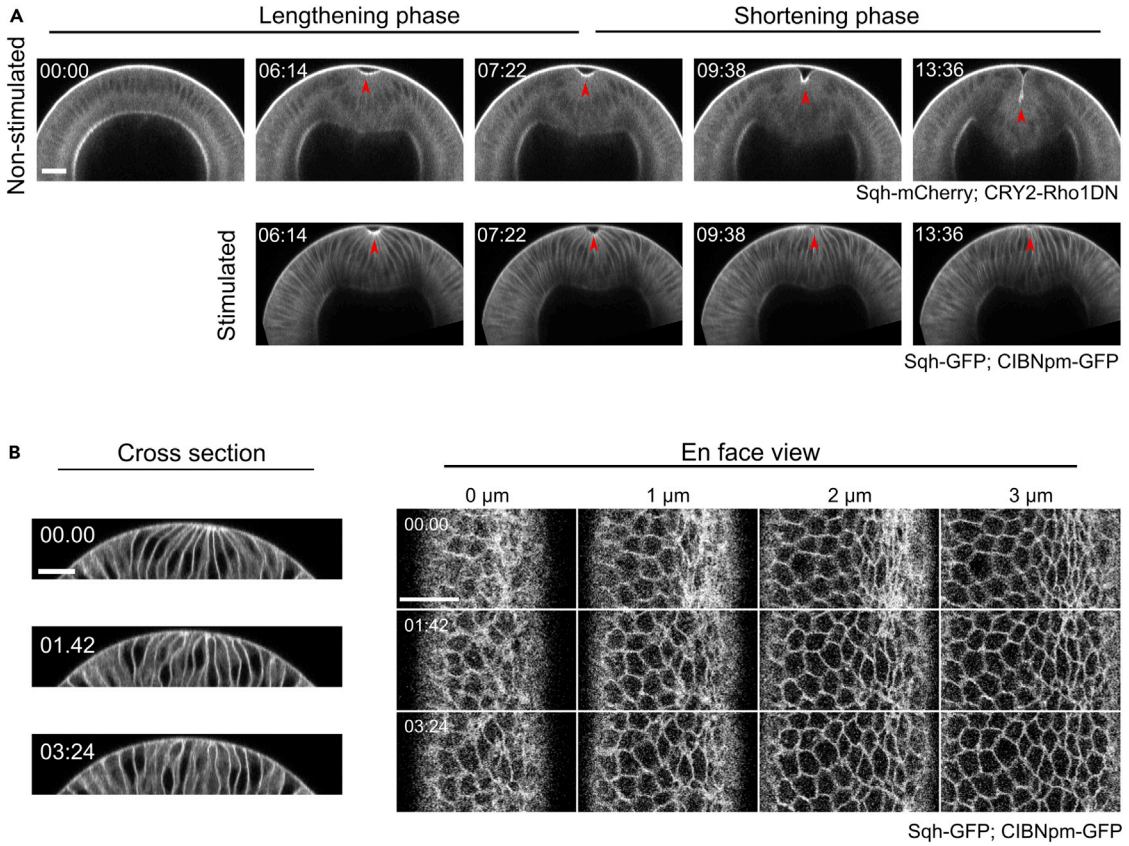

Figure 9. Acute inhibition of actomyosin during early stage of ventral furrow formation results in tissue relaxation

(A) Still images from multiphoton movies showing a control embryo (top panels) and a stimulated embryo (bottom panels). For the stimulated embryo, the first frame corresponds to the time point immediately after stimulation. Tissue relaxation of the constricted area after stimulation is indicated by red arrowheads.

(B) *En face* view of a stimulated embryo showing the apical area relaxation and diminish of apical myosin. 0 min corresponding to the onset of apical constriction. Data acquired using the Olympus FVMPE-RS multiphoton microscope. Scale bar = 20 μm. This figure is reproduced from Guo et al.[1]

the stimulation to a specific subcellular location, the region where Rho1 is inhibited will likely be less confined due to diffusion of activated CRY2-Rho1DN. Therefore, inhibition of Rho1 at a specific subcellular location will require development of new CIBN anchors that has confined localization at the target subcellular location.

Finally, a similar strategy can in theory be used to acutely inhibit other small GTPases, such as the Rho family GTPases Cdc42 and Rac, as the dominant negative form of these small GTPases have been widely used to inhibit their endogenous wildtype counterparts.[8] However, several considerations need to be taken in designing such tools. First, when designing the fusion construct between CRY2 and the dominant negative protein, the endogenous targeting signal in the dominant negative protein should be mutated. Second, an appropriate CIBN anchor that colocalizes with the GEF proteins needs to be used (or developed if not existing). This will in turn require a prior knowledge of the subcellular localization of the GEF proteins. Finally, the effectiveness of the new tool will depend on the stoichiometry between the GEF proteins and the dominant negative proteins that are targeted to the appropriate subcellular locations. Therefore, each new tool needs to be validated experimentally.

TROUBLESHOOTING

Problem 1

CRY2-Rho1DN-mCherry is localized to the cell membrane before stimulation (step 15d or step 16f).

Potential solution

The embryo has likely been stimulated prematurely. Premature stimulation could occur during the process of sample preparation, during sample transfer, during sample positioning on the microscope stage, or during pre-stimulation acquisition. Note that the optogenetic module are extremely sensitive to ambient light. It is critical to prevent any potential exposure of the sample to blue light before stimulation. The following approaches are recommended to prevent premature stimulation of the sample. Once the fly cup is set up, always keep the cup in a dark environment and protect the cup from room light when changing plates. Only use non-blue light (i.e., light at green-red range, wavelength > 532 nm) for illumination when handling the cup. When selecting embryos from the apple juice plate on the stereomicroscope, use an orange-red plastic shield between the illuminating light and the plate. Always transfer the sample in a light-proof box. When positioning the sample to the microscope stage, the room light and other light sources including computer screen should be turned off. Only use the non-blue light to illuminate the specimen when locating the embryos for imaging. During pre-stimulation image acquisition, make sure that the shutter for the 920 nm laser (multiphoton imaging) or the blue laser (single photon imaging) is closed.

Problem 2

When stimulating Opto-Rho1DN within a specific group of cells, membrane recruitment of CRY2-Rho1DN-mCherry also occurs outside of the ROI (step 15e).

Potential solution

If undesired stimulation of the optogenetic module appears outside the ROI, decrease the intensity of the stimulation laser to reduce light scattering. Ideally, the lowest laser intensity that is sufficient to induce robust membrane recruitment of CRY2-Rho1DN-mCherry should be used. The optogenetic module is highly sensitive to blue light. When a high intensity blue laser is used for stimulation, scattered light from the blue laser can cause the undesired stimulation in the regions adjacent to the ROI or even adjacent embryos.

Problem 3

The cytoplasmic signal of CRY2-Rho1DN-mCherry is low prior to stimulation, and the cell membrane recruitment of CRY2-Rho1DN-mCherry is weak after stimulation (step 15f or step 16g).

Potential solution

The expression level of the optogenetic module is too low to induce a detectable inhibitory effect. Make sure that you use a GAL4 driver line that provide adequate expression of the optogenetic module. For the experiments presented in this protocol, using a driver line that contains two copies of GAL4 transgenes (67 and 15) is critical to produce adequate level of optogenetic components in the embryo. In addition, the fly cups should be stored at 18°C for optimal expression driven by the maternal GAL4 67 and 15. In addition, make sure that the flies in the cup are well fed (with fresh yeast paste) and are less than two weeks old. The expression level of the optogenetic module may drop significantly when the flies become too old.

Problem 4

The cytoplasmic signal of CRY2-Rho1DN-mCherry is normal but no cell membrane recruitment occurs after stimulation (step 15f or step 16g).

Potential solution

Potential scenario 1: No CIBNpm or CIBNpm-GFP expression. Make sure that the starting line in the cross contains UASp-CIBNpm or UASp-CIBNpm-GFP.

Potential scenario 2: The expression of the CIBN anchor is normal. If this is the case, try increasing the laser intensity used for stimulation.

Problem 5

A milder degree of Rho1 inhibition is desired (step 15f or step 16g).

Potential solution

This can be achieved by lowering the expression level of the optogenetic module. Several ways could help with reducing the expression of optogenetic module, such as using a weaker GAL4 driver line, reducing the copy number of GAL4, or co-expressing a conditional GAL4 inhibitor protein. In addition, when the maternal GAL4 line 67; 15 are used in the experiment, store the fly cup at 25°C instead of 18°C would reduce the expression of the optogenetic module. When modulating the expression level of the optogenetic module, keep in mind that the efficiency of the tool and the expression level might not be linearly correlated. Insufficient expression of the optogenetic module could result in lack of detectable Rho1 inhibition (e.g., lack of effect on actomyosin contractility).

A milder level of membrane recruitment of the CRY2-Rho1DN can also be achieved by using a lower intensity of blue laser for stimulation. However, this approach cannot be used if imaging GFP-tagged proteins is desired in the post-stimulation acquisition step, since the laser intensity normally required to excite GFP (488-nm laser on confocal microscopes or 920-nm laser on multiphoton microscopes) to obtain a reasonable signal intensity is typically much higher than the laser intensity sufficient to cause robust simulation of Opto-Rho1DN.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bing He (bing.he@dartmouth.edu).

Materials availability

Optogenetic flies generated in this study are available by contacting the lead contact. All other materials can be purchased online, including headlamp with red light, orange-red light filter, light-proof box and black cloth.

Data and code availability

This study did not generate code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101972.

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AUTHOR CONTRIBUTIONS

H.G. and B.H. designed and performed the experiments. M.S. designed the optogenetic tool. H.G. and B.H. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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