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LOVTRAP, a Versatile Method to Control Protein Function with Light

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Abstract

We describe a detailed procedure for the use of LOVTRAP, an approach to reversibly sequester and release proteins from cellular membranes using light. In the application described here, proteins that act at the plasma membrane were held at mitochondria in the dark, and reversibly released by irradiation. The technique relies on binding of an engineered Zdk domain to a LOV2 domain, with affinity < 30 nM in the dark and > 500 nM upon irradiation between 400 and 500 nm. LOVTRAP can be applied to diverse proteins as it requires only attaching one member of the Zdk/LOV2 pair to the target protein, and the other to the membrane where the target protein is to be sequestered. Light-induced protein release occurs in less than a second, and the half-life of return can be adjusted using LOV point mutations (~2 to ~500 seconds).

Keywords

Optogenetics; LOVTRAP; Zdk; signaling; localization; dissociation

INTRODUCTION

In this unit, we provide detailed procedures for a new method to control protein activity with light, LOV2 Trapping of Protein (LOVTRAP) (Wang, et al., 2016). LOVTRAP is particularly useful because it can readily be applied to a broad range of proteins and protein activities, and because it enhances the dynamic range, or lit-dark activity difference, for the targeted proteins. In the particular iteration detailed here, proteins of interest are sequestered at mitochondrial membranes and reversibly released by irradiation, freeing them to act at the plasma membrane. In addition to manipulation of whole proteins and their mutants, we have released peptides that inhibit endogenous proteins. The approach is based upon Zdk, an engineered small protein that binds selectively only to the dark state of LOV2, the photosensory domain from *Avena sativa* phototropin 1. Either Zdk or LOV2 is anchored in

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INTERNET RESOURCES

http://www.tsienlab.ucsd.edu/Documents/REF%20-%20Fluorophore%20Spectra.xls

Dr. Roger Y. Tsien's laboratory web site which contains excitation and emission spectra of many fluorescent proteins.

http://www.chroma.com/products/fluorochrome

Web site of Chroma Technology Corp which contains spectra of many fluorochromes and optical filters. http://www.hahnlab.com

The Hahn lab website contains updates re improvements to LOVTRAP and related imaging techniques, and discussion of frequently asked questions.

the cell away from the site where the protein of interest (POI) acts. The other protein is fused to the POI. Hence, in the dark the POI is sequestered away from its site of action. Upon irradiation, the C-terminal Jα helix of LOV2 unwinds, freeing the POI to relocate to its site of action. To sequester the POI at sites other than mitochondria, similar strategies have been used, employing different subcellular localization sequences.

BASIC PROTOCOL 1: USING LOVTRAP TO CONTROL PROTEIN ACTIVITY WITH LIGHT

There are two possible configurations for the LOVTRAP system: The LOV2 domain can be attached to mitochondria and Zdk fused to the POI), or Zdk can be attached to mitochondria and the LOV2 domain fused to the POI. Currently there are three Zdks (Zdk1, Zdk2 and Zdk3) with different affinities for LOV2 (Table 1), and there are several LOV2 mutants with different recovery kinetics (Table 2). The advantages and disadvantages of selecting either LOV2 or Zdk for attachment to the POI will be discussed in the “Basic Protocol”section, and the use of different Zdk and LOV2 mutants will be discussed in the “Critical Parameters” section.

In strategy I, the POI can be fused to either the N- or C-terminus of Zdk, while LOV2 is anchored on mitochondria. This configuration minimizes the amount of free protein present in the dark, because LOV at the mitochondria can be expressed in excess over Zdk-POI. This can be valuable where downstream events are sensitive to small changes in POI concentration.). Upon irradiation, the amount of protein released will strongly depend on the ratio of LOV2:Zdk. Using a non-optimal ratio can cause little POI to be released upon irradiation. (see Critical parameters and troubleshooting). In contrast, Strategy II (POI attached to LOV2) is robust over a larger range of Zdk:LOV2 ratios, but there will always be some LOV-POI present in the dark. For strategy II, LOV2 must be fused to the C-terminus of the POI, because the C-terminus of LOV2 must be free for binding to Zdk 1. We have discovered other Zdk proteins (Zdk2 and Zdk3) that bind to different sites on LOV; these may ultimately overcome this disadvantage, but we have not explored that possibility. For both strategies, we have found that optimal response occurs when the protein attached to the mitochondria (Zdk or LOV) is present in 4-10 fold excess over the POI.

Materials

Plasmid vectors for mammalian cell expression: e.g. pTriEx (Novagen), pcDNA (Invitrogen), etc.

DNA encoding residues 1-35 of TOM20 (NCBI Access code: NP_055580) for mitochondrial localization(Kanaji et al., 2000).

> It is possible that the POI can undesirably interact with proteins at the mitochondria. In such cases, the POI can be sequestered at other loci such as the ER or Golgi apparatus. DNA encoding the desired localization signal should be used.

DNAs encoding residues 404-546 of the *Avena sativa* phototropin1 LOV2 domain (*Genbank access code: AAC05083*), the Zdk binding protein (Genbank access code: KX429612 for Zdk1, KX429613 for Zdk2, and KX429614 for Zdk3), appropriate fluorescent proteins (e.g. mVenus, mCherry, etc.) and the protein of interest.

The above DNAs can be obtained from Addgene (IDs: 81009, 81010, 8101,1 81012, 81041, 81057).

Mammalian cells lines to be studied

The LOVTRAP system has been used succesfully in mammalian cells lines including HeLa cells, Cos-7 cells and HEK 293 cells. Feasibility in other cell lines can be checked by comparing the localization of POI using dark state and lit state LOV mutants, or looking for localization changes induced by light.

Cell culture medium

Imaging medium

FuGENE 6 (Promega) or other transfection reagent

Coverslips appropriate for the studies to be performed (e.g. coated with fibronectin for cell adhesion in the example here)

Inverted fluorescence microscope driven by MetaMorph 7.5. The scope is equipped with

Ludl Electronics Products MAC5000 controller and programmable filter wheels for microscope automation

100W mercury arc lamp

Band pass filters: ET430/24X (activation), ET572/35X (RFP excitation) and HQ620/60M (RFP emission) from Chroma Technology Corporation, FF-520/15 (YFP excitation) and FF-565/24 (YFP emission) from Semrock Inc.

Dichroic mirrors: 440/500/580 dichroic mirror (for mCherry) and T545LP dichroic mirror (for mVenus) from Chroma Technology Corporation

For the spectra of the filters and dichroic mirrors, see figure 1

Neutral density filters with 1% and 3% transmittance

40X, 1.3 N.A. oil-immersion objective

Construction of the plasmids

1. Generate two DNA fragments using the TOM20 fragment, the LOV2 domain, Zdk, the fluorescent protein and the POI, fused as described for one of the two strategies described above. Clone them into the mammalian expression vector (Figure 2).

If the POI can be modified at the C-terminus, either caging strategy I or II can be used. Otherwise, only strategy I works. See

Critical Parameters and Troubleshooting. For a cross-reference of molecular biology techniques, see APPENDIX 3A.

Expressing the LOVTRAP caging system in adherent cells

2. Coat the coverslips with 10μg/ml fibronectin and seed the cells on them (about 50% confluency) one day before transfection.

For basic cell culture techniques, see Chapter 1; for detailed protocols for preparing fibronectin coated coverslips, see UNIT 14.11. (Hodgson et al. 2010)

3. Premix the two LOVTRAP plasmids at a ratio of 5-10:1 (mitochondrial targeting protein in excess to sequester POI maximally) and transfect the DNA with FuGENE 6 or other transfection reagents following the manufacturer's protocols. Cover the plate with Aluminum foil after this step.

4. Grow the cells in the dark for about 20 hours.

Alternately, cells can be transfected and cultured in tissue culture dishes, then transferred to the coverslips 4 hours before imaging. However, this will require trypsinizing and plating cells in the dark to avoid activation of the POI by ambient light. A red safe light can be used for TC procedures.

Imaging the cells

5. Mount the coverslips into chambers and add live cell imaging medium (specified in “Materials” above). Image the cells using a fluorescence microscope with a 40X objective and appropriate excitation and emission filters (Figures 3A, C). Time-lapse images have been taken using the MetaMorph Multi-dimensional Acquisition module from Molecular Devices (Figure 4).

6. The release of proteins from sequestration is complete in less than a second (Wang et al., 2016), but the POI take substantially longer to return to the sequestered state (seconds to minutes, depending on the variant of LOV2 used, see section “*Kinetics of the system*”). To avoid photodamage, we typically do not irradiate continuously but instead use pulsatile irradiation, i.e., irradiate for 5 seconds or shorter to produce the maximal amount of free protein, then leave the sample in the dark while the LOV domain relaxes to the dark form over seconds to minutes (depending on the LOV mutant used). When we want the protein to remain free, we irradiate at regular intervals to maintain a sufficient free concentration. We obtain images of the cells during one of the periods when irradiation is not occurring. In a typical experiment with wild-type LOV2, shown in Figure 3B, irradiation pulses are 5 seconds long, and the dark intervals between them are 5 seconds. For mutants that take longer to return to the dark state, the period between irradiation can be lengthened to minimize photodamage. Photo-activation has been automated using a MetaMorph

journal (Figure 4D), provided below in the section *Metamorph journal for photo-activation*.

7. If transmitted light is required (e.g. for DIC or phase contrast images), a red long pass filter should be insert in the transmitted light path.

In some cases, the light from bright computer monitors can be a concern. This can easily be solved by keeping enough distance between the monitors and the sample and turning the monitor away from the sample. Some users have placed a black cardboard cone around the sample on the microscope.

SUPPORT PROTOCOL 1: Use of FRAP equipment

Fluorescence recovery after photobleaching (FRAP) systems (see UNIT21.1) can be adapted to activate LOVTRAP. FRAP requires much higher laser power than LOVTRAP, as it is used to photobleach everything in the region of interest as quickly as possible. LOVTRAP would be used at lower powers to avoid both photodamage and activation of neighbor cells. Most laser spots show a Gaussian distributi on so the area irradiated effectively increases as power increases (Figure 5).

Materials

Plasmid vectors for mammalian cell expression (described as in Basic Protocol 1)

Mammalian cells lines to be studied

Cell culture medium

FuGENE 6 (Promega) or other transfection reagent

Coverslips appropriate for the studies to be performed

FRAP-3D imaging system from MAG Biosystems

The FRAP-3D system is a widefield imaging system for studying FRAP in living cells. The system is equipped with

Inverted fluorescence microscope driven by MetaMorph 7.5.

Ludl Electronics Products MAC5000 controller and programmable filter wheels for microscope automation

Galvo mirror-based FRAP head

Laser launch module with 473 nm laser line

100W mercury arc lamp

Band pass filters: ET430/24X (activation), ET572/35X (RFP excitation) and HQ620/60M (RFP emission) from Chroma Technology Corporation, FF-520/15 (YFP excitation) and FF-565/24 (YFP emission) from Semrock Inc.

Dichroic mirrors: 440/500/580 dichroic mirror (for mCherry) and T545LP dichroic mirror (for mVenus) from Chroma Technology Corporation

For the spectra of the filters and dichroic mirrors, see figure 1

Neutral density filters with 1% and 3% transmittance

40X, 1.3 N.A. oil-immersion objective

Prepare the samples

1. The cells transfected with LOVTRAP plasmids are prepared according to Basic Protocol 1.
2. Grow the cells in the dark for about 20 hours.

Calibrate the Galvo mirror

3. Adjust the laser intensity to the proper level

   The laser power needs to be determined empirically so that only the cell of interest is activated.
4. Bring the laser beam to the center of the field of view using Galvo mirror control arrows (Figure 6A)
5. Calibrate the galvo mirrors by scanning a 6×6 matrix in the field of view (Figure 6B).
6. Using the “Stack Arithmetic” tool to bring all the 36 laser spots to one image. Check that all the points are within the field of view.

   If any point is out of the field of view, center the position of the laser beam slightly differently using Galvo mirror control arrows and do the calibration again.

Image the cells

7. Mount the coverslips into chambers and add imaging medium. Image the cells using appropriate excitation and emission filters.
8. Define the region of interest (ROI) to cover the cells that need to be activated.

   Make sure the laser shutters are closed when adding the ROI. Otherwise, the cell will be irradiated and activated.
9. Acquire images using the MetaMorph Multi-dimensional Acquisition module.

REAGENTS AND SOLUTIONS

Cell culture media

Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose and sodium pyruvate without L-glutamine (Corning Cellgro, Cat#15-013-CV), 500mL

100X GlutaMax Supplement (Gibco, Cat#35050-61), 5mL

Standard Fetal Bovine Serum (Hyclone, Cat#SH30088.03, Lot#AXK49939), 50mL

Depending on the cell-lines, Bovine Calf Serum should be used instead of Fetal Bovine Serum.

Imaging media

Ham's F-12 Kaighn's Modification with L-Glutamine without phenol red (Caisson, Cat#HFL12-500ML), 50mL

1M HEPES Buffer solution (Gibco, Cat# 15630-106), 1.25mL

Standard Fetal Bovine Serum (Hyclone, Cat#SH30088.03, Lot#AXK49939), 250μL

The final concentration of Fetal Bovine Serum depends on your experiment.

COMMENTARY

Background Information

Over the past several years, there has been a revolution in the development of tools to control proteins with light. After control of ion channels (Lima and Miesenböck, 2005; Zemelman, Lee, Ng, and Miesenböck, 2002), optogenetics was extended to cytosolic proteins (Wu et al., 2009; Levskaya et al., 2009; Kennedy et al., 2010; Strickland et al., 2012). Current photo-regulation systems have important limitations. Approaches where light-responsive domains reversibly block the active site require case-by-case protein engineering (Wu et al., 2009). Photo-induced dimerization systems can be “leaky”; (background activity is present before the protein has been activated by light) and the extent of lit/dark difference varies from system to system. The expression level of photomodulated proteins must sometimes be carefully adjusted to appropriately modulate downstream effectors (Levskaya et al., 2009).

LOVTRAP has several specific advantages: It has fast (subsecond) activation kinetics as well as tunable de-activation kinetics. The light-responsive element in LOV is an endogenous flavin cofactor incorporated upon expression. The amount of protein released can be adjusted by varying the light intensity. Zdk has the potential to trap the dark state conformation, increasing the difference between the dark and lit state and thereby reducing ‘leakiness’. LOVTRAP should be broadly applicable, as many different proteins can be fused to the system.

Critical Parameters and Troubleshooting

The effect of the ratio between the two components of the LOVTRAP system— The mitochondria-anchored molecules should be expressed in excess over the free component, to sequester the POI completely. As discussed above, the ratio between the two components has different effects in the two strategies described in the Basic protocol. A more quantitative analysis is provided in the original description of LOVTRAP (Wang et al,

2016). Briefly, the concentration of free POI, as a function of LOV:Zdk ratio in the two methods, is given by the following equations:

$$[POI]_{unbound}=\frac{-(mn-1)[POI]_{tot}-Kd+\sqrt{\{(mn-1)[POI]_{tot}+Kd\}^2+4Kd[POI]_{tot}}}{2}$$

Equation 1, for stragtegy I

$$[POI]_{unbound}=\frac{-(m+n-1)[POI]_{tot}-Kd+\sqrt{\{(m+n)[POI]_{tot}+Kd\}^2-4mn[POI]_{tot}^2}}{2}$$

Equation 2 for stragtegy II

where m is the fraction of LOV2 molecules that are in the closed conformation, n is the ratio of the two components, and Kd is the dissociation constant between Zdk and LOV2 in the closed conformation. In the dark, m_{dark}=0.984; while in the light, m_{light}=0.09 (Yao et al., 2008). In **Strategy I**, the POI can only be released partially by light, especially when protein expression ratios are high (n>10). However, the release of any protein in the dark is minimized (essentially zero at the appropriate protein ratios). The protein released is usually more than enough as the POI can be over-expressed. If it is important that the protein be released completely, and if the POI can be modified on its C-terminus, **Strategy II** can be a better choice.

Equation 1 and 2 are based on a simplified model to demostrate the effects of expression ratio s(Wang et al, 2016). However, in reality, protein-protein interactions in cells are complicated by crowding and structured cellular environments (Norris and Malys, 2011).

Choice of Zdk

Among the three Zdks, Zdk1 has the best dynamic range, i.e., nanomolar affinity with the dark-state LOV2 and negligible affinity with Lit-state LOV2 (Table 1). However, Zdk1 requires the C-terminus of LOV2 to be free for binding, which limits its use, and requires attachment to the N-terminus for Strategy II. In Strategy II, if the POI has to be fused to C-terminus of LOV2, Zdk2 can be used instead. However, because the affinity between Zdk2 and lit-state LOV2 is 731 nM, only a small portion of the POI will be released with light.

Kinetics of the system

When irradiated, LOV and Zdk dissociate with subsecond kinetics, but the return to the dark state occurs substantially more slowly. By introducing point mutations in LOV2, this return rate can be tuned for different biological applications (Table 2). Shorter relaxation times can be used for precise control of kinetics, while longer relaxation time enable the maintenance

of substantial amounts of active protein with only periodic pulses of light, lessening photo-damage for long activations.

Activation light and filters

The LOV2 domain has a broad absorption peak which spans 400-500 nm. Any light within this region can activate the LOVTRAP system. The ability to activate LOV falls off very rapidly above 500 nm, enabling the use of YFP and longer wavelength fluorescent proteins without activating the caged POI. Light at the shorter wavelengths within the LOV excitation spectrum is more toxic to the cell, while light at longer wavelengths overlaps with the excitation spetra of some fluorescent proteins, leading to their fluorescence emission and/or potential bleaching. Bleaching is usually not an issue as the LOV2 domain is quite sensitive to light, so weak irradiation can be used to activate it. In our lab, we have used an inverted microscope equipped with a 100W mercury arc lamp, ET430/24X band pass filter and 3% neutral density filter for activation. This setting gave 150μW intensity at the sample when using a 40X, 1.3 N.A., oil-immersion objective (routine settings for imaging CFP in our lab).

Choice of fluorescent proteins and filters

Due to spectral overlap, CFP and GFP are not compatible with LOVTRAP. Orange and red fluorescent proteins are good choices (Figure 1A). For multiplexing, YFP can be used, but the excitation filter needs to be carefully selected to avoid activating LOV2 during image acquisition (Figure 1B).

For more information about fluorescent proteins, see UNIT 21.5.

Selection of fusion protein structure and linkers—The order in which proteins are fused, and linkers used, can affect the function of the POI. Hence the fusions need to be validated empirically. There are several potentially acceptable combinations of the LOVTRAP proteins (see Figure 2). The linkers must be flexible and long enough so that fusion will not affect the binding of the POI to its effectors. On the other hand, a longer linker is more susceptible to proteases in the cells, and proteolysis can increase the basal level of POI activity in the dark. We have successfully used GSGGSGSGGT linkers for multiple constructs in Hela cells. If a longer sequence is preferred, a GSTSGSGKPGSGEGSTKG linker (Whitlow et al., 1993) is an option. Although we have used these linkers successfully in HeLa cells, proteolysis has been observed by others (personal communication) in NIH3T3 cells. Proteolysis may well be cell type dependent. Cleavage was more severe when using cells stably expressing LOVTRAP than when transient transfection was used. This is reasonable given that transient transfection exposes the constructs to endogenous proteases for only a few hours before imaging. These problems may be ameliorated using an inducible expression system, (e.g. Tetracycline-Controlled Transcriptional Activation, Gossen, et al, 1995) in stable cell lines.

Reversibility of the system—Although the LOVTRAP system itself is highly reversible (e.g. a fluorescent protein can undergo cycles of sequesteration and release for more than 10 cycles), the reversibility of the system with a given POI may be dependent on the nature of the protein. The RhoGTPases that we studied could be activated and inactivated repeatedly

despite the fact that movement between the plasma membrane and mitochondria likely involved the celluar prenylation machinery (Gao, et al, 2009) and/or interaction with guanine dissociation inhibitors (GDI, Cherfils & Zeghouf, 2013). Some users have reported that the POI moves irreversibly to the site of action, presumably due to irreversible binding at the target site.

Filter settings—The LOV2 domain is very sensitive to light. Any unnecessary exposure of the cells to light should be avoided. This is especially critical prior to imaging. For imaging, one can place a red filter in the transmitted light path, being careful to guard the sample from exposure to stray or reflected light. In cases when YFP is used, the excitation filter needs to be red-shifted from those typically used for YFP (Figure 1B) to avoid activation of the LOV2 domain while imaging YFP. Such filters are not optimal for YFP imaging, so use of longer wavelength fluorophores is preferred.

Anticipated Results

In the dark, the POI is expected to be located at the mitochondria (Figure 7), so one would expect to see fluorescence with a tubular morphology typical of mitochondria in a given cell type. However, this may be obscured if dyes or other fluorophores overlap with the spectra of the fluorescent proteins being used. Upon blue light irradiation, the POI is released into the cytosol leading to a diffuse distribution, usually complete within a few seconds after irradiation (Figure 7). It is normal that some protein remains on mitochondria upon irradiation, and more unreleased protein would be expected when strategy I is used (Figure 7). In some cases, the mitochondria need to be over-exposed to see release of the POI. The system is reversible, with POI relocating to mitochondria when the blue light is turned off (Figure 7). By monitoring a position in the cell away from the mitochondria, one can assay the release and return of the fluorescent protein (Wang, et al, 2016). With proper choice of linker, the biological activity of the POI will be triggered by irradiation.

If one is not familiar with mitochondrial morphology, oral cell type with unusual mitochondrial morphology is used, the cells can be stained with Mito Tracker dyes from Invitrogen to visualize and characterize the mitochondria (Catlog #M7514, M22426 or M22425).

If abnormal mitochondrial morphology is observed, mitochondrial function assays can be conducted. Mitochondrial membrane potential and mitochondrial superoxide generation can be tested by using flow cytometry with DilC1(5) (Invitrogen, Cat# M34151) and MitoSOX Red (Invitrogen, Cat# M36008). (Wang et al, 2016; Mukhopadhyay, 2007). In our applications, these tests revealed no unusual mitochondrial behavior.

Time Considerations

Expression of the LOVTRAP proteins in transiently transfected cells requires 20-28 hours after transfection. The time required for imaging will depend on the biology being studied. For our studies of Rho family activity oscillations and their effects on cell motility, we usually imaged 30 minutes before irradiation, 30 minutes with different specific irradiation protocols, and 30 minutes after the blue light was turned off. Although release of the POI is

very fast (<1s upon irradiation), in some cases there is a time lag before the released protein produces a discernable phenotype, depending on the kinetics of steps leading from protein activation to phenotype. The phenotype may depend on the duration or timing of irradiation, especially when there are competing pathways countering the effects of the POI. Some cells are healthy for hours in an open chamber on a temperature controlled stage, while others require a closed chamber with media perfusion. To minimize blue light exposure while maintaining the POI in a free state, it is very helpful to activate the proteins using pulsatile irradiation, as described above. The light pulse can be a second or less, as the protein is fully released within this time. The dark period will vary depending on which LOV2 mutant is used, and how much of an activity drop is acceptable during the dark period. Mutations can be used to adjust the half-life of return to the sequestered state from seconds to minutes (see section *Choice of LOV2 mutants* above).

Appendix: MetaMorph journals for photo-activation

MetaMorph (from Molecular Devices) is a program frequently used for microscope automation (Schmitz and Gerlich, 2009). The journal we have written to control the timing of photoactivation and coordinate it with stage movements and autofocus is provided here:

Journal 1: Turn on blue light for 5 seconds from timepoint #61 to #120. For wild-type LOV2, the time interval should be set to 10 seconds in MetaMorph MDA acquisition.

Photoactivation

IF MDA.Status.TimePointNum <61 THEN

1: Shutter-Close()

ELSE

IF MDA.Status.TimePointNum <121 THEN

2: Component Control(Component Name = Olympus Filter Cube, Position = 3)

3: Component Control(Component Name = Excitation - Ludl Wheel 2 - TIRF, Position = 7)

4: Component Control(Component Name = Intensity - Ludl Wheel 1- TIRF, Position = 4)

5: Component Control(Component Name = Arc lamp - Ludl Shutter 1- TIRF, Position = −1)

6: Delay(5, SECONDS)

7: Component Control(Component Name = Arc lamp - Ludl Shutter 1- TIRF , Position = 0)

8: Component Control(Component Name = Excitation - Ludl Wheel 2- TIRF , Position = 6)

9: Component Control(Component Name = Intensity - Ludl Wheel 1-TIRF , Position = 1)

ELSE

10: Shutter-Close()

END IF

END IF

Journal 2: Switch the blue light 5-second-on/5-second-off from timepoint #61 to #120. The time interval should be set to 30 seconds in MetaMorph MDA acquisition

Photoactivation

IF MDA.Status.TimePointNum <61 THEN

1: Shutter-Close()

ELSE

IF MDA.Status.TimePointNum <121 THEN

2: Component Control(Component Name = Olympus Filter Cube, Position = 3)

3: Component Control(Component Name = Excitation - Ludl Wheel 2 - TIRF, Position = 7)

4: Component Control(Component Name = Intensity - Ludl Wheel 1-TIRF, Position = 4)

5: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF, Position = −1)

6: Delay(5, SECONDS)

7: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF , Position = 0)

8: Delay(5, SECONDS)

9: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF, Position = −1)

10: Delay(5, SECONDS)

11: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF , Position = 0)

12: Delay(5, SECONDS)

13: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF, Position = −1)

14: Delay(5, SECONDS)

15: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF , Position = 0)

16: Component Control(Component Name = Excitation - Ludl Wheel 2-TIRF , Position = 6)

17: Component Control(Component Name = Intensity - Ludl Wheel 1-TIRF , Position = 1)

ELSE

18: Shutter-Close()

END IF

END IF

Journal 3: Irradiate the cells with 3.3mHz blue light from timepoint #61 to #120. The time interval should be set to 10 seconds in MetaMorph MDA acquisition

Photoactivation

This Journal will irradiate the cell with 3.3mHz blue light from timepoint #61 to #120

1: SWITCH=MOD((MDA.Status.TimePointNum-60),30)

IF (MDA.Status.TimePointNum >60)* (MDA.Status.TimePointNum <121) THEN

IF (SWITCH<6) * (SWITCH > 0) THEN

2: Component Control(Component Name = Olympus Filter Cube, Position = 3)

3: Component Control(Component Name = Excitation - Ludl Wheel 2 - TIRF, Position = 7)

4: Component Control(Component Name = Intensity - Ludl Wheel 1-TIRF, Position = 4)

5: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF, Position = −1)

6: Delay(5, SECONDS)

7: Component Control(Component Name = Arc lamp - Ludl Shutter 1-TIRF , Position = 0)

8: Component Control(Component Name = Excitation - Ludl Wheel 2-TIRF , Position = 6)

9: Component Control(Component Name = Intensity - Ludl Wheel 1-TIRF , Position = 1)

ELSE

10: Shutter-Close()

END IF

11: Shutter-Close()

END IF

For different microscopes, the component Name and filter positions can be different. Please check with your microscopy specialist for the correct component name and filter positions in MetaMorph.

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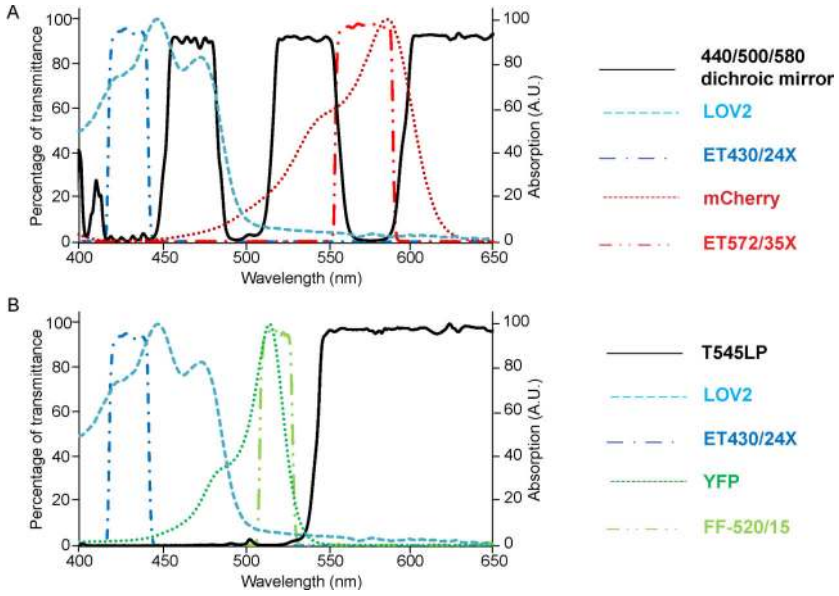


Figure 1.

Spectra of the proteins and filters. A) Absorption spectra of LOV2 and mCherry; Transmittance spectra for the filters ET430/24X, ET572/35X and the 440/500/580 dichroic mirror. B) Absorption spectra of LOV2 and YFP; Transmittance spectra for the filters ET430/24X, FF-520/15 and the T545LP dichroic mirror. Note: Absorption spectra of LOV2 and transmittance spectra for filters ET430/24X, ET572/35X, FF-520/15, 440/500/580 dichroic mirror and T545LP dichroic mirror were measured using an Agilent 8453 UV/VIS spectrophotometer. Excitation spectra of mCherry and YFP are from the website of Dr. Roger Y. Tsien's laboratory (http://www.tsienlab.ucsd.edu/Documents/REF%20-%20Fluorophore%20Spectra.xls).

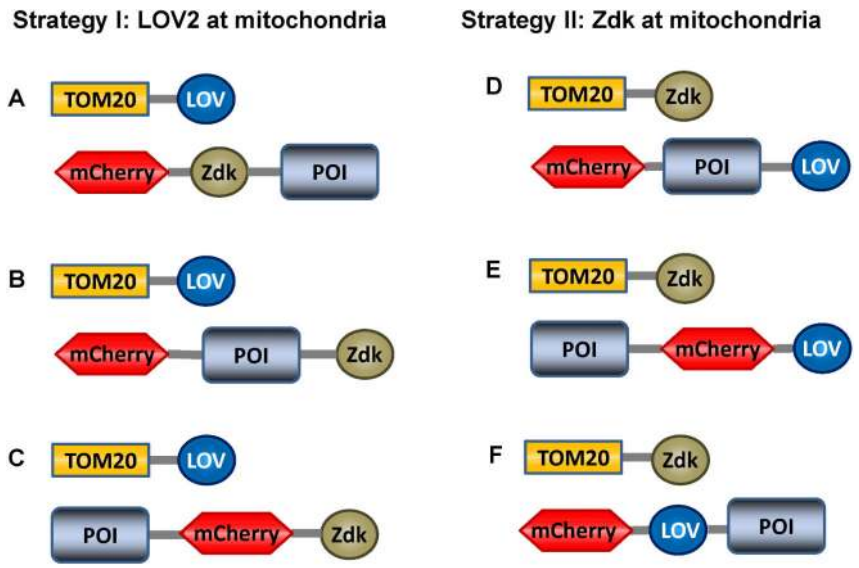
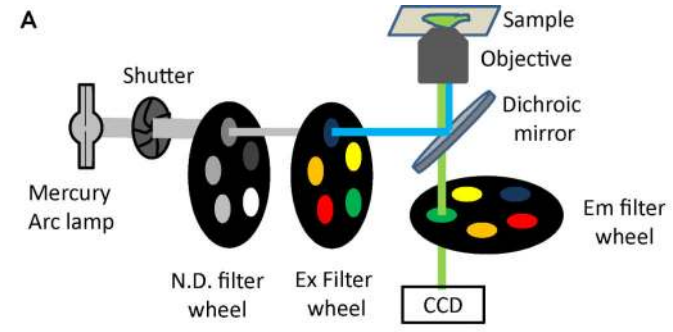


Figure 2.

Fusion proteins that can be used in the LOVTRAP system. For Zdk, amino- and carboxy-terminal fusions can be used. For LOV2, only an amino-terminal fusion is functional if Zdk1 is used.



C Metamorph journal variety values and their function

Component	Position	Function
Olympus Filter Cube	3	Switch to 440/500/580 dichroic mirror
Excitation - Ludl Wheel 2 - TIRF	7	Switch to ET430/24X Excitation filter (blue light)
Excitation - Ludl Wheel 2 - TIRF	6	Switch to ET572/35X Excitation filter (mCherry)
Intensity -Ludl Wheel 1 - TIRF	4	Switch to 3% neutral density filter
Intensity -Ludl Wheel 1 - TIRF	1	Switch to 1% neutral density filter
Arc lamp - Ludl Shutter 1 - TIRF	-1	Open Arc lamp shutter
Arc lamp - Ludl Shutter 1 - TIRF	0	Close Arc lamp shutter

Figure 3.

The protocol for photoactivation interspersed with acquiring images. A) Photo-activation light shares the same light path as fluorescence excitation. B) The timing of blue light irradiation (blue line), relative to other operations (red bar), all controlled by a MetaMorph journal. C) The positions of the components used for photo-activation are shown in the table. They are the same as those that can be used for excitation of CFP.

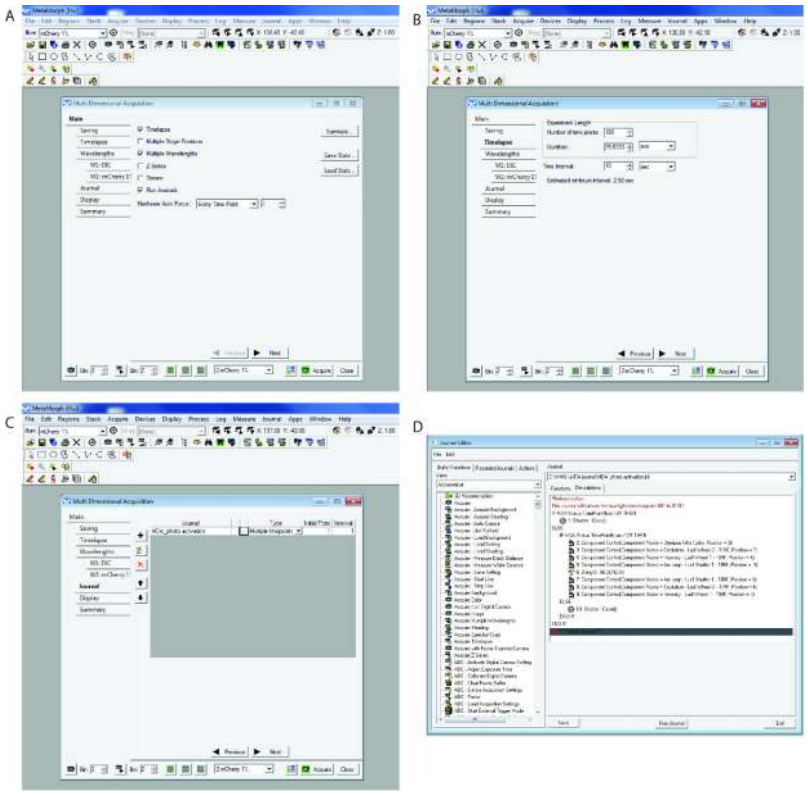


Figure 4.

Acquiring images using the MetaMorph Multi-dimensional Acquisition module. To obtain timelapse images, A) the check box “Timelapse”, “Multiple wavelengths” and “Run journals” were checked. B) The time interval was set to 10 seconds, and 180 time points were imaged for a total of about 30 minutes. C) The cells were irradiated with blue light between 10 and 20 minutes using the journal described in D).

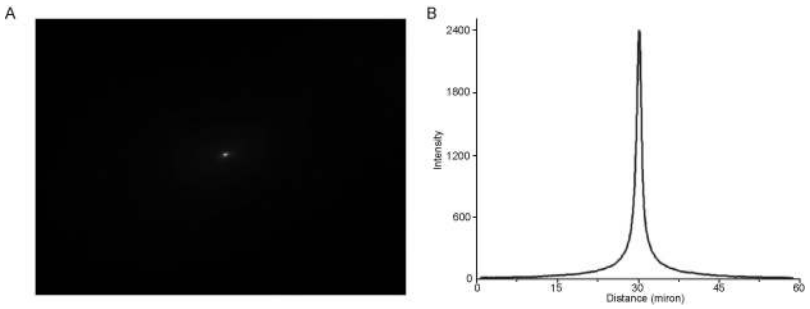


Figure 5.

Profile of the laser beam used to activate LOVTRAP in a FRAP system. A) Image of the laser spot. B) Linescan of the laser spot in A).

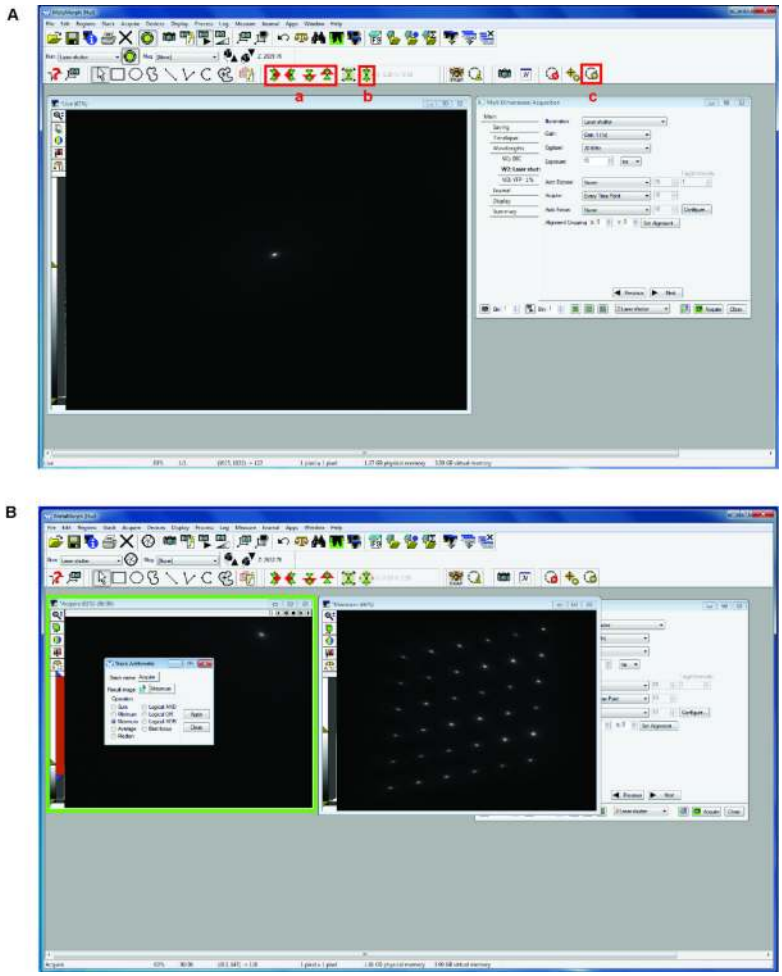


Figure 6.

Activation of LOVTRAP using the FRAP-3D imaging system from MAG Biosystems. A) MetaMorph interface of the FRAP-3D system. For the highlighted buttons: a, Galvo mirror control arrows; b, Calibrating; c, Adding region of interest.

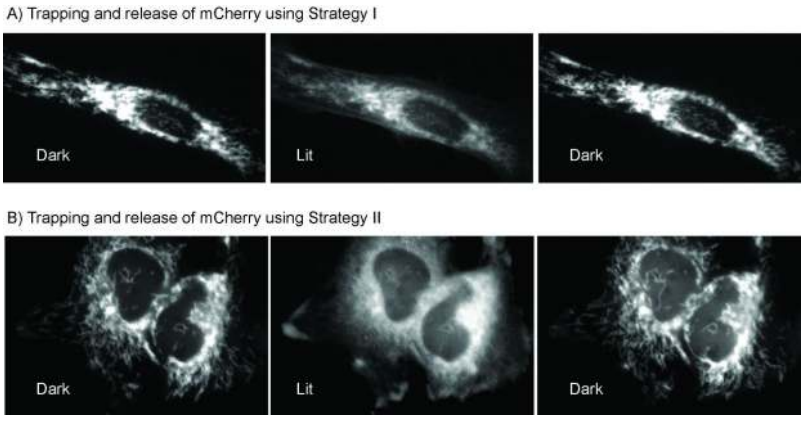


Figure 7.
Expected images using strategy I (A) and strategy II (B).

Table 1

Affinity between different Zdks and LOV2 variants (Wang et al, 2016).

Zdk	LOV2 C450A	LOV2 I539E
Zdk1	26.2 nM	>4 μM
Zdk2	17 nM	761 nM
Zdk3	11.4 nM	537 nM

Table 2

Recovery kinetics of LOVTRAP using LOV2 variants (Wang et al, 2016).

LOV2 mutation	$t_{1/2}$ (seconds)
Wild type	18.5
I427T	1.7
V416T	5.0
I427V	5.5
V416I	239
V416L	496