Online Methods

Plasmids, constructs used

For PIF-BFP, PIF was PCR amplified from pAL175[8] and subcloned with TagBFP (a kind gift from the Vale Lab) into the pMSCV-neomycin retroviral vector (Clontech) using custom designed overhangs using the AarI enzyme (Fermentas). All other plasmids used the pHR as the lentiviral backbone (kindly provided by John R. James). For the Phy construct, the mammalian codon-optimized PhyB, mCherry, and the CAAX from KRas fusion were subcloned into the pHR lentiviral vector backbone using MluI/XhoI (kindly provided by John R. James). For iSH2-YFP-PIF, the interSH2 domain from p85 was PCR amplified from CF-iSH (a kind gift from the Meyer Lab) and was enzymatically assembled[15] with YFP and PIF into pHR. Similarly, the PH domain from Akt was enzymatically assembled with Cerulean into pHR to make the PHAkt-Cerulean probe.

Retroviral constructs were transfected with TransIT-293 (Mirus Bio) into 293-GPG cells[16] (which stably express the required packaging proteins to produce retrovirus) that had been plated at 70% confluency in 6 cm dishes. After transfection, media was changed twice; first at 16 h- and again at 48 h- after transfection. Retroviral supernatant was collected at 72 h after transfection. All lentiviruses were produced by co-transfecting the pHR plasmids along with the vectors encoding packaging proteins (pMD2.G and p8.91) using TransIT-293 into HEK-293 cells at ~70% confluency in 6-well plates. Viral supernatants were collected 2 days after transfection. Both retro- and lentiviruses were used for transduction immediately or stored at 4 degrees for up to two weeks.

Retroviral and lentiviral transduction

NIH-3T3 cells (ATCC) were cultured in 10% bovine calf serum (UCSF Cell Culture Facility) in DMEM (Invitrogen) supplemented with penicillin, streptomycin and glutamine at 37°C with 5% CO_2 in a humidified incubator. For viral transduction, NIH-3T3 cells were plated in 6-well dishes to achieve ~70% confluency at the time of infection. Viral supernatants were passed through a 0.22 micron filter to exclude dead cells and particulate matter. For lentiviral transduction, 1 mL of viral supernatant was added directly to cells. For retroviral transduction, the viral supernatant was supplemented with 8 micrograms/mL of polybrene (Sigma). Viral media was replaced with normal growth media 24 h post infection. For cell lines stably expressing multiple signaling components, viral transductions were performed sequentially: first Phy, then PIF, then PHAkt-Cerulean. Cells were then subject to fluorescence activated cell sorting on a FacsAria2 (Beckton-Dickinson) to enrich for positive cells.

Preparing cells for imaging

For imaging experiments, 35 mm glass bottom petri dishes (MatTek Corp.) were coated for 1 h with 0.08 mg/mL fibronectin (which we prepared from whole porcine blood), then washed twice with PBS. 180,000 cells were plated on each dish in normal growth media and allowed to adhere for at least 30 min. For PIF-BFP experiments, we incubated cells for 30 min in a solution of 4 μM phycocyanobilin (PCB, extracted from *Spirulina*[17]; 4mM stock was pre-diluted into conditioned media and added to dishes in the dark or under a green safelight). PCB-containing media was then replaced with imaging media consisting of 2% FBS (Invitrogen) in mHBSS (150mM NaCl, 4mM KCl, 1mM $MgCl_2$, 10mM glucose, 20mM Hepes pH 7.2).

For PI3K recruitment experiments, cells were serum starved for at least 3 h in 2% fatty acid free BSA (Sigma) in DMEM. Cells were incubated with PCB for at least 30 min before exchanging into imaging media (2% fatty acid free BSA in mHBSS).

Microscopy

To tune light inputs for optogenetic control, we used one 650 and two 750 nm LEDs (Lightspeed Technologies). For these devices, light emission intensity scales linearly with the applied voltage (from 0 to 5 V). To apply light inputs specifically to defined regions of the cell, we used a custom dual-input digital micromirror device (DMD; Andor Technologies). ON pixels (regions to be stimulated with activating light) were illuminated with both 650 and 750 nm light, while OFF pixels were exposed to the second 750 nm light source at a constant intensity. To implement software-based voltage control of light intensity, we connected both ON pixel LEDs to the analog outputs of a DT9812 board (Data Translation) and set their voltages using custom MATLAB code (**Supplementary Software**).

Images for light recruitment assays were taken at room temperature on a Nikon Eclipse Ti inverted microscope equipped with a motorized laser TIRF illumination unit, a 100X PlanApo TIRF 1.49 NA objective, and an electron microscopy charge-coupled device (EM-CCD) camera (Evolve, Photometrics). 405 nm, 440 nm, 488 nm, 514 nm and 561 nm laser wavelengths (LMM5, Spectral Applied Research) were used for excitation.

Our Nikon Eclipse Ti microscope has two stacked dichroic turrets, which we used to achieve independent control of both light stimulation and measurement. The upper turret contained a 620 nm shortpass filter for exposing cells to the DMD light or was left empty (non-DMD imaging). The lower turret contained the TIRF imaging dichroics (Chroma) and was used to deliver the TIRF laser lines. In addition to the shortpass dichroic's rejection of 650 nm and 750 nm light, we used a 625 nm sputtered shortpass emission filter (Chroma) to block DMD light from the camera when acquiring TIRF membrane fluorescence images. Thus, imaging could be performed while cells were simultaneously stimulated with ON/OFF light. The microscope, dichroic positions,

filters, camera, and lasers were all controlled using the open-source Micromanager software package (UC San Francisco) using custom MATLAB code (**Supplementary Software**).

Image processing

We removed the shortpass emission filter from the light path to identify the precise location of the 9 μM x 9 μM square ON pixel region illuminated by the DMD (see **Fig. 1d** for a typical result). The location of this region was automatically identified using standard image processing tools (Sobel edge detection, image dilation to find horizontal and vertical lines, and hole-filling to identify the closed square region) in MATLAB's Image Processing Toolbox. Recruitment was quantified as the mean intensity of pixels within the ON pixel region.

For some analyses it was useful to consider the normalized recruitment level for an individual cell. In these cases, we used measurements of the mean fluorescence in response to 1V 750 nm illumination as the minimum fluorescence $F_{\min}$. Conversely, the mean intensity in the presence of 1V pure 650 nm illumination was used as the maximum recruitable fluorescence $F_{\max}$. Normalized recruitment was computed using the equation

$$F_{Norm} = \frac{F - F_{\min}}{F_{\max} - F_{\min}}.$$

For analyses in which background-subtracted fluorescence values were used, we subtracted the minimum fluorescence $F_{\min}$ from each fluorescence measurement F using the formula

$$F_{Sub} = F - F_{\min}.$$

Optimizing imaging conditions for data acquisition and feedback control

Although the peak wavelengths affecting PhyB photoisomerization are in the red/infrared range, both PhyB states are also capable of absorbing light at other frequencies. To ensure that

our imaging conditions do not perturb the Phy activity state, we measured the effect on PhyB photoisomerization and PIF recruitment binding in response to various imaging excitation wavelengths. We exposed cells expressing PIF-YFP and Phy-mCherry-CAAX to laser light at 5 illumination wavelengths and compared to the recruitment elicited by our 650 nm light source. We found that a 30 s exposure to GFP, YFP, and RFP imaging wavelengths (488, 514, and 561 nm, respectively) led to an increase in PIF-YFP membrane recruitment, where the effect was more pronounced at longer wavelengths (**Supplementary Fig. 1a**). It should be noted that this experiment represents a worst-case scenario, as 30 s exposures are much longer than those delivered during normal imaging (typically 100 ms per acquired image) and no 750nm light is present to counteract imaging-based activation of PhyB. While GFP and YFP fluorophores can still be used for quantitative imaging of the Phy/PIF system under conditions of low excitation light levels, the frequent imaging required for feedback control makes these undesirable choices for this work.

In contrast, CFP and BFP imaging wavelengths (440 and 405 nm, respectively) had no measurable effect on the Phy-PIF interaction. Thus, for all subsequent experiments involving frequent imaging (such as those implementing feedback control), we restricted ourselves to imaging CFP- and BFP-based fluorophores. We further verified that frequent BFP imaging does not affect either the steady state or transient response of the Phy/PIF module (**Supplementary Fig. 1b**).

Implementing any feedback control strategy requires frequent sampling of the system's output to make the appropriate input adjustments. To this end, we sought to identify conditions under which acquiring frequent TIRF images would not lead to fluorophore photobleaching. We

found we were able to acquire nearly 400 PIF-BFP TIRF images without significantly affecting the minimal or maximal range of recruitment (**Supplementary Fig. 1c**).