Published in final edited form as:

Methods Mol Biol. 2020 ; 2108: 281–293. doi:10.1007/978-1-0716-0247-8_24.

Optogenetics: Rho GTPases Activated by Light in Living Macrophages

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Abstract

Genetically encoded optogenetic tools are increasingly popular and useful for perturbing signaling pathways with high spatial and temporal resolution in living cells. Here, we show basic procedures employed to implement optogenetics of Rho GTPases in a macrophage cell line. Methods described here are generally applicable to other genetically encoded optogenetic tools utilizing the blue-green spectrum of light for activation, designed for specific proteins and enzymatic targets important for immune cell functions.

Keywords

Rho GTPases; Optogenetics; Photoactivatable proteins; LOV2

1 Introduction

Optogenetic manipulation of living cells during imaging experiments enables perturbation of a pathway while in direct observation of the resulting cellular behavior. This is a powerful approach because the specific perturbation and the resulting modulation of the cellular phenotype can be quantified immediately and continuously in single cells during live

imaging. In addition to optogenetic perturbations, recent advances in fluorescent biosensors for detecting protein activations and posttranslational modification statuses could be used either in combination within a set of imaging studies or directly and concurrently with optogenetic perturbations to provide researchers with the ability to “probe” and “perturb” in living cells for the first time. The most common tools available in the field thus far have been based on the light-oxygen-voltage (LOV) 2-J α system [1–8], cryptochrome-2 (CRY2), and channelrhodopsins [2, 9]. These tools require blue-green light for photoactivation, and exhibit different quantum efficiencies and half-lives of light-based conformational switching as the key mechanism of these optogenetic applications.

In our works, we have focused on studying the Rho family GTPase functions in living cells, including breast tumor cells [10–13] and macrophages [14–16], and applied optogenetic tools to directly perturb pathways important in cell invasion and motility [12]. Here, we outline a procedure for performing optogenetic imaging experiments in macrophage cell line, using photoactivatable (PA)-GTPase system [4]. We also note that approaches outlined here can be generalized to most optogenetic systems which can be activated by the blue-green spectrum of light, targeting Rho GTPase pathways. These approaches could include multicomponent dimerizer systems such as the LOVTRAP [1, 17], and the extrinsic order/disorder-based optogenetic systems wherein LOV-domain is directly inserted into the catalytic interface of a molecule to modulate enzymatic function [18]. In this report, we will present a primary application example of the PA-Cdc42 system in a macrophage cell line.

2 Materials

2.1 Cell Culture and Transfection

1. Cell lines: RAW/LR5 monocyte/macrophage cell line [19] (*see* Note 1).
2. RPMI medium with L-Glutamine 300 mg/L, supplemented with 10% newborn calf serum (NCS), penicillin (100 IU)/streptomycin (100 μ g/mL).
3. Opti-MEM medium.
4. Plasmids: The photoactivatable GTPases (PA-Cdc42, PA-Rac1) [4] and GEFs [17, 18] in pTriEX-backbone or in other mammalian expression plasmids. Empty pCDNA or other appropriate empty mammalian expression plasmid which will be used as a transfection control. Appropriate control mutant versions of PA-GTPase, including C450A mutant (dark mutant). PA-GTPases [4] and PA-GEF based on LOVTRAP [17] or direct insertion of LOV2 in the GEF-domain [18], are available from Addgene.
5. FuGENE HD transfection reagent.
6. DPBS, calcium and magnesium free: 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl, 1.15 g/L Na₂HPO₄ (anhydrous).
7. 10 mM EDTA in DPBS, made from diluting 0.5 M EDTA (pH 8) in DPBS.
8. 6-Well tissue culture plates.
9. 12-Well tissue culture plates.

10. 15 and 50 mL polypropylene tubes.
11. 6 and 10 cm cell culture dishes.

2.2 Imaging Experiments

1. 25 mm Round coverslips #1.5 thickness.
2. BWD buffer [15, 19]: 20 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM NaHCO₃, 1 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4.
3. BWD buffer containing 5% fetal bovine serum (FBS).
4. Live cell imaging chamber [11, 20, 21], Attofluor chamber system (Invitrogen) or other compatible live-cell chamber system that can accommodate 25 mm coverslips, or CellView glass bottom culture dishes (Greiner-Bio-one), or Mattek dishes ([Mattek.com](https://www.mattek.com)).
5. Mineral oil.
6. 200-proof ethanol.
7. Fluorescence inverted microscope capable of timelapse fluorescence imaging, fitted with photoactivation illuminator (IX2-RFAW: Olympus) (*see* Note 2) (Fig. 1).
8. Optionally, a fluorescence inverted microscope capable of timelapse imaging of Förster resonance energy transfer (FRET) biosensors [21], fitted with photoactivation illuminator (IX2-RFAW: Olympus).
9. Bandpass filter for photoactivation of LOV2 domain: FF01-466/40–25 (Semrock) (Fig. 2a).
10. Custom dichromatic notch filter for the photoactivation illuminator system: ZT457DCRB, 3 mm thickness (Chroma Technology) (Fig. 2b).
11. 16 mm Round, mounted pin-hole inserts of various sizes for the fluorescence microscope (Melles Griot).
12. Optionally, an Olympus Fluoview1000 multi-photon microscope system.

3 Methods

3.1 Transient Transfection of Optogenetic Components in RAW/LR5 Cells

For transfection of optogenetic components, individual optimizations of transfection conditions are required to achieve best results. As an example, we will use our protocol for the PA-Cdc42 (*see* Note 3) [4].

1. (Day 1) Plate RAW/LR5 cells in a 12-well plate the day before transfection so that they will be approximately 60–80% confluent for transfection the next day.
2. (Day 2) Let FuGENE HD (*see* Note 4) and OptiMEM come to room temperature (RT), ~10 min.

3. Prepare FuGENE HD transfection mix (DNA:FuGENE HD ratio 1:3). In 100 μ L of OptiMEM, add 1 μ g PA-Cdc42 (*see* Notes 5 and 6), vortex for 10 s; add 3 μ L of FuGENE HD, and pipet to mix (do not vortex). Incubate at RT for 15 min. The transfection mix should be scaled up or down based on surface area of the tissue culture vessel, if different cell numbers are required.
4. While the transfection mix incubates, rinse cells once with DPBS and add 500 μ L of complete medium for transfection.
5. Add transfection mix dropwise to the plate. Swirl gently to mix.
6. Incubate the plate for 2–3 h (*see* Note 7) at 37 °C and 5% CO₂.
7. After incubation, transfer the medium from cells to a 15 mL tube to collect cells that may have detached during the incubation. Then lift adherent cells by adding 10 mM EDTA/DPBS to the well and incubate for 5 min at 37 °C and 5% CO₂. Tap gently to lift cells and transfer to the same 15 mL tube. Rinse the dish once with complete medium to collect all cells and add again to the 15 mL tube. Spin cells at $300 \times g$ for 3 min.
8. Aspirate the supernatant and resuspend the cell pellet in 2 mL of complete medium.
9. Set up sterile 25 mm round coverslips in 6-well plate (*see* Note 8). Plate at 4×10^4 cells/coverslip (*see* Note 9). Alternatively, Mattek or CellView chambers can be used.
10. Let cells recover on the coverslips overnight at 37 °C and 5% CO₂.
11. (Day 3) Next day, set up for optogenetic experiments.

3.2 Imaging and Optogenetic Activation of PA-GTPase

For live cell imaging and optogenetic activation, optimization of illumination condition is required to achieve the best response.

1. Mount the coverslips containing cells onto live-cell imaging chamber (Attofluor chamber or the sealed chamber system) [21]. Alternatively, CellView or Mattek dishes can be used as well, and add BWD buffer with 5% FBS to sufficiently fill the chambers (*see* Note 10).
2. Using a 60 $\times$ magnification objective lens (60 $\times$ DIC N/A 1.45), set up for Köhler illumination and differential interference contrast (DIC) imaging.
3. In a cell-free field of view, open the photoactivation shutter momentarily and determine the location of the illumination spot. If the location of the spot is suboptimal (i.e., not well centered in the field of view), move the pin-hole by adjusting the positional adjustment screws at the photoactivation illuminator. Once the spot is centered in the field of view, mark this spot using the region tool and save such a region so that it can be loaded onto the image during image acquisition (*see* Note 11).

4. Observe and quickly identify in mVenus (515 nm excitation, 528 nm emission) fluorescence, cells positive for PA-Cdc42 expression after transfection (*see* Notes 12 and 13).
5. Set up for timelapse imaging in DIC (*see* Note 14). It is not necessary to image mVenus in the timelapse sequence as this is only a marker of expression of PA-Cdc42.
6. Locate an appropriate cell in the field of view, load the region that indicates the location of the photoactivation spot, and then move the stage so that the cell is appropriately moved into the photoactivation spot (*see* Note 15).
7. Start the timelapse experiment, usual frame rate for RAW/LR5 cells is 10 s intervals or shorter. Establish a stable basal condition by imaging for some time without photoactivation (*see* Note 16).
8. Photoactivation is initiated by pulsing the photoactivation light at 457 nm on/off at specific intervals (*see* Notes 17–20) (Fig. 3).

4 Notes

1. RAW/LR5 cell line can be readily made to express FRET biosensors for Rho GTPases [15, 22], including those that fluoresce in near infrared (NIR) [23]. This is advantageous because NIR FRET biosensor can be used together with other optogenetic tools that require blue/green light for photo-uncaging [23]. The description of biosensors based on CFP-YFP FRET and NIR FRET systems is beyond the scope of this work, but RAW/LR5 cells stably expressing the tet-OFF tTA are available [15]; thus, inducible NIR-FRET biosensor cell line can be readily produced following previously published [22] protocols.
2. If interested in imaging FRET biosensor measurements at the same time as performing optogenetic manipulation, the microscope should be fitted with optimized imaging components for biosensor measurements, in addition to the photoactivation module for the optogenetic tools [21].
3. PA-Cdc42 as published in [4] contains an F56W mutation. This mutation is found in the β 3 sheet in front of Switch II domain important for effector binding. The analysis in [4] revealed that tryptophan at this location was required to form a stable salt-bridge with the LOV2-J α in the dark-state. This enhanced the stability of the caged state of PA-Cdc42. Rac1 natively contains a tryptophan at this location. A caveat, as noted in [4], is that because of this Rac1-like mutation in PA-Cdc42, the downstream effector signaling may be biased toward the Rac1-like pathway as opposed to Cdc42. Indeed, we find that repeated photoactivation of PA-Cdc42 in macrophages leads to cell protrusions that are more lamellipodia-like. Thus, users are cautioned to note the potential consequences of these mutations that may be present in these constructs which could produce spurious effects.

4. FuGENE HD and Opti-MEM must be at room temperature, or transfection efficiency will be significantly impacted.
5. Macrophage cell lines are challenging to transfect in general. We have had the most success with FuGENE HD, giving a transfection efficiency of approximately 10%. However, the transfection efficiency also varies greatly depending on the cDNA. The amount of DNA and the DNA:FuGENE HD ratio have been optimized for RAW/LR5 cells. For other cell types, DNA and FuGENE HD amounts should be optimized.
6. PA-Rac1 and PA-Cdc42 require optimization of expression levels. Too much expression leads to constitutive activation and loss of optogenetic control. Therefore, we advise that the amount of DNA should be titrated down, using an empty pCDNA to maintain the same amount of DNA transfected at all times. LOVTRAP-GEF system also requires optimization of transfection ratios. We have found that 10:1 ratio of pTriEX-N-TOM20-LOV2 and pTriEX-mVenus-Zdk1-VAV2 DHPHC1 [1, 17] produces good optogenetic activation in living cells.
7. Overnight incubation of the cells increases transfection efficiency, while cell health is adversely affected and pre-activation can also be a problem. For a simple protein expression analysis an overnight incubation is tolerable. However, for downstream cellular assays we routinely opt for shorter transfection times, in the order of 2–3 h.
8. Keep stock of 25 mm round coverslips in 200-proof ethanol and flame them to sterilize.
9. 4×10^4 cells/coverslip is a starting cell density for imaging. Optimal plating density for imaging should be determined individually.
10. In the case of open chamber systems, mineral oil can be layered onto the BWD medium to protect it from evaporation and gas exchange. The sealed chamber system does not require the oil because it uses a clean coverslip to seal the upper side of the chamber [21].
11. The pin-hole size at the specimen plane can be calculated by the following equation:

$$\text{Exposed area on the specimen} = (\text{Pin-hole diameter})/(\text{objective magnification})$$

We are using a 200 μm diameter pin-hole insert. This translates to 3.33 μm diameter activation spot at the specimen plane under 60 $\times$ magnification. We found this size to be ideal for targeting relatively small cells such as macrophages, or small structures within larger cells such as the invadopodium in breast tumor cells [12]. Users should determine the best geometries of optogenetic activation conditions based on the size and shape of the targeted structures or the size of the targeted cells.

12. PA-Cdc42 was originally produced with mVenus as the expression tag, and PA-Rac1 with mVenus or mCherry [3]. We have changed the expression marker to mtagBFP2 for PA-Rac1 to make it compatible with fluorescence imaging conditions that require red fluorescence for other cellular markers [12].
13. Fluorescence excitation of mVenus (515 nm) will cause photoactivation of PA-GTPases that are tagged with mVenus. Thus, observations in the mVenus channel (or EGFP channel can be used as well) should be kept to an absolute minimum, just enough to quickly find cells positive for transfection. Once a cell is found, immediately switch to DIC and perform adjustments for cell placement within the field of view to target the uncaging spot in the field of view. This problem can be circumvented by switching the tag to mtagBFP2 and exciting it at 395 nm, or mCherry (585 nm) if longer wavelengths can be used. Trans-illumination light train for DIC may include a green filter by default. This should be removed and ideally replaced with a bandpass filter in red. We use a 632/32 nm bandpass glass filter for DIC imaging.
14. The choice of fluorescence wavelengths available for timelapse imaging will be limited by the requirement for PA-GTPase uncaging.
15. The location of photoactivation depends on the function of the GTPase that you want to study. In the example given in this section, we placed the spot at the edge of a cell to induce edge protrusions. In other examples in different cell type, we have placed the spot at intracellular structure that is away from the edge [12]. Therefore, adjusting the size of the pin-hole and its location becomes very important to target signaling events at specific locations.
16. We routinely observe cells for approximately 5–15 min prior to start of the photoactivation to establish a stable pre-activation baseline response.
17. Because the wild-type PA-GTPase has a 1/2-life of relaxation of approximately 43 s [3], we cycled the illumination of 1 s at 457 nm light followed by 30 s of dark state. By repeating this cycle, we achieve continuous activation of PA-GTPase [12]. It is advisable, however, to optimize this condition appropriately depending on the experimental requirements. In the case of the LOVTRAP-GEF experiments [23], we have instead irradiated the whole field of view using continuous irradiation as the 1/2-life of the LOVTRAP system based on the wild-type LOV2 was shorter (18.5 s for return to mitochondria) [1, 17]. To control the cycling of the activation light, a script can be written in Metamorph software which controls the microscope system, to automate this process during a timelapse experiment.
18. Control experiments with a dark mutant of PA-GTPase (C450A), unable to be photoactivated, should be performed under similar conditions to ascertain that the phenotype being observed is specific to optogenetic uncaging of PA-GTPase [4]. Additional control experiments could include: (a) photoactivation within regions away from the targeted zones of interest (e.g., the leading edge, cell

body, vs. nucleus); and (b) photoactivation of cells without expression of the optogenetic components to determine the effects of the light illumination.

19. PA-GTPase can be activated using multi-photon microscopy modality irradiating at 915 nm light (Fig. 4). This could be a useful approach to optogenetically target macrophages in in vivo imaging experiments.
20. Optogenetic activation of Rho-targeting GEF via LOVTRAP [1] can be used together with a compatible FRET biosensor that utilizes near-infrared fluorescent proteins [23]. This method allows direct observation of the perturbation of Rho GTPase activity during blue-green optogenetic perturbation of an upstream signal regulator (Fig. 5).

Acknowledgements

This work was supported by National Institutes of Health grant T32GM007491 to V.M., R01GM129098 and R01GM132098 to L.H. National Cancer Institute P30CA013330 for Analytical Imaging Facility support. Irma T. Hirschl Career Scientist Award to L.H. pTriEX-mVenus-PA-Cdc42 (Addgene #75263), pTriEX-mVenus-PA-Rac1 (Addgene #22007), pTriEX-PA-Rac1 C450R (Addgene #22025), pTriEX-NTOM20-LOV2 WT (Addgene #81009) and pTriEX-mVenus-Zdk1-VAV2 DH/PH/C1 (Addgene #81133) were gifts from Dr. Klaus Hahn (UNC-Chapel Hill).

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Fig. 1.

Schematic drawing of the inverted microscope used for live cell imaging and optogenetic activation of Rho GTPases. The microscope as depicted is configured for simultaneous CFP-YFP FRET biosensor imaging and DIC/red/far-red/near-infrared imaging setup. Filterwheel 1: switches the neutral density filters; Filterwheel 2: switches the excitation band-pass filters; Filterwheel 3: switches the emission band-pass filters. Microscope is equipped with ZeroDrift Compensation (ZDC) mechanism (Olympus) utilizing a 794 nm laser source, illuminating the specimen plane via an Olympus 780/30 nm notch filter. Photoactivation of PA-GTPase is performed via a pin-hole and through a 466/40 nm band-pass filter. This is combined with the main fluorescence excitation light train via a notch filter centered at 457 nm. The main fluorescence turret of the microscope contains a custom 80/20 (transmittance/reflection) mirror (Chroma). Details of the construction and specification of this microscope can be found elsewhere [21]

Fig. 2.

(a) Transmittance specification of the FF01-466/40-25 mm filter (Semrock) used for photoactivation of LOV2-Ja-based optogenetic systems. (b) Transmittance specification of the notch filter centered at 457 nm (Chroma) used to mix the photoactivation light into the main excitation light train

Fig. 3.

(a) Schematic cartoon of a PA-GTPase. A constitutively active mutant of Rho GTPase is caged by LOV2-J α in the dark state. Upon irradiation by 457 or 473 nm light, conformational shift within the LOV2-J α reversibly uncages the activated GTPase, targeting downstream effectors. (b) DIC images of a macrophage transiently transfected with PA-Cdc42 and photoactivated within a 3.3 μ m area indicated by a red circle. *PA* photoactivation. White arrows indicate protrusions. Kymograph was measured along a line drawn perpendicular to the leading edge (not shown). Scale bar = 5 μ m

Fig. 4.

Photo-uncaging of PA-Rac1 in mouse embryonic fibroblasts (MEF) using a two-photon excitation at 915 nm, shown as an example. **(a, c)** Without photoactivation; **(b, d)** with photoactivation. Global uncaging excitation was used to image the bright-field. MEFs stably expressing the PA-Rac1 were imaged on an Olympus Fluoview1000 multi-photon system. Black arrows indicate locations of lamellipodial protrusion as a function of photouncaging at 915 nm. Kymographs show the changes in edge motion (red arrow head, location of the edge) with and without two-photon-uncaging. White bar in **(a, b)** 20 μm and in **(c, d)** 5 μm

Fig. 5.

Concurrent measurement of Rac1 activity during LOVTRAP optogenetics, reproduced from Shcherbakova et al. 2018 Nature Chemical Biology [23]. (a) Schematic drawing of the LOVTRAP system. Mitochondrially targeted mtTagBFP-LOV2wt sequesters the GEF-domain of interest attached to Zdk1 molecule. Upon photoactivation with 457 nm light, Zdk1-GEF is released and acts on the target GTPase, followed by dark relaxation upon cessation of illumination. (b) An example panel of a MEF undergoing photoactivation of LOVTRAP and concurrent measurements of Rac1 activity using the NIR-Rac1 biosensor. NIR-Rac1 biosensor image sets were acquired every 10 s. 457 nm illumination (cycles of 4 s-on, 6 s-off) was started at 300 s and ended at 600 s time points. White bar = 20 μm. Pseudocolor limits are 1.0 to 1.74 (black to red). (c) Quantification of Rac1 activity measured concurrently with the LOVTRAP-TrioGEF and -VavGEF photoactivation. During the “on” phase of the 457 nm illumination, Rac1 activity levels are significantly elevated

compared to the control which received no 457 nm illumination. $n = 17$ independent photoactivation experiments for LOVTRAP-TrioGEF, $n = 10$ independent photoactivation experiments for LOVTRAP-VavGEF, $n = 10$ independent mock-photoactivation experiments for the control condition, all shown with mean $\pm$ SEM. The original Vav2 GEF LOVTRAP results in slower decay of Rac1 activity during the dark relaxation compared to the TrioGEF LOVTRAP, pointing to differential ability of GEFs to modulate Rho GTPase activities, $**p < 0.01$

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