Chapter 25

Protein Inactivation by Optogenetic Trapping in Living Cells

Hyerim Park, Sangkyu Lee, and Won Do Heo

Abstract

Optogenetic modules that use genetically encoded elements to control protein function in response to light allow for precise spatiotemporal modulation of signaling pathways. As one of optical approaches, LARIAT (Light-Activated Reversible Inhibition by Assembled Trap) is a unique light-inducible inhibition system that reversibly sequesters target proteins into clusters, generated by multimeric proteins and a blue light-induced heterodimerization module. Here we present a method based on LARIAT for optical inhibition of targets in living mammalian cells. In the protocol, we focus on the inhibition of proteins that modulate cytoskeleton and cell cycle, and describe how to transfect, conduct a photo-stimulation, and analyze the data.

Key words LARIAT, Protein inhibition, Optogenetics, Cryptochrome 2 (CRY2), CIB1, Clustering, Cytoskeleton, Cell division

1 Introduction

Perturbation of specific protein activity in a complex signaling network is an important step for elucidating many cellular, developmental, and physiological processes. Even though genetic approaches such as knockout and knockdown have been widely used as powerful tools to identify specific functions of proteins, yet they typically require a relatively long time to see the effects or can have lethal effects on embryonic development [1, 2]. At protein level, conditional approaches such as small molecule-based inhibition or targeted degradation have been utilized to control protein activities posttranslationally [3–5]. However, these techniques suffer from intrinsic limitations, such as intricate design needs for each target protein, low reversibility, off-target effects, and poor spatial resolution. Development of optogenetic tools that use genetically encoded light-sensitive proteins to control protein activities have offered opportunities to overcome these drawbacks [6–9]. These tools allow to modulate signaling pathways with rapid response, good reversibility, and high spatiotemporal resolution in mammalian cells.

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Unlike most optogenetic modules developed for protein activation, LARIAT (Light-Activated Reversible Inhibition by Assembled Trap) is a versatile method for inhibiting protein function by reversible sequestering target proteins into optically assembled clusters in living cells [10]. LARIAT utilizes multimeric proteins (MPs) and a light-mediated heterodimerization between Cryptochrome 2 (CRY2) and CIB1 [8]. The light-activated CRY2 proteins simultaneously oligomerize [9] and interconnect with CIB1-MPs through CRY2-CIB1 interaction, thereby inducing cluster formation. These clusters would serve as synthetic intracellular compartments to conditionally trap and consequently inactivate target proteins upon blue-light illumination (Fig. 1). In addition, utilizing a single-domain antibody (called "nanobody") against the intracellular green fluorescence protein (GFP) [11], we extend application of LARIAT system to inactivate various GFP-tagged proteins. Here we present a protocol to spatiotemporally control protein functions exemplified by inactivation of Vav2 and Tubulin which are involved in cell migration and mitosis, respectively. We divided this protocol into two categories: (1) inhibition of Vav2 involved in reorganization of actin cytoskeleton and (2) disruption of microtubule structure during mitosis.

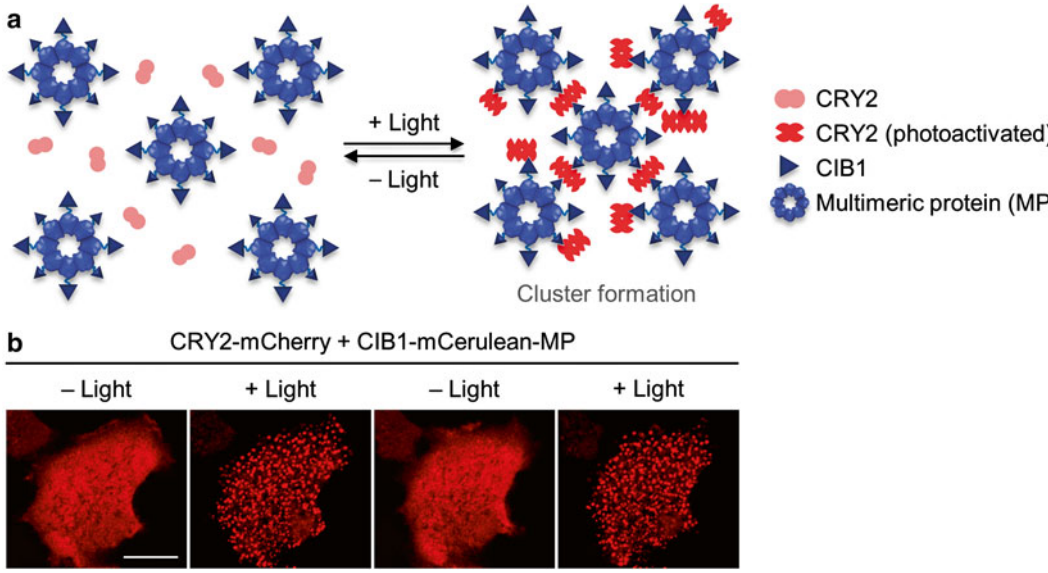

Fig. 1 Blue light-mediated cluster formation. (**a**) Schematic diagram of LARIAT. Photoactivated CRY2 binds to CIB1 conjugated with multimeric protein (MP) and induces the formation of large clusters by interconnecting CIB1-MPs. (**b**) Reversible cluster formation in a HeLa cell co-expressing CRY2-mCherry and CIB1-mCerulean-MP illuminated with 488 nm. Scale bar, 20 μm. It is reproduced with permission from *Nature Methods*

2 Materials

2.1 Plasmids (See Note 1)

1. LARIAT: Two strategies—direct conjugation of CRY2 to targets and use of CRY2-fused anti-GFP nanobody—can be applied to inhibit targets (Fig. 2).

(A) Components for inhibition of CRY2-fused target proteins

1. CIB1-mCerulean-MP (Addgene; plasmid 58366, *see* **Note 2**).
2. mCitrine-CRY2-Vav2 (*see* **Note 3**).

(B) Components for inhibition of GFP-labeled target proteins

1. CIB1-CLIP-MP (*see* **Note 4**).
2. SNAP-CRY2-V_HH(GFP) (Addgene; plasmid 58370).
3. Target proteins: EGFP-Vav2, EGFP-αTubulin (*see* **Note 5**).

(C) Fluorescence readout proteins to monitor inhibitory effect

1. mCherry-Lifeact to monitor reorganization of actin cytoskeleton [12].
2. mCherry-H2B to monitor cell cycle.

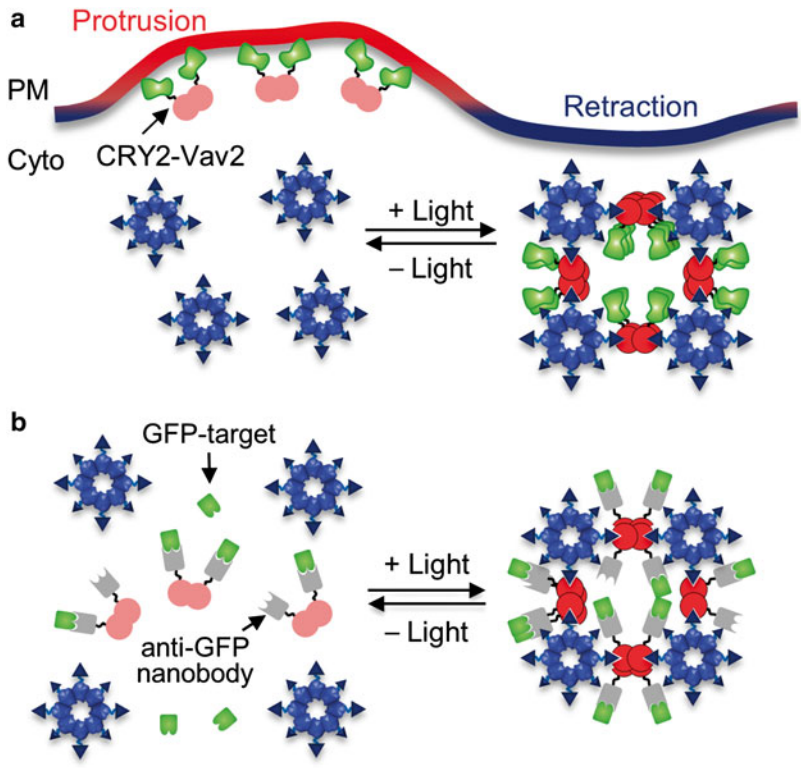

Fig. 2 Two strategies of LARIAT to inhibit target proteins. (**a**) Inhibition of CRY2-conjugated target proteins (CRY2-Vav2) by light-inducible trapping in clusters. (**b**) Inhibition of GFP-labeled target proteins by trapping via CRY2-conjugated anti-GFP nanobody (V_HH(GFP). It is reproduced with permission from *Nature Methods*

2.2 Cell Culture and Transfection

1. Plates: 96-well plastic bottom plates (ibidi), T75 flasks.
2. Tubes: 1.5-mL microcentrifuge tubes, 15-mL conical tubes.
3. Cells: HeLa cell, NIH3T3 cell.
4. Culture medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). Stored at 4 °C.
5. 0.25 % Trypsin-EDTA. Stored at 4 °C.
6. Dulbecco's Phosphate Buffered Saline (DPBS).
7. Opti-MEM (GIBCO). Stored at 4 °C.
8. 100 μg/mL poly-D-lysine solution (PDL, Sigma): dilute 5 mg of PDL in 50 mL deionized water. Stored at 4 °C.
9. Lipofectamine LTX with Plus Reagent (Invitrogen). Stored at 4 °C.
10. Neon Transfection System (Invitrogen): Neon Transfection Device, Neon Pipette, Neon Pipette Station.
11. Neon Transfection System 10 μL Kit (Invitrogen): 10 μL Neon Tips, Neon Tubes, Resuspension Buffer R, Electrolytic Buffer E. Store the remaining buffers at 4 °C.
12. Hemocytometer.
13. CO_2 incubator with 37 °C and 10 % CO_2.

2.3 Microscope

1. Nikon A1R confocal microscope mounted onto a Nikon Eclipse Ti body.
2. Objectives: 60× Plan Apochromat VC objective (Nikon), 40× Plan Fluor objective (Nikon).
3. Lasers: Nikon Multi-line Argon Laser for 457, 488, and 514 nm, Coherent Sapphire Solid Laser 561 nm (*see* **Note 6**).
4. Chamlide TC system (Live Cell Instrument): Stage-top incubator, lens warmer, humidifier, and controller placed on a microscope stage.
5. Immersion oil (Carl Zeiss).
6. Optical power meter (ADCMT).
7. Image analysis software: NIS-Elements (Nikon, ver 4.1), MetaMorph (Molecular Devices, ver 7.8), Microsoft Excel.

3 Methods

3.1 Reorganization of Actin Cytoskeleton by Inactivating Vav2

3.1.1 Cell Transfection with a Lipofectamine LTX

1. Add 100 μL of 100 μg/mL poly-D-lysine solution to wells of 96-well plate and incubate 30 min at 37 °C. Discard the remained solution and wash the wells three times with DPBS and allow the plate to dry at room temperature (RT).
2. Prepare the NIH3T3 cells that are ~80 % confluency in a T75 flask. Trypsinize and count the cells.

3. Plate the cells on the coated wells at a density of 1.5×10^4 cells per well in 200 μL of complete culture medium. Incubate overnight at 37 °C supplemented with 10 % CO_2.
4. Dilute 200 ng total amount of plasmid DNA into 20 μL of Opti-MEM in a 1.5-mL microcentrifuge tube (*see* **Note 7**). Add 0.2 μL Plus reagent in the tube (*see* **Note 8**). Mix well by pipetting and incubate at RT for 5 min.
5. Dilute 0.6 μL Lipofectamine LTX into the DNA solution (*see* **Note 9**). Mix well by pipetting and incubate at RT for 25 min.
6. Meanwhile, replace the media in the wells to 100 μL of new complete media.
7. Add 20 μL of DNA-Lipofectamine LTX mixture dropwise into the well and incubate overnight at 37 °C and 10 % CO_2.

3.1.2 Imaging and Photo-stimulation

1. At least 10 min before imaging, switch on a Chamlide TC system to maintain environmental condition in 37 °C and 10 % CO_2. Just before imaging, replace the medium with 200 μL of prewarmed Opti-MEM. Place the 96-well plate on the plate chamber.
2. Select appropriate channels for imaging specific fluorescence signals and find the cells via observing mCherry signals with a 60× oil-immersion objective (*see* **Note 10**).
3. For photo-stimulation, acquire a mCherry image first, and draw a ROI area where the photo-stimulation is to be applied. Select this ROI as "Used as Stimulation ROI" to designate a ROI area as the photo-activation area.
4. Designate 488-nm laser as stimulating light source and select laser power and illumination time (*see* **Note 11**).
5. Press the "Photo Activation" button and set the photo-activation experiment sequence to suit specific imaging needs such as Fig. 3 (*see* **Note 12**).
6. Click the "Apply Stimulation Settings" button to send the photo-activation area information and click the "Run now" button to start monitoring.
7. After the imaging is finished, take the images with other fluorescence channels to validate expression levels of CRY2- and CIB1-fused proteins.

3.1.3 Image Analysis

1. For analyzing membrane dynamics, threshold the image based on the mCherry-Lifeact signal to mask the whole cell area using "Automated Measurement" tool in the NIS-Element software and apply the threshold on all frames of the movie (*see* **Note 13**).
2. Measure the area of binary images using "ROI statistics" tool. Export raw data to Microsoft Excel software and plot the area change curve (Fig. 4a, b).

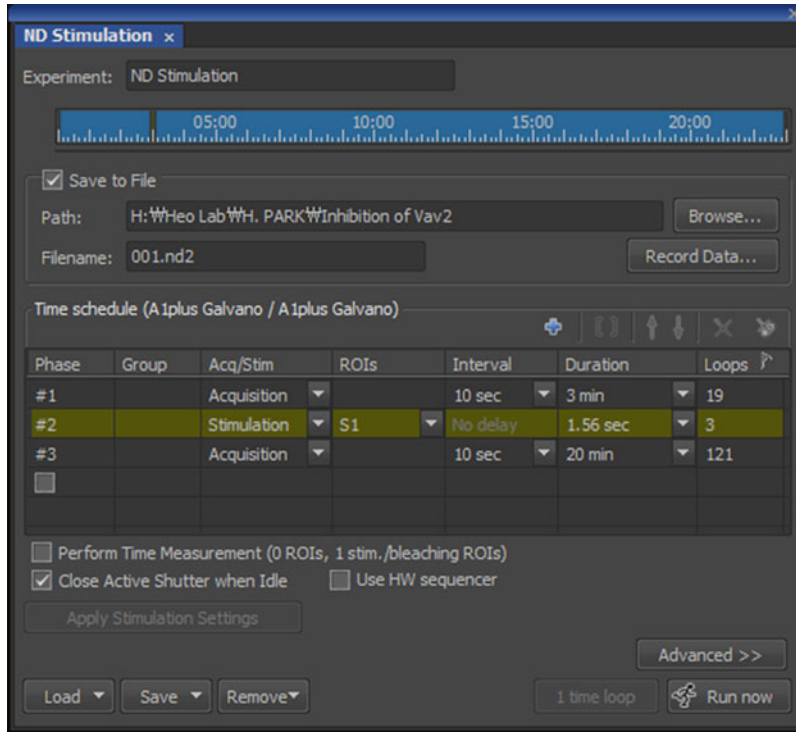

Fig. 3 Photo-activation experiment sequence for inhibition of Vav2. Interface of NIS-element AR software to set up a condition (time intervals and duration) of photo-stimulation and imaging

3. For generating kymograph, select the "Create Kymograph by Line" and draw a line across the cell body (in direction from cytoplasm to edge of the plasma membrane) (Fig. 4c).
4. For protrusion and retraction analysis, threshold the image based on the mCherry-Lifeact signal to cover the whole cell area using "Threshold Image" tool on "Measure" tap in the MetaMorph software. Select "Binary Operations" on "Process" tap and convert the mCherry images of before and after light stimulation to binary images.
5. To obtain retraction image, subtract the binary image of "after light illumination" from that of "before light illumination" using "Arithmetic" tool on "Process" tap.
6. To obtain protrusion image, subtract the binary image of "before light illumination" from that of "after light illumination" using "Arithmetic" tool.
7. Operate the "Logical AND" with the two binary images to generate image showing areas that overlapped right before stimulation.
8. Using "Color Combine" tool, combine the images presenting each regions to generate protrusion and retraction map.

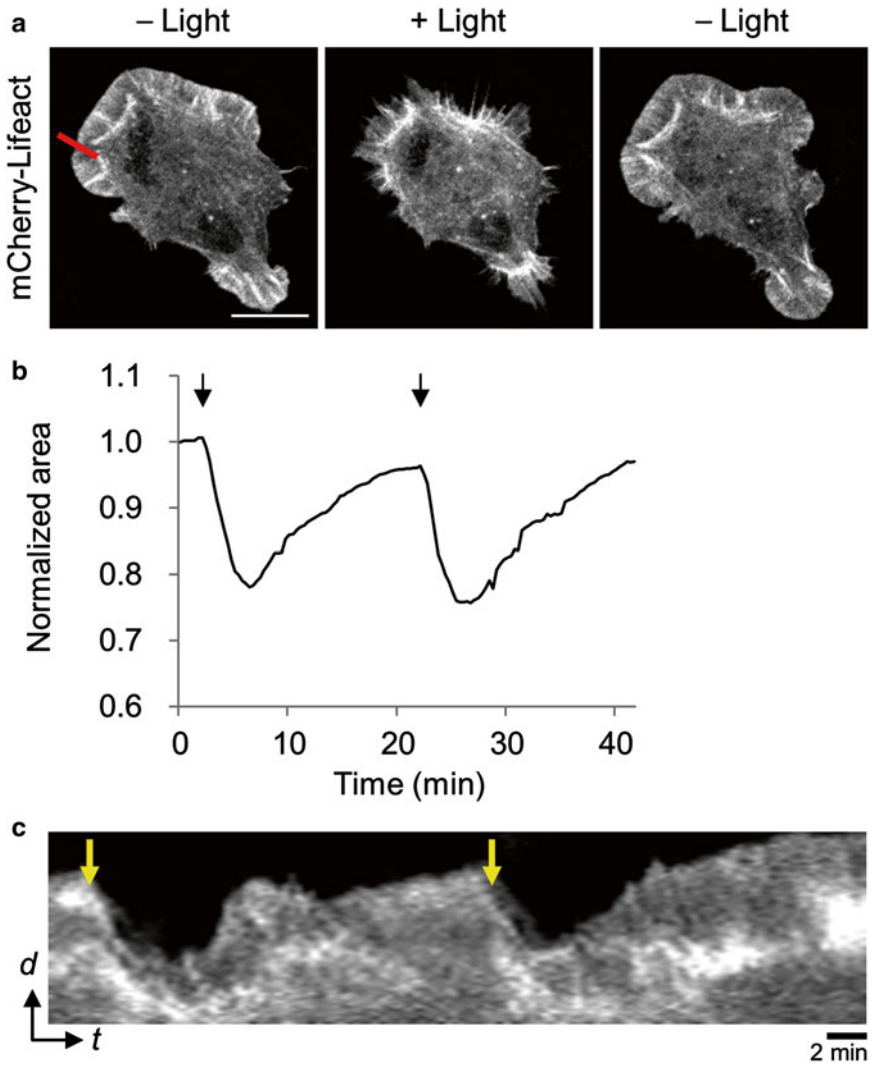

Fig. 4 Inactivation of CRY2-conjugated Vav2 by LARIAT. (**a**) Fluorescence images of a retraction of NIH3T3 cell co-expressing mCherry-Lifeact, mCitrine-CRY2-Vav2, and CIB1-mCerulean-MP upon repeated light illumination with 20-min interval. (**b**) Time-lapse measurements of cell areas. (**c**) Kymograph of Lifeact corresponding to the *red line* in panel **a**. *d*, distance; *t*, time. Scale bars, 20 μm. It is reproduced with permission from *Nature Methods*

3.2 Inhibition of Cell Cycle

3.2.1 Cell Transfection with a Neon Transfection System

1. Fill the Neon Tube with 3 mL of prewarmed Buffer E and insert into the Neon Pipette Station until having a click sound. Set the desired pulse conditions as 980 V for Voltage, 35 ms for Width, and 2 pulses for Pulses, in case of HeLa cell.
2. Prepare two 1.5-mL microcentrifuge tubes. Transfer 600 ng total amount of plasmid DNA (200 ng of SNAP-CRY2-V_HH(GFP), 200 ng of CIB1-CLIP-MP, 120 ng of EGFP-αTubulin, and 80 ng of mCherry-H2B) in one tube and 810 μL of complete culture medium in another tube.

3. Prepare the HeLa cells that are ~80 % confluency in a T75 flask. Trypsinize and count the cells.
4. Transfer 2×10^5 cells to a new 1.5-mL microcentrifuge tube and centrifuge at 7000 rpm for 0.5 min at RT. Discard the medium and wash the cell pellet with 100 μL of DPBS by centrifugation at $4500 \times g$ for 0.5 min at RT.
5. Discard the DPBS and resuspend the cell pellet with 11 μL of Resuspension Buffer R. Gently pipette the cells to avoid air bubbles (*see* **Note 14**). Transfer 10.8 μL of resuspended cells into the tube containing plasmid DNA and mix by pipetting.
6. Fill a 10 μL Neon Tip with the cell-DNA mixture. Insert the Neon Pipette with the sample into the Neon Tube placed in the Neon Pipette Station until having a click sound. Press Start button on the screen (*see* **Note 15**).
7. After "Completed" is displayed on the screen, transfer the samples from the Neon Tip into the prepared tube containing the complete culture medium.
8. Mix by pipetting and transfer 200 μL of the medium containing electroporated cells into each wells of a 96-well plate. Incubate the plate overnight at 37 °C and 10 % CO_2.

3.2.2 Imaging and Photo-stimulation

1. At least 10 min before imaging, switch on a Chamlide TC system was used to maintain environmental condition in 37 °C and 10 % CO_2. Just before imaging, replace the medium with 200 μL of prewarmed Opti-MEM. Place the 96-well plate on the plate chamber.
2. Select the 488-nm laser for observation of EGFP signals and photo-stimulation and the 561-nm laser for imaging mCherry signals. Find the cells via observing mCherry signals with a 40× objective.
3. For multipoint and time-lapse acquisition, add the positions which the acquisition is to be applied.
4. Set time-lapse parameters such as intervals and duration (*see* **Note 16**).
5. Set laser power of 488 nm to 65 μW/mm^2 for photo-stimulation and adjust laser power of 561 nm for monitoring mCherry-H2B.
6. Simultaneously, acquire images with both 488 and 561 nm by clicking the "Run now" button (Fig. 5) (*see* **Note 17**).

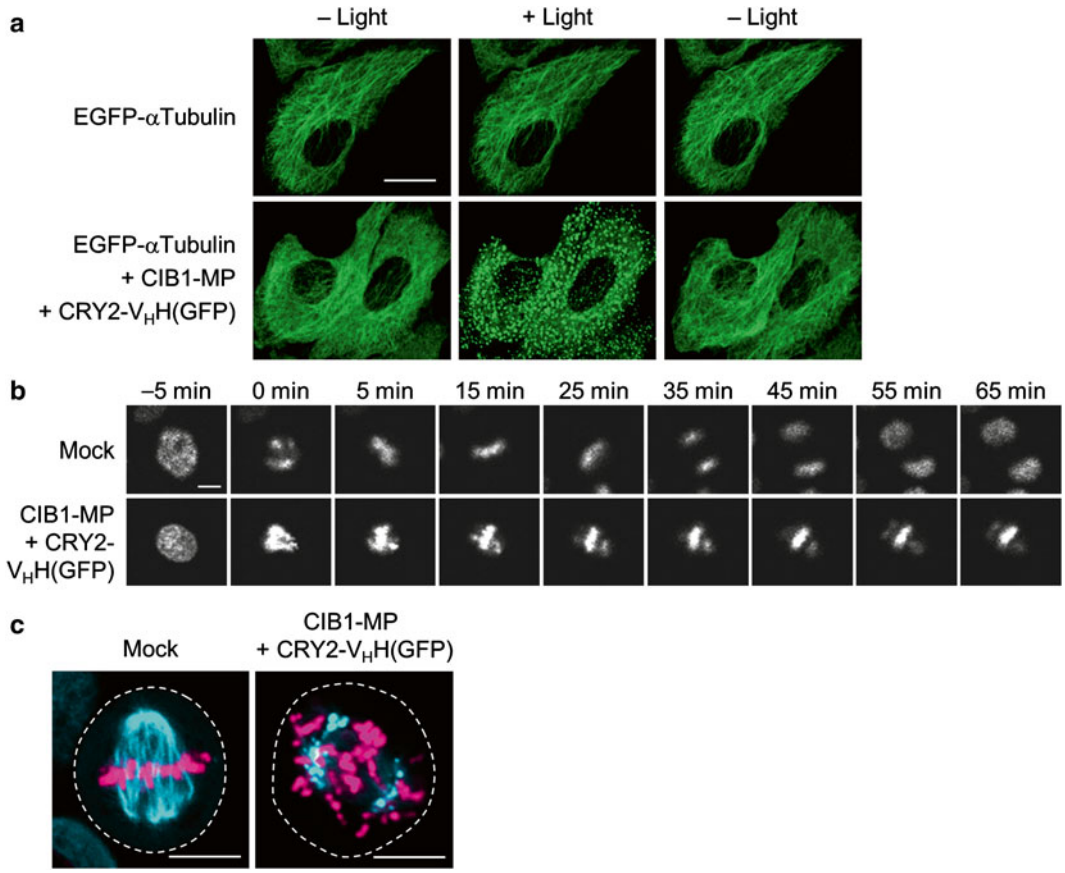

Fig. 5 Perturbation of cell cycle by trapping tubulins into clusters. (**a**) Trapping GFP-labeled tubulins into clusters by blue-light illumination. (**b**) Time-lapse images of mCherry-H2B to monitor cell division of HeLa cells co-expressing EGFP-tubulin, mCherry-H2B and each indicated fusion proteins. (**c**) Structure of mitotic spindles (*cyan*) and arrangement of chromosomes (*magenta*) in HeLa cells co-expressing EGFP-tubulin, mCherry-H2B and each indicated fusion proteins. Scale bars, 20 μm (**a**) and 10 μm (**b**, **c**). It is reproduced with permission from *Nature Methods*

4 Notes

1. Plasmids: The PCR-amplified sequence encoding association domain of CaMKIIα (amino acids 315–478) [13], indicated as MP, was cloned into pmCerulean-C1 vector (Clontech) between EcoRI and BamHI, which resulted in a plasmid encoding mCerulean-MP. In order to generate a CIB1-mCerulean-MP vector, sequence encoding CIB1 (amino acids 1–147) was inserted into the mCerulean-MP plasmid between NheI and AgeI sites. Expression plasmid for CRY2-mCherry was generated by inserting sequence encoding CRY2 into NheI and AgeI sites of pmCherry-C1 (Clontech). In order to

generate pmCitrine-CRY2-Vav2, PCR-amplified mCitrine flanked by NheI and AgeI were inserted into pmCherry-C1, original mCherry was replaced with PCR-amplified sequence encoding CRY2 at AgeI and BsrGI sites, and PCR-amplified sequence encoding Vav2 (amino acids 167–541) was excised by BspEI and XhoI and inserted into pmCitrine-CRY2.

To generate SNAP-CRY2-V_HH(GFP) vector, sequence encoding V_HH(GFP) from pcDNA3_NSlmb-vhhGFP4 (Addgene; plasmid 35579) was PCR-amplified and cloned into pmCitrine-CRY2 at XhoI and EcoRI sites, and sequence encoding SNAP (New England BioLabs) was PCR-amplified and inserted into pmCitrine-CRY2-V_HH(GFP) at NheI and AgeI sites, replacing sequence for mCitrine.

2. We used an association domain of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα), which self-assembles into multimeric protein (MP) with 12 identical subunits in LARIAT method [14]. However, you can choose other MPs to induce light-mediated interconnection. It is important to note that since labeling MPs with dimeric or weak dimeric fluorescence protein such as EGFP or EYFP variants causes spontaneous cluster formation regardless of light illumination, monomeric fluorescence proteins (mRFP, mCherry, or mCerulean) should be used [15].
3. For efficient mammalian expression, codon-optimized sequence encoding CRY2 (amino acids 1–498) was used.
4. The plasmids for CIB1 and CRY2 are conjugated with CLIP and SNAP (New England BioLabs), respectively, instead of fluorescence proteins to avoid trapping by anti-GFP nanobody. To label the CLIP or SNAP tags, we used CLIP-Cell TMR-Star (New England BioLabs) or SNAP-Cell Oregon Green (New England BioLabs), respectively. Dilute a 0.5 μL SNAP or CLIP-tag substrate in 100 μL culture medium. Replace the cell medium to the substrate-containing medium and incubate at 37 °C and 10 % CO_2 for 30 min. Wash the cells three times with complete culture medium.
5. An anti-GFP nanobody can recognize not only GFP but also other GFP variants such as CFP and YFP in living cells (Fig. 6).
6. Although we usually use the 488-nm laser for photo-activation, illumination with 405- and 457-nm light also activates LARIAT system. However, illumination with light longer than 514 nm could not induce the clustering (Fig. 7).
7. You can choose the scheme to inhibit the cytoskeletal protein among (option A) direct conjugation of target to CRY2 and (option B) use of CRY2-fused anti-GFP nanobody. In our optimal transfection condition, for option A, we usually use each 80 ng of CIB1-mCerulean-MP and mCherry-CRY2-Vav2, and 40 ng of mCherry-Lifeact. For option B, we transfect with each

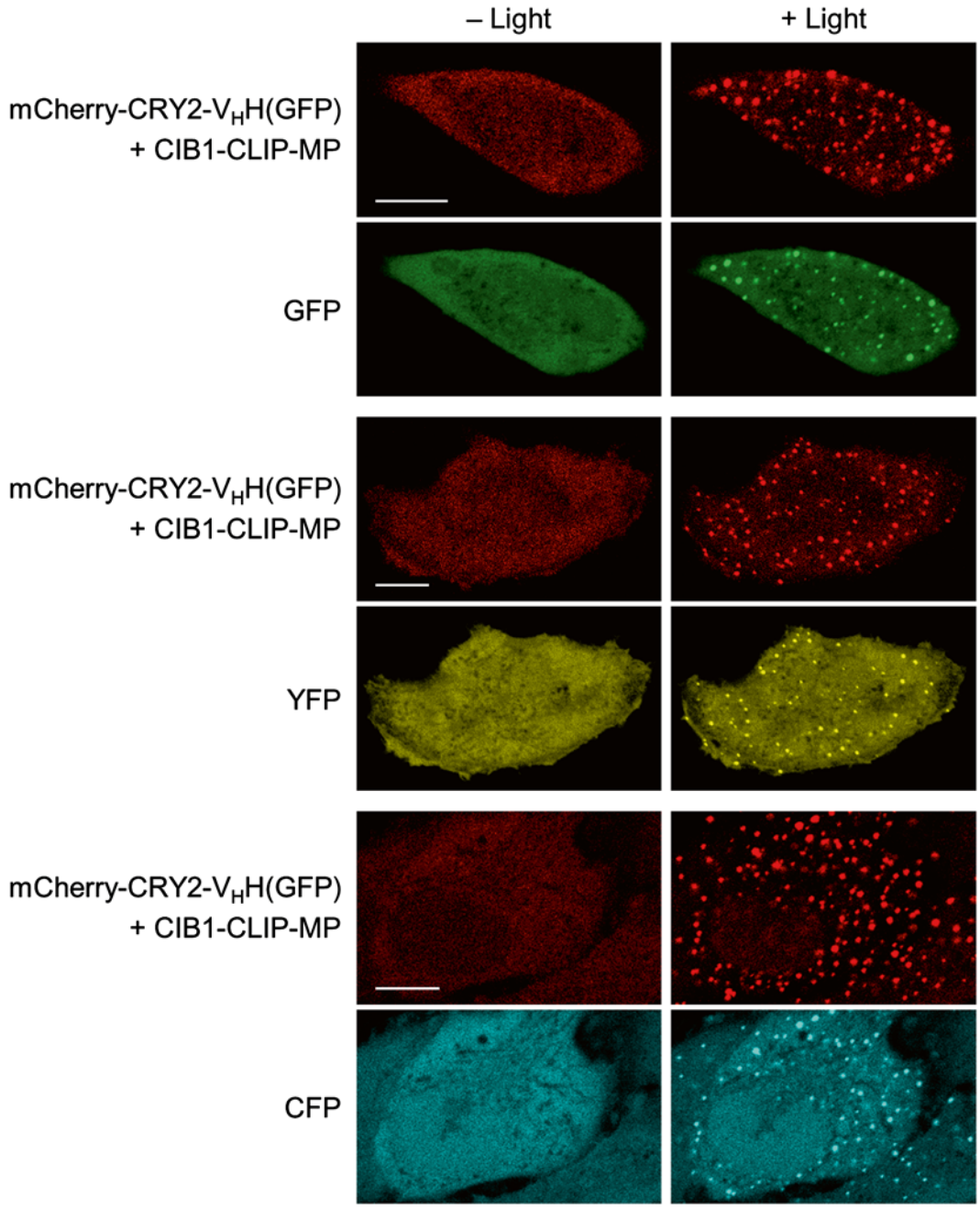

Fig. 6 Binding of anti-GFP nanobody to GFP variants. HeLa cells were co-transfected with expression plasmids as indicated. Fluorescence images were captured before and after light illumination. Scale bars, 10 μm

70 ng of CIB1-CLIP-MP and SNAP-CRY2-V_HH(GFP), 40 ng of EGFP-Vav2, and 20 ng of mCherry-Lifeact.

When we compared the morphological effects of EGFP-Vav2 and CRY2-Vav2, trapping EGFP-Vav2 with an anti-GFP nanobody showed a more effective membrane retraction than trapping CRY2-Vav2 (Fig. 8).

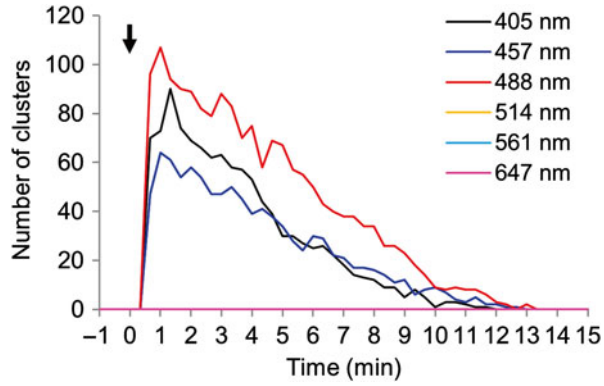

Fig. 7 Cluster formation with various wavelengths of light. A HeLa cell co-expressing CRY2-mCherry and CIB1-mCerulean-MP was illuminated by light of indicated wavelength. Cluster formation was monitored by fluorescent imaging of CRY2-mCherry in every 20 s. For quantification, clusters were defined as discrete puncta of fluorescence with criteria of fluorescence intensity (1500–4095 arbitrary units), size (>0.2 μm^2) and circularity (0.5–1.0 arbitrary units). *Arrow* indicates time point of irradiation. It is a representative data of two trials for each condition

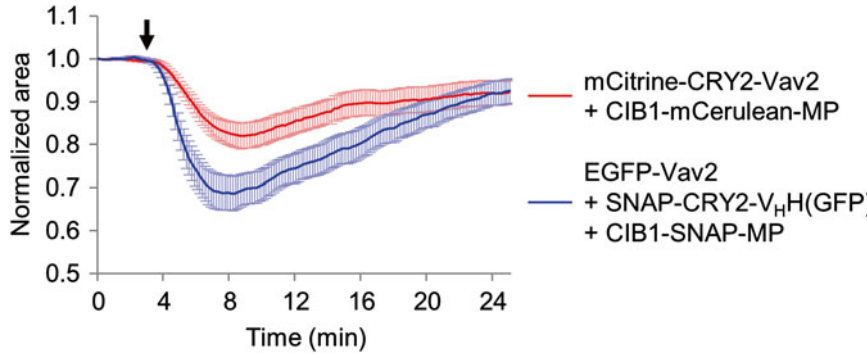

Fig. 8 Comparison of efficiencies of Vav2 inactivation by two different strategies. NIH3T3 cells co-transfected with expression plasmids as indicated, were illuminated by blue light (488 nm, 0.76 mW/mm^2). Morphological changes were monitored by fluorescent imaging of mCherry-Lifeact in every 10 s ($n=11$; Error bars, s.e.m.) *Arrow* indicates time point of irradiation. It is reproduced with permission from *Nature Methods*

8. We recommend that the ratio of DNA to Plus reagent should be 1:1.
9. We recommend the 1:3 ratio of DNA to LTX reagent to achieve high transfection efficiency.
10. Excitation of CFP, GFP, or YFP can induce CRY2-CIB1 heterodimerization and cluster formation. So, you should find the cells by monitoring red fluorescence signals to avoid perturbation of the target before photo-stimulation.

11. In most LARIAT experiments, we use a 488-nm laser for photo-activation on whole cell area. In our case, illumination for 1.5 s at 0.3 mW/mm^2 of power density was enough to induce cluster formation. For photo-stimulation on partial region of cell, adjust the laser power to stimulated area.
12. Before stimulation, it is important to monitor basal change of cell morphology for at least 1 min to convince the effect of light-inducible protein inactivation. After light illumination, at least 20 min is required to recover cell morphology to the basal state.
13. If there is any unfilled spaces on the thresholded image, especially nearby nucleus due to difference of intensity between nucleus and cytoplasm, fill the spaces by clicking "Fill holes" button to avoid loss of cell area.
14. After resuspension with Resuspension Buffer R, go to next step as soon as possible to avoid any harmful effect on cell viability and consequent reduction of transfection efficiency.
15. Keep watching the Neon Tip during electric shock to see if there is any spark that can be caused by bubbles in the tip.
16. To monitor cell division, we usually acquire images at every 5 min for 24 h. Illumination by blue light (for excitation of EGFP) at 5 min intervals is enough to maintain clusters.
17. When both CIB1-CLIP-MP and SNAP-CRY2-V_HH(GFP) are coexpressed on a cell, cluster formation can be determined by observing EGFP signals. So, clustering EGFP signal reflects that CIB1- and CRY2-fused proteins are expressed and participate in cluster formation.

Acknowledgements

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