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Optogenetic perturbation of the biochemical pathways that control cell behavior

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Abstract

Optogenetic tools provide a level of spatial and temporal resolution needed to shed new light on dynamic intercellular processes. In this chapter we outline specific protocols for applying these tools to cell motility (optogenetic cofilin), apoptosis [optogenetic Bcl-like protein 4 (Bax)], and protein kinase-mediated signaling pathways [optogenetic cAMP-dependent protein kinase (PKA)]. The activity of these optogenetic species is regulated by the light-mediated dimerization of a cryptochrome/Cib protein pair, which controls the intracellular positioning of the protein of interest. The light induced recruitment of cofilin to the cytoskeleton is utilized for directed migration studies and filopodial dynamics. Light-triggered migration of Bax to the outer mitochondrial membrane induces cellular collapse and eventual apoptosis. Finally, the light-mediated movement of PKA to specific intracellular compartments offers the means to assess the consequences of PKA activity in a site-specific fashion via phosphoproteomic analysis.

1. Introduction

Optogenetic systems are composed of genetically encoded, light-activatable proteins that can control biochemical pathways at specific subcellular locations. These light inducible proteins have the capacity to probe the temporal and spatial contributions of specific proteins of interest in a biochemical pathway. Traditional methods for understanding protein signaling (overexpression, pharmacological manipulation, etc.) can often be complicated by issues of compensation, lethality of suppression, non-specificity, and off-target effects. Optogenetics offers a degree of selectivity that overcomes these limitations (Lee, Larson, & Lawrence, 2009; O'Banion & Lawrence, 2018). They furnish control over such variables such as the timing of activity, the magnitude of activity, and the cellular and subcellular location of activity (Hughes & Lawrence, 2014; Kennedy et al., 2010).

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The optogenetic proteins described in this chapter have been used to probe and/or perturb the pathways that modulate motility, apoptosis, and cell signaling. They are based on the light-triggered recruitment of bioengineered proteins to preassigned subcellular sites using a cryptochrome (Cry) photoreceptor (Kennedy et al., 2010). Cryptochromes are photolyase-like flavoproteins that mediate blue light regulation of gene expression in *Arabidopsis* (Yu, Liu, Klejnot, & Lin, 2010). The *Arabidopsis* genome encodes at least seven blue light receptors, including Cry1 and Cry2 (Cashmore, 2003; Sancar, 2003; Shalitin, Yu, Maymon, Mockler, & Lin, 2003). Our studies employed the light-driven association of the Cry2 photoreceptor with its binding partner (Cib1) to induce recruitment of proteins of interest to specific subcellular compartments.

These optogenetic species provide the means to examine dynamic biological behavior with exquisite spatial and temporal resolution (O'Banion & Lawrence, 2018). In our hands, optogenetic control has provided mechanistic insight into cofilin-mediated regulation of cell motility and filopodial formation (Hughes & Lawrence, 2014), new tools for exploring Bax-triggered induction of apoptosis (Hughes et al., 2015), and examining the consequences of PKA activity at specific cellular compartments (O'Banion et al., 2018).

2. Spatial and temporal control of subcellular cofilin activity

An optogenetic cofilin (optoCofilin) offers the ability to regulate actin cycling in a light-dependent fashion. Cofilin is a member of the cofilin/actin-depolymerizing factor (ADF) family of proteins. It depolymerizes actin filaments, supplies a pool of monomeric actin for steady-state polymerization (Carlier et al., 1997), and plays a role in severing actin filaments to create new barbed ends that polymerization requires. An optoCofilin provides spatiotemporal control of motility and lamellipodia formation (Hughes & Lawrence, 2014). By contrast, conventional techniques (e.g., overexpression of constitutively active or inactive cofilin mutants) do not afford the means to explore the dynamic elements associated with these cellular activities. We've directed optoCofilin to the cytoskeleton in a spatially targeted fashion, which can be applied to assays of filopodial (Jacquemet, Hamidi, & Ivaska, 2015) and lamellipodial organization (Vitriol, Wise, Berginski, Bamburg, & Zheng, 2013) and therefore cellular motility (Hughes & Lawrence, 2014).

2.1 Light-mediated directed cell motility

Actin binding proteins (ABPs) are vital components of the complex biochemical machinery that determine the direction and duration of cellular migration (dos Remedios et al., 2003). By utilizing light to recruit and concentrate ABPs to regions of the F-actin cytoskeleton located near the cell's leading edge, it is possible to provoke protrusive behavior and subsequent cellular motility in the direction of the protrusion. Light-directed cell motility is accompanied by an increase in the area of the lamellipodia and movement of the cell in the direction of the localized light pulse. The following protocol describes the light-activated recruitment of an actin-binding impaired cofilin to the F-actin cytoskeleton in MTLn3 cells (Hughes & Lawrence, 2014).

2.1.1 Equipment and materials

1. Olympus FV1000 point scanning confocal with an IX81 microscope base, equipped with lasers and filter sets for 488 and 559nm light stimulation
2. Fluoview software with Stimulus window
3. 60× oil immersion objective lens
4. Opaque heat, humidity, and atmosphere (5% CO₂) controlled microscope enclosure
5. 37°C tissue culture incubator with 5% CO₂ and 95% humidity
6. 35mm glass bottom dishes (Mattek)
7. MEM alpha (Gibco 12561) supplemented with 5% FBS and 1% Pen-Strep
8. MEM alpha (no Phenol Red; Gibco 41061) supplemented with 5% FBS and 1% Pen-Strep
9. DPBS with calcium and magnesium (Gibco LS14040133)
10. Trypsin-EDTA (0.05%) (Gibco 25300054)
11. Mammalian expression plasmids: Cofilin^{S3A/S120A}-Cry2-mCh, Cry2-mCh, LifeAct-Cib-GFP prepared in MIDI prep quality or higher. (The vectors for the pCry2-mCherry-N1 backbone used in the Cry2-mCh constructs and the phCMV-FSR-GFP used to generate the LifeAct-Cib-GFP are available from Clontech (632523) & Genelantis (P013400), respectively.
12. JetPrime transfection reagent (PolyPlus transfection 114–07)
13. MTLn3 cells (Accession no. CVCL_8136)

2.1.2 Protocol

Day 1

1. Wash (DPBS) and trypsinize (Trypsin-EDTA) MTLn3 cells grown to 70–80% confluency in MEM alpha/5% FBS/1% Pen-Strep in a T75 vented tissue culture flask.
2. Count cells, plating 75,000 per 35mm Mattek dish. Supplement dish with enough MEM alpha media to cover the entire surface of the dish (1–2mL).

Day 2

3. After overnight incubation (16–24h) in a 37°C tissue culture incubator, visually inspect cells for adherence to the surface of the glass bottomed dish. If cells are not fully adherent, then transfection should be postponed.
4. Transfect the cells with plasmid DNA encoding the optogenetic constructs. For dual plasmid transfections in 35mm dishes, plasmid DNA was combined in a 1:1 ratio (500ng per construct) and diluted in 200μL of JP buffer. 2.4μL of JetPrime reagent was then added and the solution vortexed for 10s. After incubating for

10min at room temperature, the transfection complexes are added to the cells for 4h, after which the media should be aspirated and fresh MEM alpha with FBS added. After replacing the media, dishes should be wrapped in foil and returned to the tissue culture incubator.

Day 3

5. In a dark room under a red safe light, exchange the cell culture media with pre-warmed, phenol red free MEM alpha media supplemented with 5% FBS. Keep the cell culture media wrapped in foil during transportation to and from the tissue culture incubator. At this point, the microscope should be powered on and the microscope incubator should be pre-warmed for at least 1h.
6. At 60× magnification (oil immersion objective) locate the focal plane of the cells with the Texas Red/mCh light source. It may be useful to keep on hand a Mattek dish of mCh-expressing cells (live or fixed) in order to place the objective in the correct plane prior to adding the sample of interest.
7. Scan the dish for cells expressing the fluorescent mCh marker. Cells selected for light-directed motility experiments should be suitably bright and fully adherent, with a clearly defined leading edge. Once a cell has been selected for analysis, utilize the Fluoview region of interest (ROI) tool to define a circular ROI near the leading edge of the cell. The size of the ROI, the laser power, and the scan speed will need to be optimized so that localized illumination of the cell does not result in wholesale recruitment of the ABP across the entire cell. For the microscope used in the cofilin experiments, a scan rate of 12μs/pixel and 488nm laser at 10% power is sufficient for localized recruitment of optoCofilin to F-actin at the leading edge. After the initial recruitment with the 488nm laser, cells can be imaged every minute with the Texas Red/mCh channel in order to track changes in cell shape and light-directed migration. If desired, the ROI can be moved to the cell's new coordinates at the end of the time course, and F-actin recruitment can be re-stimulated, and the cell trajectory monitored again at the desired time interval.

Image analysis

8. In FIJI/ImageJ, directed cell migration can be tracked by manually outlining the cell body at each acquisition time point. For example, if the cells are imaged every minute for 10min post-localized recruitment, then 10 different sets of coordinates will be manually generated. Once the cell has been manually outlined, the X, Y coordinates of the cell centroid (ANALYZE→SET MEASUREMENTS→CENTROID) can be generated with the command (CTRL +M). Once centroid data have been generated for all of the analyzed cells, each cell trajectory should be individually centered by subtracting the initial X, Y coordinates from each subsequent set of coordinates in the trajectory. Normalized coordinates can then be plotted using a graphing software package such as SigmaPlot and compared to the trajectories of control cells (for example, cells co-transfected with Cry2-mCh and LifeAct-Cib-GFP).

2.2 Filopodia induction protocol

Filopodia are among the dynamic cellular structures (others include stress fibers and lamella that are routinely generated and recycled during cytoskeletal remodeling). These visceral, spiky, and actin-rich protrusions decorate the cell membrane and are involved in cell adhesion as well as migration (Jacquemet et al., 2015) and are characterized by their protrusion beyond the lamella. Increased filopodia formation has been directly linked to increased cofilin activation in cells (Sumi, Matsumoto, Takai, & Nakamura, 1999). Additional ABPs are also involved in filopodia generation and recycling, and thus experiments to determine filopodial content are an important part of characterizing the activity of any ABP fused to a light responsive protein. The following protocol describes the generation and quantification of filopodia upon complete recruitment of an impaired actin-binding cofilin to the F-actin cytoskeleton, as described in Hughes and Lawrence (2014) (Fig. 1).

2.2.1 Equipment and materials

1. Inverted Olympus IX81 microscope equipped with a Hamamatsu C8484 camera, and FITC, Texas Red, and Cy5.5 filter cubes (Semrock)
2. Metamorph software
3. 60× magnification oil immersion Plan S-Apo objective
4. Opaque heat, humidity, and atmosphere (5% CO₂) controlled microscope enclosure
5. 37°C tissue culture incubator with 5% CO₂ and 95% humidity
6. 35mm glass bottom dishes (Mattek)
7. MEM alpha (Gibco, 12561) supplemented with 5% FBS and 1% Pen-Strep
8. MEM alpha (no Phenol Red; Gibco, 41061) supplemented with 5% FBS and 1% Pen-Strep
9. DPBS with calcium and magnesium (Gibco, LS14040133)
10. Trypsin-EDTA (0.05%) (Gibco 25300054)
11. Mammalian expression plasmids: cofilin^{S3A/S120A}-Cry2-mCh (optoCofilin), Cry2-mCh, and LifeAct-Cib-GFP, prepared in MIDI prep quality or higher
12. JetPrime transfection reagent (PolyPlus transfection 114-07)
13. MTLn3 cells (Accession no. CVCL_8136)

2.2.2 Protocol

Day 1

1. Wash (DPBS) and trypsinize (Trypsin-EDTA) MTLn3 cells grown to 70–80% confluency in MEM alpha/5% FBS/1% Pen-Strep in a T75 vented tissue culture flask.

2. Count cells, plating 75,000 per 35mm Mattek dish. Supplement dish with enough MEM alpha media to cover the entire surface of the dish (1–2mL).

Day 2

3. After overnight incubation (16–24h) in a 37°C tissue culture incubator, visually inspect cells for adherence to the surface of the glass bottomed dish. If cells are not fully adherent, then transfection should be postponed.
4. Transfect cells with plasmid DNA encoding the optogenetic constructs. For dual plasmid transfections in 35mm dishes, plasmid DNA was combined in a 1:1 ratio (500ng per construct) and diluted in 200µL of JP buffer. 2.4µL of JetPrime reagent was then added and the solution vortexed for 10s. After incubating for 10min at room temperature, the transfection complexes are added to the cells for 4h, after which the media should be aspirated and fresh MEM alpha with FBS added. After replacing the media, dishes should be wrapped in foil and returned to the tissue culture incubator.

Day 3

5. In a dark room under a red safe light, exchange the cell culture media with pre-warmed, phenol red free MEM alpha media supplemented with 5% FBS. Keep the cell culture media wrapped in foil during transport to and from the tissue culture incubator. At this point, the microscope should be powered on and the microscope incubator should be pre-warmed for at least 1h.
6. At 60× magnification (oil immersion objective) locate the focal plane of the cells with the Texas Red/mCh light source. It may be useful to keep on hand a Mattek dish of mCh-expressing cells (live or fixed) in order to place the objective in the correct plane prior to adding the sample of interest.
7. Scan the dish for cells expressing the fluorescent mCh marker. Cells selected for filopodia-forming experiments should be suitably bright and fully adherent. In this experiment, both Texas Red/mCh and FITC/GFP channels are imaged at each time point (once every 60s over 10min). Exposure times will need to be optimized for the experiment, as the mCh fluorescence is typically significantly less bright than the GFP fluorescence. In this experiment, the GFP channel exposure time is 100ms, while the mCh exposure time is 300ms.

Image analysis

8. Filopodia are manually counted at the beginning of the time course and at the end of the time course for each cell analyzed using the FITC channel images of the LifeAct-Cib-GFP construct. Filopodia are distinguished by their size (2–10µm) and their extension beyond the lamella, which differentiate them from other cytoskeletal features such as focal adhesions. The fold change in filopodia (10min versus 0min) is then calculated for each cell in the analysis and the average net fold change calculated for each experimental condition.

3. Optogenetic control of apoptosis

Under physiological conditions the regulation of cell fate is tightly controlled. An engineered optoBax has been developed which is recruited to the outer mitochondrial membrane (OMM) in a light-triggered fashion, thereby offering spatiotemporally precise method to initiate apoptosis.

3.1 Light-triggered cellular collapse/apoptosis

Cellular collapse, characterized by a loss of cellular adhesion and membrane blebbing, is one of the hallmarks of apoptosis. The recruitment of Bcl-2 family proteins such as Bax to the OMM results in mitochondrial outer membrane permeabilization (MOMP) (Dewson & Kluck, 2009). The contents released from the resulting pores in the OMM include cytochrome C and Smac1/Diablo. The presence of these proteins in the cytosol initiates the biochemical cascade resulting in caspase activation and ultimately cell death. As such, experiments in which optogenetic Bcl-2 family proteins undergo sustained recruitment to the OMM with light are an important test of their intended biochemical activity. In this protocol, we describe cellular collapse assays in HeLa cells with a cytosolic Bax mutant fused to a light responsive Cry2 protein, as reported in Hughes et al. (2015).

3.1.1 Equipment and materials

1. Inverted Olympus IX81 microscope equipped with a Hamamatsu C8484 camera, and FITC, Texas Red, and Cy5.5 filter cubes (Semrock)
2. Metamorph software
3. 60× magnification oil immersion Plan S-Apo objective
4. Opaque heat, humidity, and atmosphere (5% CO₂) controlled microscope enclosure
5. 37°C tissue culture incubator with 5% CO₂ and 95% humidity
6. 35mm glass bottom dishes (Mattek)
7. DMEM (Gibco 41965039) supplemented with 10% FBS and 1% Pen-Strep
8. DMEM (no Phenol Red; Gibco 31053028) supplemented with 10% FBS and 1% Pen-Strep
9. DPBS with calcium and magnesium (Gibco LS14040133)
10. Trypsin-EDTA (0.05%) (Gibco 25300054)
11. Mammalian expression plasmids: Cry2-mCh-Bax^{S184E}, Cry2-mCh, and Tom20-Cib-GFP, prepared in MIDI prep quality or higher
12. JetPrime transfection reagent (PolyPlus transfection 114–07)
13. HeLa cells (ATCC CCL-2)

3.1.2 Protocol

Day 1

1. Wash (DPBS) and trypsinize (Trypsin-EDTA) HeLa cells grown to 80–90% confluency in DMEM/10% FBS/1% Pen-Strep in a T75 vented tissue culture flask.
2. Count cells, plating 100,000 per 35mmMattek dish. Supplement dish with enough DMEM media to cover the entire surface of the dish (1–2mL).

Day 2

3. After overnight incubation (16–24h) in a 37°C tissue culture incubator, visually inspect cells for adherence to the surface of the glass bottomed dish. If cells are not fully adherent, then transfection should be postponed.
4. Transfect the cells with plasmid DNA encoding the optogenetic constructs. For dual plasmid transfections in 35mm dishes, plasmid DNA is combined in a 1:1 ratio (500ng per construct) and diluted in 200µL of JP buffer. 2.4µL of JetPrime reagent is then added and the solution vortexed for 10s. After incubating for 10min at room temperature, the transfection complexes are added to the cells for 4h, after which the transfection media are aspirated and fresh DMEM with FBS added. After replacing the media, dishes should be wrapped in foil and returned to the tissue culture incubator.

Day 3

5. In a dark room under a red safe light, exchange the cell culture media with pre-warmed, phenol red free DMEM media supplemented with 10% FBS. Keep the cell culture media wrapped in foil during transport to and from the tissue culture incubator. At this point, the microscope should be powered on and the microscope incubator should be pre-warmed for at least 1h.
6. At 60× magnification (oil immersion objective) locate the focal plane of the cells with the Texas Red/mCh light source. It may be useful to keep on hand a Mattek dish of mCh-expressing cells (live or fixed) in order to place the objective in the correct plane prior to adding the sample of interest.
7. Scan the dish for cells expressing the fluorescent mCh marker. Cells selected for cellular collapse experiments should be suitably bright, fully adherent, and show no signs of light-independent mitochondrial localization. *Tip: mitochondrial accumulation may begin to occur if the cells are exposed to the Texas Red channel for too long. As a result, exposure times should be minimized.* In this experiment, both Texas Red/mCh and FITC/GFP channels are imaged at each time point (once every 120s for the duration of the experiment), with the typical total experimental length no longer than 60min. While exposure times will need to be optimized for the Texas Red channel (will range from 100 to 300ms), the FITC channel should be minimized to reduce the potential for light induced

cytotoxicity. In this experiment, the FITC/GFP channel exposure time was restricted to 10ms.

Image analysis

8. Cellular collapse is characterized by the loss of adherent cell morphology and the appearance of membrane blebbing. Although this can occur as early as 15–20min into the experiment, the timing of cellular collapse can vary based upon transfection level of the pro-apoptotic optogenetic proteins. Quantification of the timing of cellular collapse can be easily accomplished by importing the acquired images into ImageJ by placing the time lapse images from a single optical channel into the same folder, then choosing File→Import→Image Sequence and selecting the filename of the first image in the series. A timestamp can then be added to the images by choosing Image→Stacks→Label, then selecting the 00:00 label format and entering the appropriate image interval (in seconds). The collapse timepoints of individual cells in each field of view can then be determined by visual inspection.

3.2 Small molecule inhibition of light-driven apoptosis

Numerous small molecules and peptides are known to be inhibitors of apoptosis (e.g., the caspase inhibitor Z-VAD-FMK and the calpain inhibitor MDL-28170). However, inhibitors that specifically target Bcl-2 mediated MOMP are less common, and screening for these compounds with an optogenetic cell death system represents a potential avenue for drug discovery. In this protocol, we describe an assay for the inhibition of optogenetic apoptosis with a Bax-channel blocker (Bonbrun et al., 2003), a compound with well characterized anti-MOMP activity, as reported in Hughes et al. (2015).

3.2.1 Equipment and materials

1. Inverted Olympus IX81 microscope equipped with a Hamamatsu C8484 camera, and FITC, Texas Red, and Cy5.5 filter cubes (Semrock)
2. Metamorph software
3. 60× magnification oil immersion Plan S-Apo objective
4. Opaque heat, humidity, and atmosphere (5% CO₂) controlled microscope enclosure
5. 37°C tissue culture incubator with 5% CO₂ and 95% humidity
6. 35mm glass bottom dishes (Mattek)
7. DMEM (Gibco 41965039) supplemented with 10% FBS and 1% Pen-Strep
8. DMEM (no Phenol Red; Gibco 31053028) supplemented with 10% FBS and 1% Pen-Strep
9. DPBS with calcium and magnesium (Gibco LS14040133)
10. Trypsin-EDTA (0.05%) (Gibco 25300054)

11. Mammalian expression plasmids: Cry2-mCh-Bax^{S184E}, Cry2-mCh, and Tom20-Cib-GFP, prepared in MIDIprep quality or higher
12. JetPrime transfection reagent (PolyPlus transfection 114–07)
13. HeLa cells (ATCC CCL-2)
14. Bax-channel blocker (Santa Cruz Biotechnology sc-203524)

3.2.2 Protocol

Day 1

1. Wash (DPBS) and trypsinize (Trypsin-EDTA) HeLa cells grown to 80–90% confluency in DMEM/10% FBS/1% Pen-Strep in a T75 vented tissue culture flask.
2. Count cells, plating 100,000 per 35mm Mattek dish. Supplement dish with enough DMEM media to cover the entire surface of the dish (1–2mL).

Day 2

3. After overnight incubation (16–24h) in a 37°C tissue culture incubator, visually inspect cells for adherence to the surface of the glass bottomed dish. If cells are not fully adherent, then transfection should be postponed.
4. Transfect the cells with plasmid DNA encoding the optogenetic constructs. For dual plasmid transfections in 35mm dishes, plasmid DNA is combined in a 1:1 ratio (500ng per construct) and diluted in 200μL of JP buffer. 2.4μL of JetPrime reagent is then added and the solution vortexed for 10s. After incubating for 10min at room temperature, the transfection complexes are added to the cells for 4h, after which the transfection media are aspirated and fresh DMEM with FBS added. After replacing the media, dishes should be wrapped in foil and returned to the tissue culture incubator.

Day 3

5. In a dark room under a red safe light, exchange the cell culture media with pre-warmed, phenol red free DMEM media supplemented with 10% FBS and the desired concentration of Bax channel blocker. For example, a final concentration of 20μM of Bax channel blocker in 2mL of DMEM can be with the addition of 2.2μL of an 18.5mM stock solution of Bax channel blocker (Santa Cruz sc-203524) in DMSO. The quantity of DMSO added to the cell media is critical, as the non-small molecule containing control sample must have an equivalent volume of DMSO in the sample media. After addition of the compound-containing cell media, cells are then allowed to incubate 1h in a 37°C humidity-controlled tissue culture incubator prior to imaging. If the experimental goal is to analyze a series of samples at different inhibitor concentrations, then the treatment of samples with the small molecule must be staggered so that the pre-incubation time is the same (i.e., 1h) for all samples in the experiment.

6. At 60× magnification (oil immersion objective) locate the focal plane of the cells with the TxRed Red/mCh light source. It may be useful to keep on hand a Mattek dish of mCh-expressing cells (live or fixed) in order to place the objective in the correct plane prior to adding the sample of interest.
7. Scan the dish for cells expressing the fluorescent mCh marker. Cells selected for cellular collapse experiments should be suitably bright, fully adherent, and show no signs of light-independent mitochondrial localization. *Note: at higher concentrations of Bax channel blocker (>10 μM), cell morphology will become noticeably more rounded, making image analysis more difficult.* In this experiment, cells are imaged to determine the minimum time required for cellular collapse in the presence of the inhibitor. Therefore, it may be most practical to start with the lowest concentration of inhibitor first and subsequently employ higher concentrations. Cells should be imaged with a protocol identical to that in Section 3.1. In this experiment the inhibitor effect of Bax channel blocker was found to be titratable from 1 to 10 μM.

Image analysis

8. Image analysis should be conducted for each concentration of inhibitor (including the no-inhibitor control) utilizing the method detailed in Protocol step 8 in Section 3.2.

4. Optogenetic regulation of the cAMP-dependent protein kinase

An optogenetic cAMP-dependent protein kinase (optoPKA) offers the ability to regulate enzymatic activity at localized intercellular regions. Because of its ubiquitous expression, compartmentalized cellular signaling is often difficult to assess with traditional gain of function or loss of function experiments (Koschinski & Zaccolo, 2017; O'Banion et al., 2018). Targeted recruitment of optoPKA to the OMM or the plasma membrane has been described by O'Banion et al. (2018), enabling spatially delimited downstream pathways to be queried. Since PKA is known to be a critical mediator of many intracellular processes (apoptosis, lipid metabolism, mitochondrial energy transfer, epithelial-mesenchymal transfer), the strategy holds potential for application across many pharmacological and physiological systems.

4.1 Light induced phosphorylation and subsequent identification of endogenous substrates

Optogenetic proteins kinases allows for the discovery of new enzymatic substrates and the confirmation of known endogenous kinase substrates at specific subcellular locations. The following protocol outlines a strategy for understanding organelle specific protein phosphorylation patterns elicited by the photoactivation of optoPKA. Recruitment of the catalytic subunit of PKA (designated as “C” in Cry2-mCh-C^{W196R/Y204A}) to specific intracellular sites is triggered using a 470nm LED light (O'Banion et al., 2018). Site-specific recruitment of optoPKA induces unique protein phosphorylation signatures as assessed by phosphoproteomics.

4.1.1 Equipment and materials

1. Blue LED light source (Home Depot PAR38 Blue LED Flood Lamp Model #469072)
2. Six-well tissue culture plates (Greiner Bio-One 657–160)
3. 37°C tissue culture incubator with 5% CO₂ and 95% humidity
4. DMEM (Gibco 41965039) supplemented with 10% FBS and 1% Pen-Strep
5. DPBS with calcium and magnesium (Gibco LS14040133)
6. Trypsin-EDTA (0.05%) (Gibco 25300054)
7. Mammalian expression plasmids: Cry2-mCh-C^{W196R} (Addgene #104548), Cry2-mCh-C^{W196R/Y204A} (Addgene #104546), and Cry2-mCh-C^{W196R/F327A} (Addgene #104547), and Cib-GFP-CAAX (Addgene #26867), prepared in MIDI prep quality or higher
8. JetPrime transfection reagent (PolyPlus transfection 114–07)
9. HEK293T cells (ATCC CRL-3216)
10. Forskolin (Sigma-Aldrich F3917; 10mM stock solution in DMSO)

4.1.2 Protocol

4.1.2.1 Initial screening for endogenous PKA substrates

Day 1

1. HEK293T cells maintained in DMEM/10% FBS/1% Pen-Strep were plated in six-well tissue culture plates at a density of 130,000 cells per well.

Day 2

2. Cells are transfected with 1µg of total DNA per well (500ng Cib-GFP-CAAX with 500ng Cry2-mCh-C^{W196R}, Cry2-mCh-C^{W196R/F327A}, Cry2-mCh-C^{W196R/Y204A}, or Cry2-mCh) using the JetPrime transfection reagent (2µL JetPrime Polyplus reagent: 200µL JetPrime buffer), following the manufacturer's protocol. The cell media were changed to complete media at 4h post-transfection and the cells stored overnight, in a humidified, light-protected, tissue culture incubator (37°C; 5% CO₂).

Day 3

3. In a dark room under a red safety light, cells are washed 1× with 2mL DPBS, then serum starved in 2mL PBS for 15min in a humidified tissue culture incubator (37°C; 5% CO₂), followed by light exposure (2min; blue LED flood lamp; continuous illumination: 470nm LED flood light (PAR38; Westinghouse 0315100; 1.7mW power)) or darkness (2min). Forskolin treated cells (non-transfected control cells also seeded on Day 1) are incubated with 20µM forskolin in DPBS for 2min. Post-light exposure or forskolin treatment, DPBS is aspirated, and cells are lysed with 400µLM-PER (ThermoScientific) containing

1× Halt Protease and Phosphatase Inhibitor cocktail. Cells are then lysed in the dark for 4min at room temperature, prior to transferring to 1.7mL centrifuge tubes and centrifuged (5 min; 1000×g; 4°C). 15µL of the resulting supernatants are then diluted 1:1 with Laemmli sample buffer containing BME, then boiled for 5min at 95°C prior to analysis by western blot.

4. Western Blot: Samples are separated by SDS-PAGE [4–12% Tris-Glycine gradient gel (Bio-Rad)], then transferred to PVDF membrane overnight (20V; 4°C). The transfer membrane is then blocked (5% BSA; TBS-Tween) for 1h prior to overnight incubation with the primary antibody for the recognition of all consensus PKA phosphorylation sites, Phospho-PKA Substrate antibody (Cell Signaling; 9624S), diluted 1:1000 in blocking buffer.

Day 4

5. After overnight incubation, the membrane is washed (35m; TBS-Tween) and placed in secondary antibody (Cell Signaling; 7074P2), diluted 1:3000 in blocking buffer, for 2h at room temperature. After washing membrane (4×5min; TBS-Tween), blot is developed with chemiluminescent substrate (Bio-Rad Clarity Western ECL substrate) for 5min prior to imaging. For complete analysis, blots are stripped (ThermoScientific; RESTORE buffer) according to package instructions and re-probed with GAPDH (Cell Signaling; 5174S) and mCh (Abcam; 167453) antibodies.

Analysis: Phosphoprotein bands of interest are selected based on the following criteria: increased phosphorylation over background in the presence of forskolin, and, for the samples transfected with optogenetic constructs, increased phosphorylation in the presence of light versus non-illuminated control cells. Bands are quantified using densitometry analysis in FIJI/ImageJ (Analyze→Gels→Select Lanes; Analyze→Gels→Plot Lanes).

Day 5

6. Lysates from cells transfected with Cry2-mCh-C^{W196R} (30µL; 4mg/mL lysate concentration as determined by Bradford assay) are electrophoresed on a 4–12% Tris-Glycine gradient gel. A region of the gel containing the band(s) of interest identified by the western blot (Day 4) was excised with a sterile scalpel or razor blade. The band was then sent to a proteomics facility for phospho-proteome isolation and characterization (Michael J. Hooker Proteomics Core Facility). *Abbreviated phosphoproteomics protocol:* bands are in-gel digested with trypsin overnight. The resulting peptides are enriched for phosphopeptides using TiO₂, and the TiO₂ elution for each sample is analyzed by LC/MS/MS (Easy nLC 1000-Qexactive HF). Samples are eluted over a 45min gradient from 5% to 35% B, where mobile phase A=0.1% formic acid and mobilephase B=acetonitrile with 0.1% formic acid. The top 15 most intense ions are chosen for HCD fragmentation, and data are processed using Proteome Discoverer 1.4. Finally, data are searched against a reviewed Human Uniprot database using Sequest (10ppm precursor ion mass tolerance; 0.02Da product ion mass tolerance; up to

two missed trypsin cleavage sites; carbamidomethylation of Cys is set as a fixed modification; oxidation of Met, deamidation of Asn and Gln, and phosphorylation of Ser, Thr, Tyr are set as variable modifications). Peptide false discovery rates of 5% are used to filter all results.

Analysis: The following data show the top seven “hits” identified from the isolated gel band. For confirmation of the band of interest, antibodies for the top scoring PKA-substrate phospho-proteins were purchased and the lysates re-probed by western blot (COPE: Abcam, ab180946. Emerin: Cell Signaling, 5430S. KCTD5: Abcam, ab118145. SMN1: Cell Signaling, 12976S; RING finger: Cell Signaling, 5754S). In the follow-up western blot, only the band revealed by the Abcam COPE antibody matched the electrophoretic mobility of the unknown phospho-protein of interest.

Protein ID	%Abundance (Rel. peak area)	MW (kDa)
Coatamer subunit epsilon OS = Homo sapiens GN = COPE PE = 1 SV = 3— [COPE_HUMAN]	100.00	34,482
Mitochondrial import receptor subunit TOM34 OS = Homo sapiens GN = TOMM34 PE = 1 SV = 2—[TOM34_HUMAN]	88.69	34,559
Emerin OS = Homo sapiens GN = EMD PE = 1 SV = 1—[EMD_HUMAN]	48.06	28,994
Ubiquitin-conjugating enzyme E2 J1 OS = Homo sapiens GN = UBE2J1 PE = 1 SV = 2—[UBE2J1_HUMAN]	39.26	35,199
RING finger and CHY zinc finger domain-containing protein 1 OS = Homo sapiens GN = RCHY1 PE = 1 SV = 1—[ZN363_HUMAN]	38.26	30,110
BTB/POZ domain-containing protein KCTD5 OS = Homo sapiens GN = KCTD5 PE = 1 SV = 1—[KCTD5_HUMAN]	8.78	26,093
Survival motor neuron protein OS = Homo sapiens GN = SMN1 PE = 1 SV = 1— [SMN_HUMAN]	5.77	31,849

5. Illumination methods for optogenetic studies

Illumination methods for optogenetic experiments vary widely, ranging from fiber optic implants for in vivo experimentation in animal models, to microscope-based laser lines and filter sets, to the use of off-the-shelf light sources from the local hardware store. Some illumination methods are analogous to the surgeon’s scalpel, enabling precise spatial resolution at the subcellular level and the targeting of individual organelles (μm -level), while others resemble a cudgel, and are useful in experiments requiring broad illumination of a large surface area, such as a tissue section or flask of cells. In this section, we describe different light sources, and their utility in different classes of optogenetic experiments. Fig. 2 outlines considerations with which we design our illumination approach for optogenetic assays.

6. Summary

In this chapter we outline cellular assays which take advantage of a modified protein of interest fused into a cryptochrome based optogenetic system. We describe an optoCofilin that is used to probe and manipulate the biochemical pathways that drive cell motility. Light-

triggered site-specific recruitment of optoCofilin initiates protrusive behavior and subsequent cellular motility in the direction of the protrusion. We have also developed an optogenetic version of the pro-apoptotic factor bcl-2-like protein 4 (Bax), which is engineered to migrate to the OMM in a light-dependent fashion, thereby triggering cellular collapse and apoptosis. Finally, we have designed an optogenetic protein kinase (optoPKA) that enables the activation of signaling pathways with exquisite spatial resolution, thereby allowing for the identification of downstream substrates.

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Abbreviations

ADF	cofilin/actin-depolymerizing factor family proteins
Bax	Bcl-like protein 4
Cry2/Cib	cryptochrome 2 Cib protein pair
MOMP	mitochondrial outer membrane permeabilization pore
OMM	outer mitochondrial membrane
optoBax	optogenetic Bcl-like protein 4
optoCofilin	optogenetic cofilin
optoPKA	optogenetic cAMP dependent protein kinase
PKA	cAMP-dependent protein kinase A
ROI	region of interest

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Fig. 1.

Cofilin-induced filopodia in MTLn3 cells. (A) F-actin network of an MTLn3 cell transfected with Cofilin^{S3A/S120A}-Cry2-mCh/LifeAct-Cib-GFP prior to (*left*) and 10min after (*right*) illumination. (B) F-actin network of a Cry2-mCh/LifeAct-Cib-GFP transfected MTLn3 cell prior to (*left*) and 10min after (*right*) illumination. Numerous filopodia are formed in the cofilin-transfected cells following light stimulation [highlighted in (A)]. *Reprinted with permission from Hughes, R. M., & Lawrence, D. S. (2014). Optogenetic engineering: Light-directed cell motility. Angewandte Chemie (International Ed. in English), 53(41), 10904–10907. doi:10.1002/anie.201404198.*

	Programmable LED Array	Microscope Based Light Source	Hand Held / Mountable LED Lamp
Description	<ul style="list-style-type: none"> • Programmable LED arrays can be assembled in-house from components that are readily available from online suppliers. • At the core of LED array designs is a programmable driver such as the Arduino UNO board. Numerous Arduino-based systems have been reported in the literature, with detailed plans, schematics, and source code. • LED arrays can be customized for different illumination patterns for different plate footprints. 	<ul style="list-style-type: none"> • Microscopes excel at providing precise information at the cellular level, the primary workhorses of cellular optogenetics. • Software packages for most commercially available scopes contain features for timing illumination events and for collecting data over different time scales, from experiments lasting a few minutes to experiments lasting several days. • Single cell illumination experiments are typically conducted on scanning confocal microscopes, while multi-cell illumination experiments can be conducted with both confocal and wide-field techniques. 	<ul style="list-style-type: none"> • Off the shelf light sources are available in a wide variety of wavelengths of light. Flood lamps, holiday lights, and even laser pointers have been repurposed as light sources in optogenetic experiments. • In experiments where the time domain does not require absolute precision, these light sources can be as good or better than engineered LED arrays. • Good entry level tools for conducting large scale optogenetics work with cells or tissues.
Recommended Use	Fixed Cellular Analysis Immunostaining	Single Cell illumination Live cell microscopy Live cell pharmacology Life cell reporter assay	Western Blot Fixed Cellular Analysis Immunostaining
Cost	\$\$	\$\$\$\$\$	\$

Fig. 2.

A summary of illumination methods for optogenetic studies.