Chapter 1

Optogenetic Control of Ras/Erk Signaling Using the Phy–PIF System

Alexander G. Goglia, Maxwell Z. Wilson, Daniel B. DiGiorno, and Jared E. Toettcher

Abstract

The Ras/Erk signaling pathway plays a central role in diverse cellular processes ranging from development to immune cell activation to neural plasticity to cancer. In recent years, this pathway has been widely studied using live-cell fluorescent biosensors, revealing complex Erk dynamics that arise in many cellular contexts. Yet despite these high-resolution tools for measurement, the field has lacked analogous tools for *control* over Ras/Erk signaling in live cells. Here, we provide detailed methods for one such tool based on the optical control of Ras activity, which we call "Opto-SOS." Expression of the Opto-SOS constructs can be coupled with a live-cell reporter of Erk activity to reveal highly quantitative input-to-output maps of the pathway. Detailed herein are protocols for expressing the Opto-SOS system in cultured cells, purifying the small molecule cofactor necessary for optical stimulation, imaging Erk responses using live-cell microscopy, and processing the imaging data to quantify Ras/Erk signaling dynamics.

Key words Optogenetics, Signal transduction, Single-cell dynamics, MAP kinase, Ras, Erk

1 Introduction

The Ras/Erk signaling cascade is one of the most intensely studied protein kinase networks in cell biology, responding to a variety of external stimuli by modulating cell growth, survival, and differentiation. In recent years, advances in live-cell biosensors have revolutionized our ability to measure Erk activity in single cells, both in vitro [1–4] and in vivo [5–7]. These studies have uncovered a wealth of dynamic responses, including Erk responses that exhibit switchlike activation [8, 9], oscillations [3], and traveling waves propagating from cell to cell across tissues [5, 10].

The discovery of this rich world of Erk dynamics begs a question: what patterns of Erk activity determine a cell's response? We might imagine that certain genes respond selectively to a specific frequency of Erk oscillations or to the total "area under the curve" of Erk activity. Erk dynamics may also vary significantly between identically

Aik-Choon Tan and Paul H. Huang (eds.), *Kinase Signaling Networks*, Methods in Molecular Biology, vol. 1636,
DOI 10.1007/978-1-4939-7154-1_1,

treated cells. Yet some dynamic features may vary more between cells than others; and these noise-resistant features could be relied upon by the cell to accurately sense the environment [11].

Knowledge of the role played by such signaling dynamics has been limited by a lack of tools to selectively control Ras/Erk activity. Extracellular ligands often bind tightly (and therefore cannot be washed on and off with high temporal resolution), and receptor internalization can desensitize cells to subsequent stimuli. Cellular optogenetics offers one solution to this challenge by engineering light-responsive proteins that can be expressed in cells to control specific pathways of interest, such as Ras/Erk [12–14]. These light-inducible systems possess two unique features: (1) they are specific to only the pathway under optogenetic control, and (2) they allow for true dynamic control because input intensity can be precisely tuned in real time. Recent advances by our group and others have yielded a number of engineered signaling modules that can be controlled with spatially and temporally precise beams of visible light [14–22]. While many excellent optogenetic systems have been developed, this article will focus on the light-inducible "Phy/PIF" interaction system and its important advantages for use in cell signaling. Of currently available optogenetic tools, the Phy/PIF system provides the fastest dynamic control, the broadest dynamic range, and the highest light-sensitivity (thereby limiting phototoxicity) [23].

In addition to studying how Erk dynamics control cellular responses, optogenetic inputs could also be used to dissect how Erk dynamics themselves are generated. Ras/Erk signaling is subject to complex feedback regulation [24–27], crosstalk from other signaling pathways [28], and frequent mutation (pathway components are among the most commonly mutated nodes in human cancers) [29, 30]. By simultaneously stimulating Ras activity and measuring Erk responses in the presence of different extracellular cues, drugs, or mutations, we might learn how each condition affects transmission of signals through the pathway. In this chapter, we will describe how to use the Phy/PIF optogenetic system to control Ras/Erk signaling in cultured mammalian cells.

The Phy/PIF system involves light-dependent association of fragments of two *Arabidopsis thaliana* proteins normally involved in stem elongation: phytochrome B (Phy) and phytochrome interaction factor (PIF) 6 [31, 32]. Photoactivity of Phy depends on its ligation to the small molecule chromophore phycocyanobilin (PCB), which must be provided exogenously to cells of non-photosynthetic organisms. Red (650 nm) light induces a conformational change in PCB-bound Phy that causes PIF to bind in a matter of seconds (Fig. 1). In the absence of further light input, this interaction persists for hours, while administration of infrared (750 nm) light reverts Phy to its inactive conformation and promotes rapid dissociation of the Phy-PIF heterodimer [17]. One of

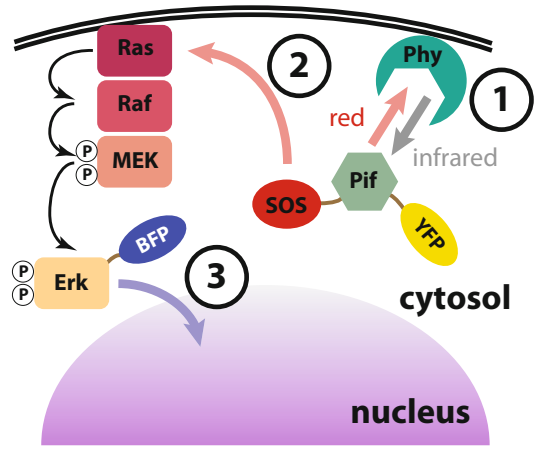

Fig. 1 Using the Phy/PIF optogenetic system to control Ras/Erk activity. Red light (650 nm) input promotes membrane-bound Phy heterodimerization with cytosolic YFP-PIF-SOScat, causing Ras activation and nuclear import of BFP-Erk. Infrared light (750 nm) reverses the Phy-PIF interaction

the great strengths of the Phy/PIF system is the speed with which Phy changes activation states in response to light input, inducing or terminating Phy-PIF binding within seconds of receiving the appropriate light input. Similarly, this system is highly robust and can be switched on/off hundreds of times with no detectable loss of signal and no cellular toxicity. The tremendous light sensitivity of this system minimizes the likelihood of phototoxicity, as it is activated/inactivated using dilute red (650 nm, 20 μmol/m^2/s) and infrared (>750 nm, 300 μmol/m^2/s) light, respectively [17].

Here, we describe a Phy/PIF-based optogenetic system that can be used for the light-dependent control of Ras/Erk signaling. The Phy/PIF interaction is used to control Ras pathway activation by expressing the constituent Phy and PIF protein domains as fusions with components of MAPK signaling [14] (Fig. 1). In our system, the PIF component (residues 1–100) is expressed as a fusion with a variant of the catalytic domain of SOS2 (referred to as SOScat), which is a Ras GTP exchange factor [33]. Two key features of the SOScat variant are that it is constituently active but defective in membrane localization, as the ability of full-length SOS2 to activate Ras depends on transient plasma membrane recruitment [14]. Next, the Phy component (residues 1–621) is targeted to the plasma membrane using a C-terminal linker followed by the CAAX membrane localization sequence from KRas [16, 34]. Thus, Ras/Erk signaling is placed under fine spatiotemporal control based on light-dependent recruitment of cytoplasmic PIF-SOScat to membrane-bound Phy-CAAX. This pair of Phy/PIF fusion constructs is referred to as "Opto-SOS" [14].

To provide a parallel readout of downstream Ras pathway activity in live cells, the light-activatable Opto-SOS system can be coupled with a fluorescently tagged reporter of downstream kinase activity. Such reporters are designed to alternate nucleocytoplasmic localization based on pathway activity, allowing downstream signaling to be monitored over time with live-cell confocal microscopy. We commonly use one of the following two reporters: FP-Erk, which is a fluorescent protein-bound Erk fusion protein, or Erk KTR-FP, which is an Erk kinase translocation reporter (KTR) bound to a fluorescent protein. In the case of the former, pathway activation causes FP-Erk to be transported into the nucleus based on MEK-dependent phosphorylation [1, 11, 35]. Alternatively, the Erk KTR is an Erk-specific substrate that undergoes nuclear export upon Erk-mediated phosphorylation [2]. Combining either reporter individually with Opto-SOS in a single cell line enables complete dynamic control and characterization of Ras/Erk signaling in live cells (Fig. 2). Furthermore, these data represent signal processing in single cells, allowing assessment of both population-level and cell-to-cell variability. Because the activity of many cell signaling processes can be controlled based on the spatial and physical proximity of pathway components (e.g., membrane recruitment of SOScat), the Phy/PIF optogenetic system has a great variety of potential applications. Indeed, there is a growing literature demonstrating its use for light-based control of

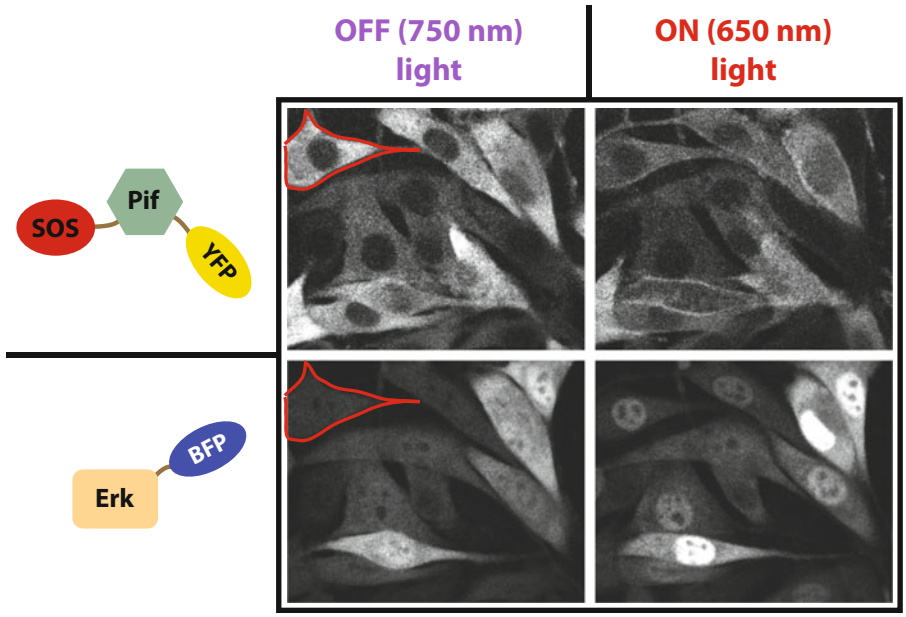

Fig. 2 NIH3T3 cells expressing the Opto-SOS system. Representative images of YFP-PIF-SOScat (*upper*) and BFP-Erk (*lower*) from Opto-SOS NIH3T3 cells, showing light-inducible cytoplasmic depletion of SOScat and nuclear import of BFP-Erk. Red-outlined cell is further analyzed in Fig. 4

phosphoinositide 3-kinase (PI3K) [15], Ras-related C3 botulinum toxin substrate 1 (RAC1) [17], Ras homolog gene family member A (RhoA) [17], cell division control protein 42 homolog (Cdc42) [17, 36], actin assembly [36], and organelle targeting [37].

2 Materials

2.1 Establishing Opto-SOS + Erk Reporter Cell Lines

1. HEK 293T LX cells.
2. pCMV-dR8.91 packaging plasmid (Trono lab, École Polytechnique Fédérale de Lausanne, Switzerland).
3. pMD2.G envelope plasmid (Addgene plasmid #12259).
4. Transfer vector containing Opto-SOS (Addgene plasmid #50851).
5. Transfer vector containing a compatible downstream reporter: e.g., BFP-Erk (Addgene #50848) or Erk KTR (Addgene #59150).
6. Adherent cells or cell line of interest (e.g., NIH3T3, PC12, MDA-MB-231, etc.) to be transduced.
7. Dulbecco's modified Eagle medium and standard culture medium for cells to be transduced.
8. Fetal bovine serum (FBS).
9. Penicillin/streptomycin (P/S).
10. Miniprep reagents.
11. NanoDrop or equivalent spectrophotometer.
12. Reduced serum medium (e.g., Opti-MEM).
13. Transfection reagent (e.g., FuGENE).
14. Standard tissue culture equipment.
15. Sterile 10 mL syringe and attachable 0.45 μm filter.
16. Polybrene (hexadimethrine bromide).
17. HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

2.2 Purifying Phycocyanobilin

1. Phycocyanobilin.
2. Methanol (CH_3OH), HPLC grade.
3. 5 μm phenyl-hexyl 100 Å, LC column.
4. Acetonitrile (CH_3CN), containing 0.1% (v/v) formic acid, for HPLC.
5. Milli-Q water.

6. Preparative HPLC system, with photodiode array detector and automated fraction collector.
7. Rotary evaporator distillation unit.
8. Dimethyl sulfoxide, HPLC grade.

2.3 Imaging Opto-SOS Cells

1. Fibronectin bovine protein suspended at 1 mg/mL in Milli-Q water.
2. Dulbecco's phosphate buffered saline (D-PBS).
3. Standard tissue culture equipment.
4. Adherent cultured Opto-SOS cell line (i.e., cells expressing both Opto-SOS and a compatible downstream reporter).
5. Phenol red-free imaging medium (e.g., DMEM).
6. HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).
7. Purified phycocyanobilin (PCB), 1000× stock.
8. 96-well glass-bottom plates.
9. Mineral oil, light, white, high purity grade.
10. Benchtop centrifuge equipped with swing bucket rotor and attachments for microplates.
11. Confocal microscope, preferably with an environmental control incubation chamber. Our group uses a Nikon Eclipse Ti spinning disk confocal microscope (Nikon Instruments, Melville, NY, USA), with a 40× oil objective (Nikon Instruments, Melville, NY, USA), iXon EMCCD camera (Andor Technology, Belfast, UK), CSU-X1 spinning disk confocal scanner unit (Yokogawa Electric, Tokyo, Japan), and Monolithic Laser Combiner 400 (Keysight Technologies, Santa Rosa, CA, USA).
12. 650 and 750 nm bandpass filters.

2.4 Data Processing to Measure Erk Dynamic Responses

1. Computer with ImageJ (free software) and basic mathematics computing software such as MATLAB (the Mathworks, Natick, MA).
2. YFP-SOScat and BFP-Erk images collected over time from Opto-SOS cells, as in Subheading 3.3.

3 Methods

3.1 Establishing Opto-SOS + Erk Reporter Cell Lines

Establishing stable cell lines with our Phy/PIF-based optogenetic Ras/Erk system has proven to be a highly general approach, as we have had success in a wide variety of cell lines, including everything from primary mouse and chick neurons, to human carcinoma-derived cell lines, to NIH-3T3 fibroblasts. Transient co-transfection of Opto-

SOS and a downstream reporter may be sufficient for simpler experiments that require a short timescale, but we have found that generating stable cell lines increases the proportion of cells co-expressing both constructs and has a high rate of success with minimal additional work required.

3.1.1 Preparing Reagents

1. Prepare complete growth medium for HEK 293T LX cells by supplementing standard DMEM with 10% FBS (v/v) and 10,000 U/mL P/S.
2. Prepare the necessary quantities of plasmid DNA for each required plasmid using a standard miniprep protocol, and measure the resulting DNA concentrations. For each desired virus, the following quantities of plasmid DNA are required: pCMV-dR8.91, 1.33 μg; pMD2.G, 0.17 μg; and desired transfer vector, 1.5 μg.
3. Immediately prior to transfection, warm the Opti-MEM reduced serum medium to 37 °C, and bring the FuGENE HD transfection reagent to room temperature.

3.1.2 Lentivirus Production

1. 12 h prior to transfection, seed one well of a 6-well tissue culture dish with HEK 293T LX cells such that the cells reach a confluency of 50–60% at the time of transfection. Cells should be grown at 37 °C, 5% CO_2 in 2 mL/well of complete growth medium (DMEM, 10% FBS, 1% P/S).
2. Prepare a sterile 1.5 mL tube containing 150 μL of warm Opti-MEM, which will serve as the base of the transfection mixture.
3. Begin assembling the transfection mixture by adding appropriate volumes of the two helper plasmids (i.e., pCMV-dR8.91 and pMD2.G) and of your transfer vector (containing either Opto-SOS or the desired downstream reporter): pCMV-dR8.91, 1.33 μg; pMD2.G, 0.17 μg; and transfer vector, 1.5 μg. Briefly (<5 s) vortex the tube or pipette up and down to mix the reagents.
4. To complete the transfection mixture, carefully add 9 μL of the FuGENE HD reagent by immersing the pipette tip to the bottom of the tube and then slowly dispensing the FuGENE directly into the center of the Opti-MEM/plasmid solution. Mix the final transfection solution by gently and slowly pipetting the liquid up and down three times.
5. Allow FuGENE/DNA complexes to form by incubating the transfection mixture at room temperature for 15 min.
6. Gently draw up the entire volume of transfection mixture using a pipette, and add it dropwise over the entire surface area of a single well of 293T LX cells without bringing the pipette tip into contact with the media. Gently swirl the plate to mix and return the plate to the 37 °C incubator.

7. Allow cells to generate lentivirus for 48–52 h post-transfection. There is no need to change media or disturb the cells until lentivirus is collected.
8. For each well containing transfected 293T cells, use a sterile syringe to draw up all growth medium in the well (which contains the newly produced lentivirus), then attach a 0.45 μm filter to the tip of the syringe, and expel the growth medium through the filter into a sterile collection tube. This filtration step ensures that no floating cells or debris will contaminate future cells infected with the newly formed lentivirus.
9. Tubes containing lentivirus can be stored at 4 °C for the short term (i.e., time measured in days) but must be frozen at −20 °C or preferably −80 °C for long-term storage (*see* **Note 1**).

3.1.3 Lentiviral Transduction of Target Cell Line

1. Seed one well of a 6-well tissue culture dish with the desired recipient cell line. Cells should be plated at a density such that they will not overgrow the well in 48 h. Grow the cells in 2 mL of complete growth medium.
2. To each lentiviral stock, add 5 μg/mL Polybrene and 50 μM HEPES buffer (*see* **Note 2**).
3. Typically, 100 μL of lentiviral stock is required to transduce one well of a 6-well dish containing 50% confluent adherent cells. However, this volume will depend on many conditions, including viral titers obtained and the cell line of interest. Lentiviral stock should be added dropwise over the whole surface area of the well, and the plate should be gently rocked to mix.
4. Incubate the cells in the presence of lentivirus at 37 °C for a minimum of 8 h and maximum of 48 h. After this point, the cells can be transferred to new tissue culture dishes, and the media can be changed, though this is only necessary if they have outgrown their current culture conditions.
5. To confirm lentiviral transduction, at 48 h post infection (hpi), cells can be assessed for expression of YFP or BFP using a confocal or fluorescence microscope. Cells are unlikely to be expressing an appreciable amount of fluorescent protein prior to 48 hpi.
6. For cell lines exhibiting low transduction efficiency (i.e., those with a low percentage of total cells expressing desired constructs), fluorescence-activated cell sorting (FACS) can be used to enrich for cells that co-express both constructs.

3.2 Purifying Phycocyanobilin

In many cases, phycocyanobilin (PCB) purchased from commercial vendors is suitable for experimental protocols using the Phy/PIF system. However, at high levels of Phy expression, we have encountered problems with contaminants in this commercial prep that lead

to constitutive Phy activity (even in the inactive, 750 nm exposed state), as well as high levels of fluorescence in red and near-infrared wavelengths. Fortunately, the contaminants that cause both effects can be removed using high-performance liquid chromatography (HPLC), thereby generating a highly active, pure PCB product. The following steps detail an HPLC protocol that can be used to purify commercial PCB for use with the Opto-SOS Phy/PIF system.

3.2.1 HPLC Purification of PCB

Warning: Phycocyanobilin is highly light sensitive. Ensure that all purified samples are kept in the dark and that all purification steps take place in low-light conditions (*see* **Note 3**).

1. Dissolve 50 mg of phycocyanobilin in 100% HPLC grade methanol.
2. Load the entire phycocyanobilin/methanol solution onto a 5 μm phenyl-hexyl 100 Å column.
3. Run the loaded column on HPLC with a constant flow rate of 12 mL/min at 400 bar, with the following gradient:
   (a) At $t = 0$ min: 70% H_2O/30% acetonitrile with 0.1% formic acid.
   (b) At $t = 5$ min: 70% H_2O/30% acetonitrile with 0.1% formic acid.
   (c) At $t = 45$ min: 40% H_2O/60% acetonitrile with 0.1% formic acid.
   (d) At $t = 50$ min: 100% acetonitrile with 0.1% formic acid.
   (e) At $t = 55$ min: 100% acetonitrile with 0.1% formic acid.
4. Collect the fractions corresponding to the phycocyanobilin peaks, as shown in Fig. 3. An initial early solvent peak will appear first, followed by a double peak that contains both our desired PCB product and a second molecule that is difficult to separate but does not affect the quality of Phy/PIF translocation. Next will be a small peak containing a molecule that leaves Phy in a constitutively active (i.e., PIF-bound) conformation even in the absence of light. It is important to remove this molecule. Finally, the last peak contains a compound that is extremely autofluorescent in red and near-infrared wavelengths.
5. Remove organic solvent from the fractions through rotary evaporation, with the evaporation flask pressure vacuumed to 40 torr and heated to 8–10 °C. Cover the entire rotary evaporation apparatus with foil, to avoid exposing the sample to light during this step.

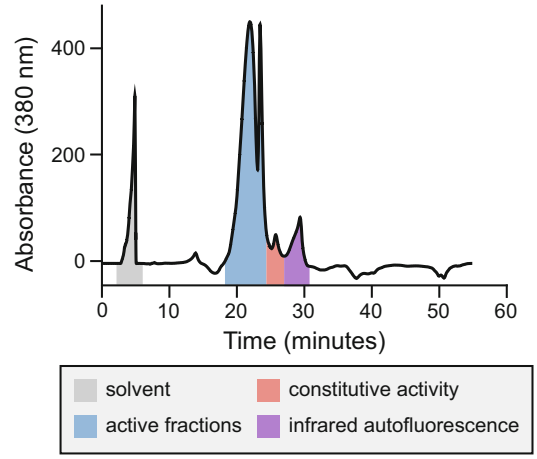

Fig. 3 HPLC purification of PCB. A trace of the absorbance at 380 nm during HPLC fractionation of commercial PCB. Four sets of peaks represent functionally distinct chemical moieties: the solvent fraction [39], two peaks containing functional PCB (*blue* peaks), a peak that upon Phy ligation induces constitutive Phy/PIF binding, and a peak that is highly autofluorescent in the red/far red wavelengths

6. (Optional) Lyophilize any remaining material to obtain pure phycocyanobilin powder.
7. Weigh and resuspend the remaining material in 100% DMSO to a final concentration of 10 mM. This will serve as a 1000× working stock of pure PCB.

3.3 Imaging Opto-SOS Cells

3.3.1 Preparing Reagents

1. Dilute stock fibronectin 100-fold (from 1 mg/mL to 10 μg/mL) in D-PBS.
2. Prepare imaging medium by supplementing phenol red-free medium with 20 mM HEPES buffer (*see* **Note 4**).
3. In low-light conditions, add purified PCB to imaging medium and dilute to 1×. Recall that PCB and PCB-containing reagents should always be carefully protected from ambient light to prevent photodegradation.

3.3.2 Fluorescence Microscopy of Opto-SOS Cells

1. Coat individual wells of a 96-well glass-bottomed plate with 10 μg/mL fibronectin, and incubate the plate at room temperature for >30 min.
2. Remove fibronectin from the wells, ensuring that the coated surface does not remain dry for long (i.e., be prepared to add cells to the well <1 min after removing the fibronectin solution).
3. Seed ~40,000 cells/well of a desired Opto-SOS cell line in 100 μL of complete growth medium. The exact ideal number

of cells per well will vary depending on the cell line, but roughly 50% confluence is typically ideal for imaging.

4. Spin the plate in a benchtop centrifuge at 210 × *g* for 1 min to ensure that the cells adhere to the fibronectin-coated glass with an even plating density. Allow the cells to adhere by incubating at 37 °C for a minimum of 2–4 h, though overnight incubation is preferred for most cell lines.
5. Carefully remove the growth medium, and wash the cells with plain imaging medium (i.e., serum-free medium *without* PCB added). This wash step removes residual growth factors and serum proteins that may be left over from the original growth medium, the removal of which will allow cells to reach a truly inactive state of Ras signaling (*see* **Note 5**).
6. In low-light conditions, add 100 μL per well of imaging medium containing 1× PCB, and incubate the cells in the dark at 37 °C for >1 h. Perform this and all subsequent steps in dark or low-light conditions (*see* **Notes 6** and **7**).
7. Prior to imaging, allow 30 min for the cells to acclimate to the microscope incubator conditions (37 °C and 5% CO_2), as temperature alterations can impact cell signaling responses.
8. For longer (>1 h) imaging protocols, add ~50 μL per well of mineral oil to prevent evaporation of imaging medium. Mineral oil should be added carefully such that the oil layer sits on top and does not mix with the underlying imaging medium.
9. Place a 750 nm bandpass filter directly in the microscope's bright-field illumination path, which is typically located on top of the microscope condenser. This is to ensure that constant 750 nm light is applied to cells, while imaging fields are first located by the user, thus keeping the Opto-SOS system in an inactivated state.
10. Use the 40× oil objective to identify locations on the 96-well plate that contain cells with strong expression of both fluorescent components (i.e., Opto-SOS and downstream reporter).
11. Select an imaging plane in the center of the nucleus so that nuclear translocation of the reporter will be maximally visible. Such a plane can be found by moving upward from the glass surface in the *z*-axis until the nucleus first becomes clearly visible. Turn on the autofocus function if it is available on your microscope to prevent focal plane drift (*see* **Note 8**).
12. Activate the Opto-SOS system by replacing the 750 nm filter with a 650 nm bandpass filter. YFP-PIF-SOScat recruitment should be visible within seconds. We have found that the timescale of reporter translocation can vary slightly between cell lines, but both BFP-Erk and KTR-BFP typically reach steady-state activation in 5–8 min. Use the microscope's YFP

imaging mode to capture images of YFP-PIF-SOScat membrane recruitment and the BFP imaging mode to record reporter translocation.

13. Replace the 650 nm filter with the 750 nm filter to deactivate the Opto-SOS system, and again image cells in the YFP and BFP channels. **Steps 12** and **13** can be repeated as frequently as required based on individual experimental protocols (*see* **Note 9**).

3.4 Data Processing to Measure Erk Dynamic Responses

In every cell line we have tested, optogenetic Ras activation induces Erk nuclear translocation that persists as long as SOScat membrane translocation is induced and can be applied repeatedly over time. The ability to restimulate the same cells using different intensities of light makes it possible for the first time to carry out measurements of Ras/Erk signal processing, such as single-cell dose-response curves (stimulating a cell with different light intensities and measuring nuclear Erk) or dynamic signal transmission (stimulating a cell with a time-varying light stimulus and measuring Erk's response over time). In this section, we describe some useful approaches for quantifying SOScat membrane translocation and nuclear Erk dynamics.

3.4.1 Measuring Erk Dynamics in Response to Light

1. Open the BFP channel time-lapse movie in ImageJ. ImageJ supports a variety of formats used by both open- and closed-source microscope software packages. Draw an ellipsoidal region in the nucleus of a cell of interest expressing BFP-Erk, and press "M" to measure its mean intensity. New regions can be drawn as the cell moves or changes shape. Advance through the movie collecting mean intensities at each timepoint. The list of cytoplasmic intensities at each timepoint will be called $B_{\mathrm{nuc}}(t)$.
2. Rewind the movie to the first timepoint, and draw a polygon or freehand region in the cytoplasm of the same cell measured in **step 1**. Again, press "M" to measure its mean intensity at each timepoint. New regions can be drawn as the cell moves or changes shape. The list of cytoplasmic intensities at each timepoint will be called $B_{\mathrm{cyt}}(t)$.
3. Rewind the movie once more, and draw an ellipsoidal region nearby, but in a region with no cells. This will measure the background intensity (i.e., autofluorescence of the medium and other sources of light). Again, press "M" to measure its mean intensity at each timepoint. New regions can be drawn as cells move over the original background region. The list of background intensities at each timepoint will be called $B_{\mathrm{bkgd}}(t)$.

3.4.2 Measuring SOScat Dynamics in Response to Light

1. Open the YFP channel time-lapse movie in ImageJ. Draw a polygonal or freehand region in the cytoplasm of the same cell of interest (as was measured in Subheading 3.4.1) expressing YFP-PIF-SOScat. Press "M" to measure its mean intensity at each timepoint. New regions can be drawn as the cell moves or changes shape. The list of cytoplasmic intensities at each timepoint will be called $\Upsilon_{\mathrm{cyt}}(t)$.
2. Rewind the movie, and draw an ellipsoidal region nearby but in a region with no cells. This will measure the background intensity (i.e., autofluorescence of the medium and other sources of light). Again, press "M" to measure its mean intensity at each timepoint. New regions can be drawn as cells move over the original background region. The list of background intensities at each timepoint will be called $\Upsilon_{\mathrm{bkgd}}(t)$.

3.4.3 Data Processing to Measure Erk Dynamics Over Time

1. Subtract the intensity of the background from the measured nuclear and cytoplasmic BFP intensities at each timepoint to generate subtracted intensity values. For example, to calculate background-subtracted nuclear Erk (denoted $\bar{B}_{\mathrm{nuc}}$), use the formula $\bar{B}_{\mathrm{nuc}}(t) = B_{\mathrm{nuc}}(t) - B_{\mathrm{bkgd}}(t)$. Repeat this calculation for all BFP images (representative traces are shown in Fig. 4d).

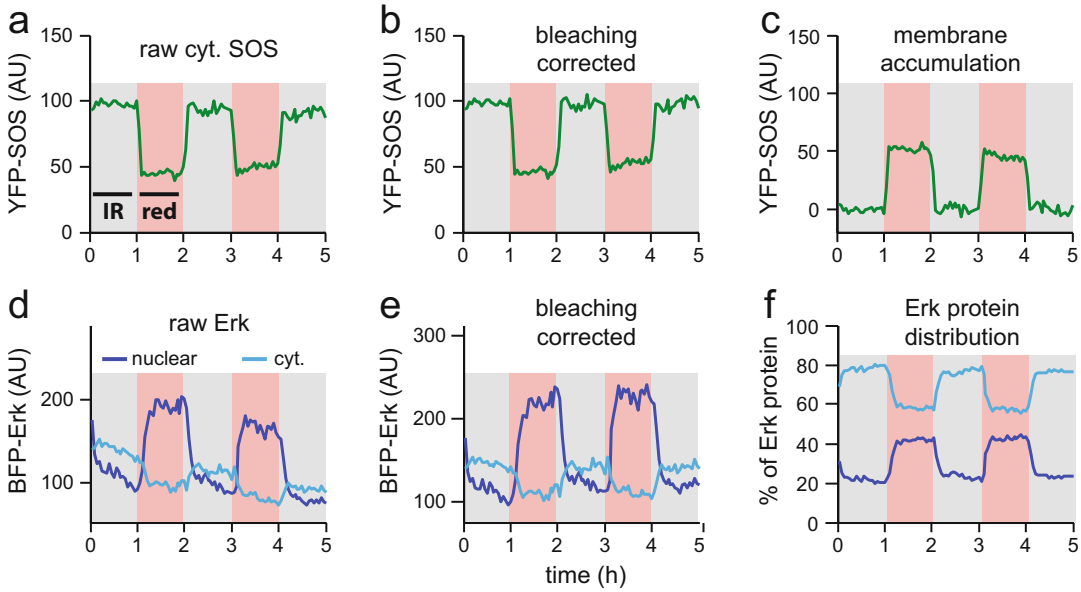

Fig. 4 Analyzing SOScat and Erk levels over time in individual cells. All data shown are for the representative cell indicated in Fig. 2, which was stimulated with alternating 1 h cycles of red and infrared (IR) light for 5 h. (**a**–**c**) Analysis of cytoplasmic SOScat levels. (**a**) Raw cytoplasmic SOScat, (**b**) bleaching-corrected SOScat, and (**c**) the calculated membrane accumulation of SOScat over time. (**d**–**f**) Analysis of nuclear and cytoplasmic BFP-Erk levels. (**d**) The raw nuclear and cytoplasmic Erk, (**e**) bleaching-corrected Erk, and (**f**) the calculated distribution Erk protein between the nucleus and cytoplasm over time are shown

We have found an easy and robust way to measure Erk nuclear-cytoplasmic dynamics that accounts for photobleaching and the movement of Erk protein between nucleus and cytoplasm over time. This measurement takes advantage of the observation that cell size, nuclear size, and BFP-Erk levels are roughly constant on the timescales of our experiments (i.e., a few hours in starvation media) and that by taking care to limit laser intensity during imaging, photobleaching is roughly linear over the course of an experiment. We can thus represent this mass conservation with the following equation:

$$V_{\mathrm{nuc}}\bar{B}_{\mathrm{nuc}}(t) + V_{\mathrm{cyt}}\bar{B}_{\mathrm{cyt}}(t) = E_T \times (1 - kt)$$

where V_{nuc} is the nuclear volume, V_{cyt} is the cytoplasmic volume, k is the photobleaching rate, and E_T is the total fluorescent Erk per cell (which is bleached at a rate kt). Solving this equation for $\bar{B}_{\mathrm{nuc}}(t)$ we can write:

$$\bar{B}_{\mathrm{nuc}}(t) = a + b \times \bar{B}_{\mathrm{cyt}}(t) + c \times t.$$

This simple linear equation is of the form $y = a + bx + ct$ that can quickly be solved for the coefficients a, b, and c that best fit the nuclear and cytoplasmic data, for example, by using the following two lines of MATLAB code for data vectors $\boldsymbol{x}$ (background-subtracted cytoplasmic Erk), $\boldsymbol{y}$ (background-subtracted nuclear Erk), and $\boldsymbol{t}$ (the timepoint number):

```
coeff = [ones(N,1) x(:) t(:)] \ y(:);
a = coeff(1); b = coeff(2); c = coeff(3);
```

2. After finding the coefficients in **step 2**, we can determine the photobleaching-corrected levels of nuclear and cytoplasmic Erk and the nuclear-cytoplasmic volume ratio for each cell. To do so, we note that $V_{\mathrm{cyt}}/V_{\mathrm{nuc}} = -b$ and the photobleaching rate $k = -b/a$. Thus,

$$\mathrm{Erk}_{\mathrm{nuc}}(t)/V_{\mathrm{nuc}} = \frac{\bar{B}_{\mathrm{nuc}}(t)}{1 + (b/a)t}; \mathrm{Erk}_{\mathrm{cyt}}(t)/V_{\mathrm{cyt}} = \frac{\bar{B}_{\mathrm{cyt}}(t)}{1 + (b/a)t}$$

3. Representative traces of photobleaching-corrected Erk are shown in Fig. 4e. After these calculations, other quantities such as nuclear-cytoplasmic ratio, nuclear fold change, or the fraction of Erk protein in the nucleus and cytoplasm can readily be quantified (e.g., *see* Fig. 4f).

3.4.4 Data Processing to Measure SOScat Dynamics Over Time

1. Subtract the intensity of the background from the measured cytoplasmic YFP intensity at each timepoint to generate a subtracted intensity. For instance, to calculate background-subtracted nuclear Erk (denoted $\bar{\Upsilon}_{\text{nuc}}$), use the formula $\bar{\Upsilon}_{\text{nuc}}(t) = \Upsilon_{\text{nuc}}(t) - \Upsilon_{\text{bkgd}}(t)$. Repeat this calculation for all YFP images (a representative trace is shown in Fig. 4a).
2. For timepoints where 750 nm light is exclusively applied and during which SOS is exclusively localized to the cytoplasm, the following equation holds:

$$\bar{\Upsilon}_{\text{cyt}}(t_{750}) = \frac{\text{SOS}_T}{V_{\text{cyt}}} \times (1 - kt_{750}).$$

Again, this is of the form $y = a + bt$, where we can solve using the following two lines of MATLAB code for data vectors $\boldsymbol{y_{750}}$ (background-subtracted cytoplasmic SOS *at only those timepoints where 750 nm light is applied*) and $\boldsymbol{t_{750}}$ (the times *for only those timepoints where 750 nm light is applied*):

```
coeff = [ones(N,1) t750(:)] \ y750(:);
a = coeff(1); b = coeff(2);
```

After finding the coefficients in **step 2**, we can correct for SOScat photobleaching at all timepoints by computing

$$\frac{\text{SOS}_{\text{cyt}}(t)}{V_{\text{cyt}}} = \frac{\bar{\Upsilon}_{\text{cyt}}(t)}{1 + \left(\frac{b}{a}\right)t}.$$

(a representative trace is shown in Fig. 4b).

3. At each timepoint, we assume that SOS lost from the cytoplasm is gained on the membrane, where it is able to activate Ras. Thus, we can approximate the SOS gained on the membrane as the SOS lost in the cytoplasm by subtracting the cytoplasmic intensity at each timepoint from the maximum cytoplasmic pool of SOS measured at times when 750 nm light is applied (a representative trace is shown in Fig. 4c):

$$\text{SOS}_{\text{membrane}}(t) \tilde{} \text{SOS}_{\text{cyt}}(t_{750})/V_{\text{cyt}} - \text{SOS}_{\text{cyt}}(t)/V_{\text{cyt}}$$

Using the above calculations, it is possible to obtain high-quality, bleaching-corrected measurements of both membrane SOS and nuclear Erk dynamics in response to time-varying light inputs, thereby enabling the study of the Ras/Erk transfer function in single cells.

4 Notes

1. It is thought that an appreciable proportion of the lentivirus transduction efficiency is lost with each freeze/thaw cycle. Thus, it is better to use lentivirus to infect cells shortly after harvest or to store lentiviral stocks in small single-use aliquots to prevent subjecting virus to multiple freeze/thaw cycles.
2. Polybrene is used to increase lentiviral transduction efficiency by reducing electrostatic repulsion between the plasma membrane and lentiviral capsid [38]. HEPES is used to buffer the pH, as fusion of the lentiviral capsid with the membrane of a target cell is enhanced at more acidic pH.
3. It is vitally important that PCB be protected from prolonged exposure to ambient light during the entire purification process and thereafter when storing the purified product. Prolonged ambient light exposure results in photodegradation of the chromophore, which compromises the ability of Phy to respond to light stimuli. Thus, high-quality PCB is crucial to ensuring the success of your experiments. Though it is not always visible to the naked eye, one sign of compromised quality is if a sample of PCB begins to change color from a rich, dark, royal blue to a more pale, purple hue.
4. When preparing imaging media for live-cell confocal microscopy experiments, it is ideal to use formulations that do not include phenol red, as this will maximize signal to noise when imaging.
5. Incubation in serum-free imaging medium allows the cells to equilibrate to growth factor-free conditions with the Ras/Erk pathway turned "off."
6. Cells are sensitive to light after the addition of PCB, and all subsequent procedures should thus be performed in the dark or very low-light conditions (e.g., turn off overhead lights in the lab, wrap all PCB-containing reagents in tin foil, etc.). These precautions are required because, in the presence of PCB, the Opto-SOS system is easily activated by ambient light.
7. When setting up sensitive experiments in which cells must be in a truly "off" Ras/Erk signaling state, it can be difficult to see what one is doing while also ensuring that ambient light does not aberrantly activate the Phy/PIF system. We have found that infrared LED lights (~750 nm) can be used to provide a degree of visibility while preparing reagents in a dark lab.

8. A confocal microscope is required for imaging the Opto-SOS system, as epi-fluorescence microscopy cannot accurately resolve PIF membrane recruitment or nucleocytoplasmic shuttling of the downstream reporter.
9. BFP can be imaged ad libitum without harming Phy/PIF performance or perturbing translocation. However, imaging of YFP will partially activate Phy, so it is prudent to limit frequency and duration of exposures.

Acknowledgments

We thank Mohammad Seyedsayamdost for assistance and advice with HPLC purification. This work was supported by the NIH National Institute of Biomedical Imaging and Bioengineering (grant DP2EB024247 to J.E.T.), the NIH National Cancer Institute (fellowship F30CA206408 to A.G.G.), and a Princeton University Dean of Research Innovation Award to J.E.T.

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