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High-throughput multicolor optogenetics in microwell plates

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Optogenetic probes can be powerful tools for dissecting complexity in cell biology, but there is a lack of instrumentation to exploit their potential for automated, high-information-content experiments. This protocol describes the construction and use of the optoPlate-96, a platform for high-throughput three-color optogenetics experiments that allows simultaneous manipulation of common red- and blue-light-sensitive optogenetic probes. The optoPlate-96 enables illumination of individual wells in 96-well microwell plates or in groups of wells in 384-well plates. Its design ensures that there will be no cross-illumination between microwells in 96-well plates, and an active cooling system minimizes sample heating during light-intensive experiments. This protocol details the steps to assemble, test, and use the optoPlate-96. The device can be fully assembled without specialized equipment beyond a 3D printer and a laser cutter, starting from open-source design files and commercially available components. We then describe how to perform a typical optogenetics experiment using the optoPlate-96 to stimulate adherent mammalian cells. Although optoPlate-96 experiments are compatible with any plate-based readout, we describe analysis using quantitative single-cell immunofluorescence. This workflow thus allows complex optogenetics experiments (independent control of stimulation colors, intensity, dynamics, and time points) with high-dimensional outputs at single-cell resolution. Starting from 3D-printed and laser-cut components, assembly and testing of the optoPlate-96 can be accomplished in 3–4 h, at a cost of ~$600. A full optoPlate-96 experiment with immunofluorescence analysis can be performed within ~24 h, but this estimate is variable depending on the cell type and experimental parameters.

Introduction

Cell regulation involves the complex interplay of different signaling pathways. Multicolor optogenetics offers a potentially powerful way to systematically analyze multi-pathway networks. Optogenetic actuators use light to control protein activity within living cells[1]. Because illumination is programmable and can be applied remotely, optogenetics can, in principle, offer data-rich experiments with precise perturbations, multiple channels of control (multiple stimulation colors), and the potential for programmable high-throughput analysis. Systematically performing such experiments, however, remains a challenge. Although complex optogenetic stimulation can be performed with a microscope, throughput is low and the types and time scales of experiments are limited. Furthermore, the ability to simultaneously manipulate multiple optogenetic tools with distinct activation spectra is challenged by spectral overlap in the available toolset. For example, although most blue-light-activatable proteins are stimulated by wavelengths shorter than ~500 nm[2], red-light-sensitive phytochrome B (PhyB) shows weak but meaningful absorbance across the visible spectrum[3]. Thus, orthogonal multiplexing of blue- and red-light-sensitive probes is difficult because blue-light stimulation will cross-activate the red-light-activatable probe (PhyB). Cross-activation can be counterbalanced by simultaneous stimulation with far-red light, which reverses PhyB activation[4,5] (Supplementary Fig. 1). However, such experiments require the simultaneous control of three illumination wavelengths.

Development of the optoPlate-96

Here, we describe the construction and use of the optoPlate-96 (Fig. 1), a scalable high-throughput platform for three-color microwell illumination of cells for high-dimensional, long-term (up to weeks) optogenetics experiments within standard cell culture incubators. The optoPlate-96 comprises

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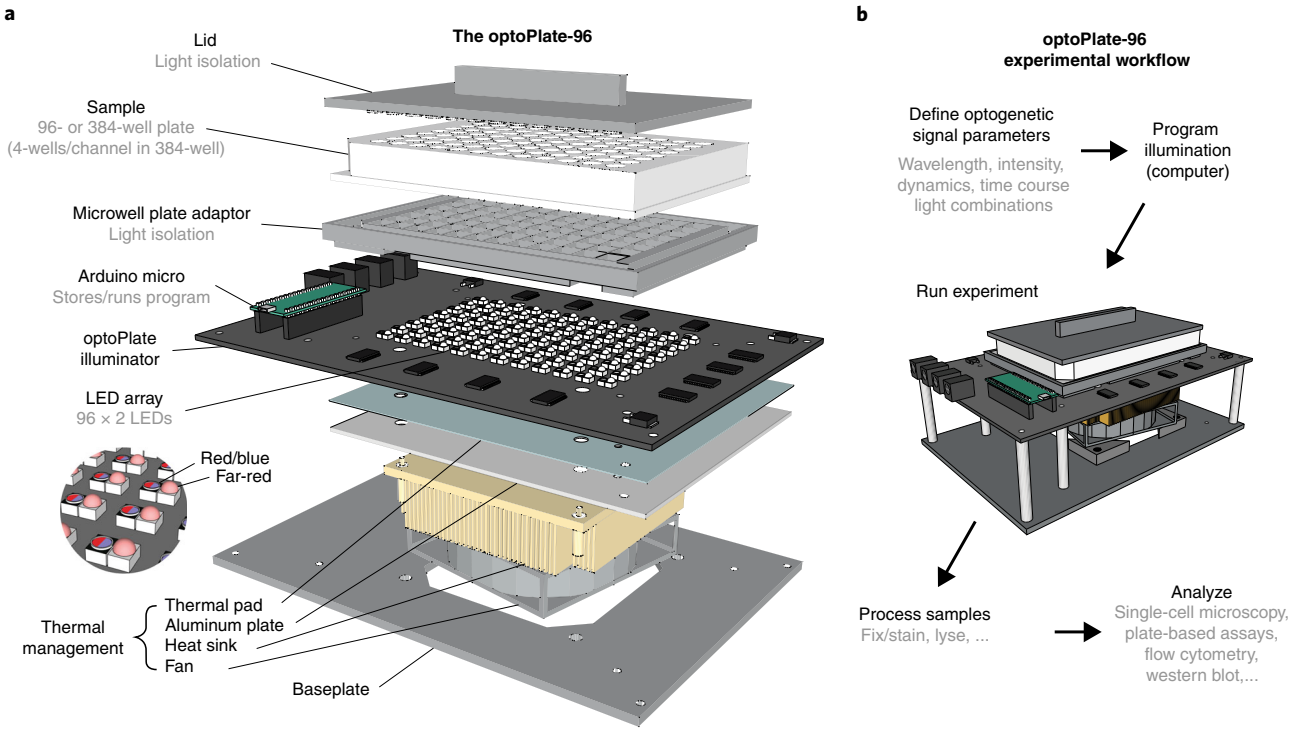

Fig. 1 | The optoPlate-96. a, An exploded view of the optoPlate-96 shows its components. The optoPlate illuminator is a 96-position LED array that can independently illuminate up to three colors per position. An adaptor enables 96- or 384-well plate illumination, and a lid prevents light contamination. An active heat sink spreads and removes heat, minimizing sample heating. **b**, Workflow for a typical optoPlate-96 experiment. Standoffs and heat sink pedestals depicted in **b** were omitted from **a** for clarity.

an LED-based illuminator formatted for microwell plates, a light-insulating adaptor and plate-fitting lid, and a thermal management system to evenly distribute and minimize sample heating (Fig. 1a). After custom manufacture (e.g., 3D printing) of necessary parts, the optoPlate-96 can be manually assembled in 3–4 h for ~$600, with minimal infrastructure required.

The optoPlate illuminator features two LED emitters in each of 96 positions corresponding to the wells in a 96-well microplate, or to 4 wells of a 384-well plate (Supplementary Fig. 2). This configuration can accommodate one single-color LED and one bi-color LED for up to three wavelengths of illumination per well (Fig. 1a). The combined 288 LED channels are driven by 12 onboard LED driver chips, each of which can drive up to 30 mA through each of 24 channels with linear, 12-bit intensity resolution (4,096 intensity levels) (Supplementary Fig. 3). An onboard Arduino Micro microcontroller stores and executes the user-defined illumination profiles during the experiment, eliminating the need for external data cables. The device is fully integrated and uses no wires, except for power cords.

We recently used the optoPlate-96 to systematically probe signal transmission properties of the Ras–Erk signaling pathway in a panel of normal and cancer cell lines[6]. 96- and 384-well stimulation coupled with single-cell immunofluorescence enabled a systematic examination of how dynamic Ras inputs impact signaling, gene expression, and cell fate decisions across normal and diseased cell contexts. Notably, the devices in this study could illuminate with only two (versus the current three) wavelengths of light, and they had no active mechanisms to dissipate heat. Heat accumulation was managed largely by applying pulsatile (versus constant) illumination.

Overview of the procedure

The optoPlate-96 can be fully assembled within 3–4 h in a standard lab, without specialized equipment. The main stages of the assembly procedure are (i) optoPlate illuminator component placement (Steps 1–12); (ii) optoPlate illuminator reflow and through-hole soldering (Steps 13–24); (iii) illuminator quality control (QC; Steps 25–32); and (iv) heat sink/adaptor/baseplate mounting (Steps 33–42). Although component placement and soldering can be automated and performed

commercially, this is expensive and slow for small scale production. Thus, we describe a protocol for placing components manually and reflow-soldering all components simultaneously in a toaster oven.

Steps 33–42 (mounting heat sink, adaptor, and baseplate) require prior manufacture of parts through common procedures, such as 3D printing and laser cutting. Where these resources are unavailable, the associated design files can be sent to contractors that specialize in on-demand prototyping at relatively low cost (e.g., Protolabs; http://www.protolabs.com).

We then describe how to perform an optoPlate experiment and analyze it with single-cell immunofluorescence. Specifically, we describe how to (i) program the optoPlate (Steps 43–47); (ii) seed optogenetically addressable cells (Steps 48–59); (iii) starve and prepare cells for the experiment (Steps 60–68); (iv) run the experiment (Steps 69–73); (v) fix and permeabilize cells (Steps 74–82); (vi) immunostain (Steps 83–91); (vii) perform automated imaging (Steps 92 and 93); and (viii) quantitate and analyze single-cell fluorescence (Steps 94 and 95).

Comparison with other methods for optogenetic stimulation in multiwell plates

There are currently few methods available for stimulation of optogenetic proteins in multiwell plates. The Light Plate Apparatus[7] offers 24 wells of stimulation with two wavelengths of illumination, a user-friendly graphical user interface (GUI) for programing, and detailed assembly instructions. Similarly, a separate optogenetic illuminator was described that allowed one or two colors of illumination that were independently programmable in 24-well plates[8]. However, these devices cannot be applied to experiments in 96- and 384-well format. Furthermore, because they can illuminate with a maximum of only two distinct wavelengths, they do not allow for orthogonal control of the most common red- and blue-light-responsive optogenetic probes in mammalian cells.

Illumination of samples in microwell plates has been previously achieved using several alternative strategies. The first strategy used an LED array in the format of a 96-well plate, similar to the optoPlate. However, this device could illuminate only one[9] or two[10] colors per well, was not adaptable to experiments in 384-well plates, and required connection to bulky data cables for operation. A second strategy used an 8×8 array of commercially available three-color LEDs that were coupled to a 96-well microwell plate with a 3D-printed adaptor[11]. However, this device illuminated only 64 individual wells, and the illumination wavelengths were limited to 470, 525, and 620 nm, which are insufficient for orthogonal control of blue- and red-light probes because of the lack of a far-red illumination. A third strategy to illuminate microwell plates was achieved by repurposing a commercial plate-reader[12]. An optical fiber was fed through the fluidics head of a Tecan Infinite M200 Pro plate reader, and illumination position and wavelength could be defined by combined programming of the plate reader and a microcontroller. However, in this approach, throughput is limited because individual wells must be stimulated sequentially. Furthermore, downstream readouts are limited to those available within a microplate reader, and these do not provide single-cell resolution. In addition, owing to the large size and cost of the Tecan microplate readers, it is challenging to parallelize plate-reader-based optogenetic stimulation for enhanced throughput. Finally, the Lumos is a commercial device that can stimulate with four separate colors in each well of 48-well plates. This device can illuminate fewer wells compared to the optoPlate-96, and it does not illuminate with the wavelengths necessary to multiplex blue- and red-sensitive optogenetic probes.

Advantages

The optoPlate-96 has several key features that enable high-throughput optogenetics experiments. The microwell-plate format allows 96 independently controlled optogenetics experiments to be performed simultaneously. Experiments in 384-well plates quadruple the number of biological samples because each of the 96 illuminator positions can activate 4 wells of a 384-well plate. The 384-well format can be used to test different media conditions (e.g., drug concentrations) within one illumination setting, or to increase the number of biological replicates of a particular condition. The 3D-printed microwell plate adaptor is essential for microwell plate experiments because it ensures that light cannot bleed through between neighboring microwells in a 96-well plate (or between neighboring groups of 4 microwells in a 384-well plate) (Supplementary Fig. 4). The low profile of the adaptor has been optimized to maximize light intensity at the sample, and the adaptor can support diffuser film inserts that homogenize the spatial distribution of light at the sample. Further light homogenization can be achieved by using taller adaptors (see 'Experimental design' section). 3D-printed lids have also been designed to eliminate light contamination from ambient or reflected light that may illuminate the sample from above (Supplementary Fig. 4). All design files necessary for printed circuit board (PCB)

manufacture, 3D printing, laser cutting, and metal machining are maintained in our online optoPlate-96 repository (http://bit.ly/OP96designFiles).

Independent control over all 288 illumination channels is coordinated through the Arduino microcontroller, which runs a custom script. Users have full control over the timing, intensity, and wavelengths of illumination for each illumination channel. Annotated scripts and the associated usage manual are maintained in the online optoPlate-96 repository (http://bit.ly/OP96arduinoScripts).

The optoPlate-96 provides flexibility in the wavelengths of stimulating light. We present three configurations (one-, two-, or three-color) that are optimized for common optogenetic tools used in mammalian cells, for instance, the iLid/sspB[13] system and the PhyB/PIF[14] system. However, other-wavelength LEDs can be substituted as required, as long as their form factor (either PLCC2 or PLCC4 packaging) and polarity are compatible with the polarity of the LED pads on the PCB (Supplementary Fig. 5).

The optoPlate illuminator can be manually assembled without specialized equipment within 3–4 h at a cost of ~$600 (although the price can be lower if the components are bought at higher volume). Because they are compact, inexpensive, and easy to assemble and use, multiple optoPlates can readily be operated side by side to maximize experimental throughput.

The optoPlate was designed to minimize heat generation, especially during light-intensive experiments. A key heat management feature is the active heat sink that comprises a thermal pad, an aluminum plate, a metal block, and a fan (Supplementary Fig. 6). Although fan speed can be controlled through the optoPlate-96 control script, we have observed no adverse effects (e.g., vibrational) from running the fan at maximum speed, and we recommend this setting for all experiments.

Limitations

Despite the thermal management features of the optoPlate-96, sample heating must always be considered in optoPlate experiments. There are many sources of heat generation on the optoPlate, including LED driver chips, voltage regulators, and the high density of components. Heat from the driver chips can be minimized by optimizing the supply voltage to the LEDs. LEDs operate at a particular voltage (V_f), and any remaining 'overhead' voltage is converted to heat by the LED drivers. Thus, the use of variable-voltage, high-current power supplies is recommended in order to tune the supply voltage to the minimum necessary to maximize light output. For this reason, four separate barrel jack connectors are provided to satisfy the diverse power needs of the optoPlate illuminator: the Arduino (7.5 V recommended), the red/blue and far-red LEDs (4 V for red/far-red and 5 V for blue), and the fan (12 V).

Another source of heating is the power dissipation inherent in LED operation. When all LED channels are driven to the maximum allowed by the driver chips, the optoPlate illuminator will draw 30 mA/channel × 288 channels × ~4 V = ~35 Watts. Because LEDs are only 40–50% efficient, heat is generated as a function of the number, intensity, and duration of operating LED illuminators. In addition, up to 8.6 A can flow through the device under maximal load, and this current flow may generate heat through electrical resistance in the small traces of the PCB.

Although the active heat sink is effective in minimizing sample heating, it cannot completely decouple the illumination profile from sample heating. Fortunately, because signaling downstream of typical optogenetic actuators (e.g., LOV2, PhyB) has activation/inactivation kinetics on the order of seconds to minutes, 'constant' optogenetic activation can almost always be achieved using pulsed light. Pulsed illumination markedly decreases heat generation at the sample, and illumination profiles can often be found that generate no sample heating (Supplementary Fig. 6). If high light flux is necessary, as in three-color experiments, we find that conditions can be optimized to minimize sample heating to no more than ~4 °C above ambient temperature. In these extreme cases, the biological sample can be maintained at 37 °C by reducing the temperature of the cell culture incubator.

We note that, in addition to minimizing sample heating, it is always advantageous to reduce total illumination of the sample to prevent phototoxicity. This is particularly important for optogenetic probes that require activating light in the blue and ultraviolet range.

Another limitation of the optoPlate-96 is the lack of a GUI to program the instrument. The illumination script is well annotated and is explained in supporting documentation, but future development of a GUI will make device programming easier.

Applications

The optoPlate-96 is designed for optogenetics experiments in a cell culture incubator over a wide range of time scales (seconds to weeks). optoPlate experiments can be analyzed with any assay compatible with microwell plates. We have most frequently coupled optoPlate stimulation to quantitative immunofluorescence, In-Cell Western, and flow cytometry assays. For adherent cells, we find that in-plate immunofluorescence readouts are optimal because they provide single-cell analytical resolution with a minimal number of disruptive cell processing steps.

In addition, the optoPlate illuminator (assembled PCB only) can be used for live-cell microscopy within an environmental chamber[15]. The thin profile of the surface-mounted PCB allows it to fit atop a 96-well plate and illuminate from above while an inverted microscope images from below the sample plate. This arrangement allows one to decouple the optogenetic stimulation light from the imaging optics of the microscope, as well as allowing simultaneous stimulation of every sample in a microwell plate with only intermittent imaging. The optoPlate is also suitable for live-cell imaging because all power and data transmission are integrated within the circuit board, providing robustness and simplicity in the device. External cables are required for only the power supplies, and these can easily be fed into the environmental chamber.

Experimental design

Choice of optoPlate configuration

The optoPlate-96 can be assembled to illuminate with one, two, or three distinct colors. If one is using only blue-light-sensitive optogenetic probes, the one-color (blue) configuration will maximize the dynamic range of stimulation. If only a red/far-red-sensitive protein will be used (e.g., PhyB/PIF[14]), the two-color configuration will be optimal to maximize the dynamic range of the far-red LED for efficient photoswitching. If blue and red probes will be used simultaneously, the three-color configuration is required to allow orthogonal stimulation of both probes. The assembly of each optoPlate configuration is identical, except for the placement and part number of (i) the individual LEDs and (ii) the resistors that define the current draw per channel. Our assembly protocol provides instructions for the construction of all three configurations. We also provide separate Arduino scripts for the operation of each of the three configurations (http://bit.ly/OP96arduinoScripts).

Choice of adaptor height and spatial distribution of illumination

The 3D-printed adaptor couples the optoPlate illuminator to the microwell plate, ensuring that light from one channel does not cross-illuminate neighboring wells. We provide design files for two versions of the adaptor that differ only in height. The short adaptor optimizes the photon flux experienced by cells, allowing less light to be used to achieve a given level of stimulation. The tall adaptor provides a more homogeneous distribution of light at the sample, albeit with diminished light intensity. The tall adaptor is particularly important for 384-well experiments, in which each of the 4 wells illuminated by a channel should experience the same light intensity. The short adaptor is best used with 96-well plates and provides maximum illumination efficiency, which can help minimize heat generation in light-intensive (e.g., three-color) experiments.

Choice of downstream assay

The optoPlate-96 is compatible with any plate-based endpoint assay and can also be adapted to stimulate 96-well plates for live-cell microscopy. In-plate immunofluorescence offers a particularly convenient assay that minimizes cell disturbance and offers single-cell resolution of multiple analytes per cell. Although traditional immunofluorescence is limited to ~3–4 optical channels, recent innovations such as cycIF[16] and iterative indirect immunofluorescence imaging (4i)[17] expand the number of analytes per cell substantially by allowing sequential rounds of immunolabeling and imaging. Note that if the optogenetic probes are fused to fluorescent proteins (FPs), these proteins must first be bleached with a bleaching solution, as described previously[16] and in this protocol.

optoPlate illuminator modifications

The optoPlate illuminator was designed to accommodate custom modifications, for example, to enable monitoring of the status of an optoPlate experiment, or to couple a temperature sensor directly to the illuminator. To make these modifications, users can communicate with the Arduino either through the USB port, or through one of three pins that is accessible on the edge of the PCB, next to the Arduino. These pins provide access to pins 13, A0, and A1 on the Arduino Micro.

Component placement for reflow soldering (Steps 1–12)

Unlike hand soldering with a soldering iron, reflow soldering allows all components to be simultaneously soldered in place through exposure to variable temperatures in an oven. This is ideal for devices with many components arranged tightly on a PCB, as is the case with the optoPlate illuminator.

Reflow soldering works by depositing a thin layer of solder paste between two surfaces to be soldered. Upon heating, the paste liquefies and joins the heated surfaces, similar to standard hand soldering. Solder paste is first deposited across the circuit board using a stencil. Then the stencil is removed and surface-mount components are placed in preparation for reflow soldering.

Note that the identity of components is written next to the footprint of each component on the PCB. These markings on the optoPlate illuminator PCB are specific to the three-color implementation.

Surface mount (reflow) soldering (Steps 13–19)

The assembled PCB with components can be rapidly soldered using a reflow oven according to the reflow profiles specified on the component spec sheets. In the absence of a reflow oven, reflow soldering can be accomplished using a standard toaster oven and oven thermometer, as described in this protocol. Note that temperatures within a toaster oven will be spatially heterogeneous, and, in our experience, successful heating protocols register different temperatures on an oven thermometer from what is recommended in the component product sheets. In fact, strict adherence to the temperatures recommended in the component product sheet may overheat and burn the PCB/components. Thus, careful attention must be paid to visual cues during reflow to ensure best results. Ideally, sacrifice one PCB and a few components to practice the reflow process. If successful, proceed to assemble and reflow the entire board.

Through-hole (hand) soldering (Steps 20–24)

Several optoPlate illuminator components are not suitable for surface mounting and must be hand-soldered. Take extreme care when hand-soldering, to ensure safety and also to prevent destruction of the surface-mounted components. Accidentally touching the LEDs with the hot soldering iron or with a hot solder wick will melt and destroy the LEDs.

Quality control (Steps 25–32)

Once all components have been soldered, it is important to verify that the illuminator board is working properly. Here, the two main objectives are to see that each LED lights up and that each LED can be independently controlled (i.e., that there is no bridging of pins—'crossed wires'—on the LED driver chips). To do this, we take advantage of the spatial orientation of each LED. The LED drivers are grouped by the color of LED that they control in the three-color optoPlate configuration (Supplementary Fig. 7). Each driver drives 24 LED channels of a single color in 24 neighboring wells. Because neighboring pins drive LEDs in neighboring wells (by row for blue/red, by column for far-red), we can identify bridged pins by programming the optoPlate to illuminate alternating rows or columns. The objective is to observe the alternating pattern of LED rows/columns that was programmed. If LEDs in 'OFF' rows/columns are ON, then the user knows that two pins have been accidentally bridged and can pinpoint the bridged pins by referring to Supplementary Fig. 7.

To simplify the testing process, we provide six scripts for QC (http://bit.ly/OP96qcScripts). These scripts will program the Arduino to illuminate the odd/even rows/columns of the blue, red, and far-red channels. These will reveal whether the optoPlate illuminator has been soldered properly or further touchup is necessary.

Full optoPlate-96 assembly (Steps 33–42)

Assembly of the complete optoPlate-96 requires mounting the heat sink to the illuminator, mounting the microwell plate adaptor to the illuminator, and mounting the illuminator/heat sink/adaptor assembly onto a baseplate. The heat sink is not essential for all applications but is important to ensure even and minimal sample heating in optoPlate experiments. Assembly requires pre-manufacture of various components (adaptor, plate lid, baseplate, heat sink standoff, thermal pad, and aluminum plate). Design files for these components are maintained in the online optoPlate-96 repository (http://bit.ly/OP96designFiles).

Programming the optoPlate (Steps 43–47)

optoPlate-96 illumination is programmed using a custom script that is uploaded to an Arduino Micro microcontroller via USB before the experiment. Annotated custom scripts and a user manual are maintained in the optoPlate-96 GitHub repository (http://bit.ly/OP96arduinoScripts). Separate scripts are provided for the one-color (blue), two-color (red/far-red), and three-color (blue/red/far-red) configurations. The scripts include a 'test mode' feature, which allows the entire illumination program to be played back at a higher speed to ensure that all LEDs are programmed as desired. A test run should be performed before each experiment. After the test run has confirmed proper LED programming, the final script should be re-uploaded to the Arduino, wherein the test mode has been disabled.

Programming a time course

Time course experiments can be run on the optoPlate, where each well represents a separate time point. These experiments are performed by staggering the start of successive wells and then fixing all wells simultaneously at the conclusion of the experiment. The provided Arduino scripts achieve time course stimulation by allowing variable time delays for each well before the start of the illumination program, as explained in the user manual.

Choice of illumination pattern

Optogenetics experiments should ideally be performed using the minimal amount of light required to achieve the desired activation. Excessive illumination can cause both phototoxicity (particularly with blue or UV light) and heating of the optoPlate illuminator. Fortunately, optogenetic probes can be maintained in the active form by using pulsed illumination, in which light pulses are on a faster time scale than the decay kinetics of the probe. The ideal pulsing parameters will depend on the specific optogenetic actuator. For example, for PhyB/PIF-based probes, we often stimulate at 500 ms ON/4.5 s OFF. However, for probes with faster kinetics of dark reversion, such as the blue-light-sensitive iLid/sspB, a higher duty cycle is required to achieve maximal activation. We recommend that for the particular probe and cell type to be used, a preliminary experiment be performed in which a range of ON and OFF pulses are examined to determine the pulsing parameters that maximize signal but minimize light usage.

Choice of power supply

Four onboard barrel jack connectors allow flexibility of input voltage supply. The voltage that supplies the Arduino (7.5 V) and the fan (12 V) should be the same for any optoPlate configuration. LED voltage supplies should be matched to the particular LEDs in a manner that maximizes LED intensity while minimizing supply voltage. For instance, we supply the blue LEDs with 5 V because we found that higher voltages provided little increased light intensity. In a similar manner, we determined that the red and far-red LEDs are ideally supplied with 4 V. Note that if you undersupply the LEDs, LED intensity may decrease as a function of increased load. For instance, a single red LED powered with 2.5 V may be brighter than if that LED were powered together with the other 95 LEDs on the board. Thus, keep in mind that it is necessary to always examine light intensity under the maximal load conditions anticipated in the experiment. Note that the barrel jacks are labeled for the components they power in the three-color optoPlate configuration. The barrel jack connector labeled 'blue/red' powers the LED emitter in the left position of each well (four pads on the PCB), whereas the barrel jack labeled 'far-red' powers the right LED emitter (two pads) (Supplementary Fig. 7).

Seeding and starving an optoPlate experiment (Steps 48–68)

For plate-based immunofluorescence experiments, we recommend using either glass-bottom or plastic-bottom plates with thin bottoms (e.g., Greiner μClear, Falcon Imaging Microplates). To obtain an even distribution of cells in a well, it is necessary to first coat the wells with an extracellular matrix protein (e.g., fibronectin) and then gently spin down the cells immediately after seeding. If experiments are performed that require serum starvation, note that full removal of medium from a well can dislodge cells. Sequential fractional washes (three-fourths volume washes) should be used to dilute serum while minimally disrupting the cells. Similarly, if media additives are required for an experiment (e.g., drugs or phycocyanobilin chromophore for PhyB activation), these should be mixed at a 2× concentration and added to the culture medium to achieve the final 1× concentration. We commonly run 96-well experiments with 100 μL of medium per well and 384-well experiments with 50 μL per well.

Running an experiment (Steps 69–73)
To begin an optoPlate experiment, the microwell plate of prepared cells is placed atop the optoPlate-96 and the 3D-printed lid is fitted atop the microwell plate. The LED and fan power supplies are connected first. Once the Arduino power supply is connected to the illuminator, the experiment has begun and a timer should be set for the desired experimental length. Once the experiment has concluded, the Arduino power cord is detached and the microwell plate is quickly taken to the cell culture hood for further processing.

Cell fixation (Steps 74–76)
After optoPlate illumination, cells should be fixed rapidly to best preserve the cellular state at the end of the experiment. To avoid cell loss during fixation, add an appropriate amount of 16% (wt/vol) paraformaldehyde (PFA) to achieve a final concentration of 4% (wt/vol). To minimize the time delay between illumination and fixation, use an electronic multichannel pipette with a multi-dispense option, if available.

Permeabilization and immunostaining (Steps 76–91)
Conditions for permeabilization may vary for specific cell types and antibodies. We have found that a good general protocol is to first permeabilize with 0.5% (vol/vol) Triton X-100 in PBS for 10 min, followed by ice-cold 100% (vol/vol) methanol for 10 min. Odyssey blocking buffer can be used as a general blocking and antibody dilution buffer. 50-µL volumes of antibody dilutions should be used to ensure coverage of the 96-well well bottom. For 384-well plates, at least 20 µL should be used per well. Wells should be washed 4–5 times after antibody incubation steps. It is not critical to incubate the samples during each wash step. If available, an automated plate washer (e.g., Biotek EL405) can be used to automate the wash steps.

Imaging and image analysis (Steps 92–95)
Automated multiwell plate imaging can be performed on any microscope with an automated stage, although dedicated high-content microscopy systems (e.g., the Thermo Fisher ArrayScan) are ideal for this purpose. Many software solutions exist to process and analyze imaging data. We describe quantification using CellProfiler[18].

Materials

Biological materials

- Cells of interest: In the examples shown in this protocol, we use NIH/3T3 (ATCC, cat. no. CRL-1658) and Lenti-X 293T (Clontech, cat. no. 632180) cell lines, but we anticipate that our approach will be compatible with any primary or cultured adherent cells in which optogenetic probes can be expressed **! CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

- 16% (wt/vol) paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15710-S) **! CAUTION** Paraformaldehyde is a volatile fixative and can cause pain if it comes into contact with skin or eyes, or if it is ingested. Always wear proper protective lab coat, gloves, and eye protection while handling it.
- PBS tablets (Thermo Fisher, cat. no. 18912014)
- DMEM, high glucose (Gibco, cat. no. 11965-092)
- FBS (Gibco, cat. no. 16140071)
- Calf serum (Hyclone, cat. no. SH30087.03)
- Penicillin–streptomycin (Gibco, cat. no. 15070063)
- 0.25% (wt/vol) Trypsin-EDTA (Gibco, cat. no. 25200056)
- Phycocyanobilin (Frontier Scientific, cat. no. P14137) **▲ CRITICAL** Commercially obtained phycocyanobilin typically contains impurities that impair optogenetics experiments. Thus, phycocyanobilin should be purified through HPLC, as previously described[19].
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Tween 20 (Sigma-Aldrich, cat. no. P1379)
- Methanol (EMD-Millipore, cat. no. AX1699M)
- Ethanol (Sigma-Aldrich, cat. no. 459844)
- Hydrogen peroxide (H_2O_2, 30% (wt/vol); Sigma-Aldrich, cat. no. 216763)

- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. 320331)
- Fibronectin (Millipore Sigma, cat. no. FC010)
- Dimethyl sulfoxide (DMSO; Fisher BioReagents, cat. no. BP231)
- Odyssey blocking buffer (Licor cat. no. 927-40000)
- Primary antibody (variable). In the examples described in this protocol, we used antibodies against phospho-Erk1(Y204)/Erk2(Y187), phospho-Erk1/2(T202/Y204), and phospho-Akt(S473) (1:400 (vol/vol) working dilution for each antibody; Cell Signaling Technologies, cat. nos. 5726, 4370, and 4060, respectively)
- Secondary antibody (variable). In the examples described in this protocol, we used goat α-rabbit Alexa Fluor 647 and goat α-mouse Alexa Fluor 488 (1:100 (vol/vol); Jackson Immunoresearch, cat. nos. 111-605-003 and 115-545-003, respectively)
- Annexin V (1:50 (vol/vol); Thermo Fisher, cat. no. A23204)
- Anti-ppErk (rabbit; 1:400 (vol/vol); Cell Signaling Technologies, cat. no. 4370)
- Anti-ppErk (mouse; 1:400 (vol/vol); Cell Signaling Technologies, cat. no. 5726)
- Anti-pAkt (rabbit; 1:400 (vol/vol); Cell Signaling Technologies, cat. no. 4060)
- DAPI (Thermo Fisher, cat. no. D1306) **! CAUTION** DAPI is a known mutagen and should be handled with care.
- Plasmids encoding optogenetic proteins (variable) **▲ CRITICAL** Any blue-responsive or PhyB/PIF-based optogenetic probe will work with the optoPlate-96 configurations we describe. In the 'Anticipated results' section, we present data generated from cells expressing either the optoSOS system (Addgene cat. nos. 50851 and 50839) or the optoPI3K system (construct available from the authors upon request). These constructs were cloned into the pHR lentiviral system, which requires additional plasmids for packaging (pMD2.G (Addgene, cat. no. 12259) and pCMV-dR8.2 (Addgene, cat. no. 8455)).
- FuGene HD transfection reagent (Promega, cat. no. E2311)

Equipment

Electronic components

- LED driver chips (12; Texas Instruments, cat. no. TLC5947DAP)
- Voltage regulators (three; 5 V; ON Semiconductor, cat. no. NCV5501DT50RKG)
- Bipolar transistor (PNP; NXP, cat. no. BC857B,215)
- Bi-color (blue/red) LEDs (96 ; Wurth Electronics, cat. no. 150141RB73100)
- Far-red LEDs (96; Marubeni, cat. no. SMT780-27)
- Blue LEDs (192; OSRAM, cat. no. LBT64GV1CA59Z) **▲ CRITICAL** Efficient inactivation of PhyB takes place over a very narrow range of far-red illumination. We tested LEDs with illumination wavelengths between 730 and 830 nm, and we found that 780-nm LEDs achieved optimal PhyB inactivation. The SMT780-27 (far-red) LEDs include a lens to focus the LED light. We found that this lens is important for optimal PhyB inactivation.
- Resistors (16; 10 kOhm, 1/4 W, case code 0805; Vishay Semiconductors, cat. no. TNPW080510K0BEEA)
- Resistors (12; 1.65 kOhm, 1/4 W, case code 0805; KOA Speer, cat. no. RN732ATTD1651B25)
- Resistors (4; 9.76 kOhm, 1/4 W, case code 0805; Vishay Semiconductors, cat. no. TNPW08059K76BEEA)
- Capacitors (12; 10 μF, case code 0805; Taiyo Yuden, cat. no. GMK212BBJ106KG-T)
- Capacitors (3; 1 μF, case code 0805; Taiyo Yuden, cat. no. UMK212B7105MG-T)
- Barrel jack terminals (4; Switchcraft, cat. no. RAPC722X)
- Female headers (2; 17-pin; 2.54 mm; Sullins Connector Solutions, cat. no. PPPC171LFBN-RC)
- Male headers (1; 4-pin; 2.54 mm; Harwin, cat. no. M20-9990446)
- Arduino Micro microcontroller (Arduino, cat. no. A000053)

Soldering supplies

- Solder paste stencil (PCB Unlimited, cat. no. STNCL-PROTO)
- Solder paste stencil kit (PCB Unlimited, add-on to cat. no. STNCL-PROTO)
- Solder paste (ChipQuik, cat. no. SMD291SNL10)
- Soldering iron (Atten, cat. no. AT-937)
- Soldering iron tip (hoof; Hakko, cat. no. T18-C2)
- Soldering iron tip (fine; Hakko, cat. no. T18-S4)

- Soldering iron tip (screwdriver; Hakko, cat. no. T18-D16)
- Solder (Chip Quik, cat. no. SMDSWLF.020) ▲ CRITICAL Either leaded or lead-free solder can be used. However, note that lead-free solder has a higher melting temperature than leaded solder and may thus require higher soldering temperatures.
- Solder flux pen (Chip Quik, cat. no. 910-CQ4LF)
- Solder wick (Chemtronics, cat. no. 60-2-10)
- Toaster oven (Proctor Silex, cat. no. 31118R)
- Oven thermometer (Cooper-Atkins, cat. no. 24HP-01-1)
- Flat-tip tweezers (TDI, cat. no. ECO-C2A-SA)
- Pointed tweezers (TDI, cat. no. TDI-AC-SA-I)
- Hot air rework station (Sparkfun, cat. no. TOL-14557)

Power supplies and cables

- Power supply (5 V, 10 A; Adafruit, cat. no. 658)
- Power supply (variable 0–18 V, 0–20 A; BK Precision, cat. no. 1688B) ▲ CRITICAL A high-current, variable-voltage power supply can be important for tuning the LED supply voltage; the minimum voltage that maximizes LED intensity should be selected. Oversupplying LED voltage can contribute to device heating.
- Power supply (7.5 V, 2 A; Phihong, cat. no. PSC15A-075)
- Power supply (12 V, 1 A; Triad Magnetics, cat. no. WSU120-1000)
- Barrel jack splitter (Adafruit, cat. no. 1351)
- USB-A to USB micro connector (Digikey, cat. no. AE10418-ND)
- Barrel jack cable assembly (Digikey, cat. no. CP-2185-ND)
- Banana plug connector (red; Digikey, cat. no. J145-ND)
- Banana plug connector (black; Digikey, cat. no. J146-ND)

Connectors/structural/other

- Threaded standoffs (2 inch; McMaster-Carr, cat. no. 93330A463)
- Press-fit inserts (2-56 thread size; McMaster-Carr, cat. no. 92394A111)
- Press-fit inserts (6-32 thread size; McMaster-Carr, cat. no. 92394A113)
- Screws (2-56, 1/4 inch long; McMaster-Carr, cat. no. 91770A076)
- Screws (4-40, 1/4 inch long; McMaster Carr, cat. no. 91249A105)
- Black acrylic (1/4 inch thick, 10 × 8 inches; TAP Plastics, cat. no. 99999)
- Light diffuser film (inventables.com, cat. no. 23114-01)

Thermal management

- Heat sink (Dynatron R30; Newegg.com, cat. no. 9SIAB944J55062) ▲ CRITICAL The optoPlate illuminator was designed to accommodate this specific heat sink.
- Hex nuts (narrow, 8-32; McMaster-Carr, cat. no. 90730A009)
- Thermal pad (Arctic Cooling, cat. no. ACTPD00004A)
- Thermal grease (Wakefield-Vette, cat. no. 120-2)

General laboratory consumables

- Tissue culture flasks (75 cm^2; Fisherbrand, cat. no. FB012937)
- Culture plates (6-well; Fisherbrand, cat. no. FB012927)
- Serological pipettes (1, 2, 5, 10, 25, and 50 mL; Fisherbrand, cat. nos. 13-678-11B, 13-675-3C, 13-676-10H, 13-676-10J, 13-678-11, and 13-678-11F, respectively)
- Pasteur pipettes (Fisherbrand, cat. no. 13-678-20D)
- Pipette tips (20–200 and 100–1,000 µL; Fisherbrand, cat. nos. 02-707-430 and 02-707-404)
- Reservoirs (Fisher Scientific, cat. no. 1422242)
- 96-well plates, glass or thin plastic bottom, black walls or white walls (e.g., Greiner Bio-One, cat. no. 655090 or 655098; Falcon, cat. no. 353219)
- ▲ CRITICAL The optoPlate-96 adaptor was designed for Greiner Bio-One µClear plates but can accommodate plates from other manufacturers as well (e.g., Falcon Imaging Microplate, cat. no. 353219). If plates from a different manufacturer will be used, the adaptor may need to be modified to accommodate manufacturer-specific plate geometries. Note that, although black-walled plates are typically recommended to minimize the likelihood of light cross-talk between wells and may be preferable for imaging, opaque white-wall plates can be used to maximize the light flux experienced by

the cells. This can be critical for three-color experiments, in which maximizing far-red intensity is important in order to counteract blue-light cross-stimulation of PhyB. Notably, we have never observed light cross talk between wells with either white- or black-walled 96-well plates.

- 384-well plates (glass or thin plastic bottom, black walls (Greiner Bio-One, cat. no. 781091) ▲ **CRITICAL** The optoPlate-96 adaptor has been optimized for Greiner Bio-One plates. If plates from a different manufacturer will be used, the adaptor may need to be modified to accommodate manufacturer-specific plate geometries.
- Parafilm (Bemis, cat. no. PM999)
- Kimwipes (Fisher, cat. no. 06-666A)
- Conical centrifuge tubes (15 and 50 mL; Fisherbrand, cat. nos. 07-200-886 and 05-539-13)
- Hemocytometer (Fisher, cat. no. 02-671-51B)
- Centrifuge tubes (1.5 mL; Fisherbrand, cat. no. 05 408 129)
- Sterile syringe filter (0.45 μm; Fisherbrand cat. no. 09 720 514)
- Sterile syringe (10 mL; Fisherbrand cat. no. 14 955 459)

General laboratory equipment

- Pipettes (2.5, 20, 200, 1,000 μL; Eppendorf, cat. no. 2231300004)
- Multichannel pipettes (12-channel, 30–300 μL; Eppendorf, cat. no. TI13690052)
- Electronic multichannel pipette (12-channel, 15–300 μL; Eppendorf, cat. no. 2231000531)
- Pipette filler (Fisherbrand, cat. no. FB14955202)
- Biosafety cabinet (Thermo Scientific, cat. no. 1300 A2)
- Tissue culture incubator (Fisherbrand Isotemp, cat. no. 11 676 600)
- Table-top centrifuge (Sorval X1R; Thermo Scientific, cat. no. 75612452)
- Vortex (Fisherbrand, cat. no. 9454FIALUS)
- Flashlight (Office Depot, cat. no. 749396)
- An automated plate washer (e.g., Biotek, model no. EL405)
- High-content microscopy system (e.g., Thermo Fisher, ArrayScan model)

Software

- optoPlate-96 GitHub repository (http://bit.ly/optoPlate96)
- Arduino (http://www.arduino.cc)
- CellProfiler (http://www.cellprofiler.org)

Reagent setup

PBS, pH 7.4

Dissolve one PBS tablet in deionized water. Store at room temperature (22–25 °C) for up to 6 months.

0.5% (vol/vol) Triton X-100 in PBS

Add 2.5 mL of Triton X-100 to 500 mL of PBS. Store at room temperature for up to 6 months.

0.1% (vol/vol) Tween 20 in PBS (PBS-T)

Add 0.5 mL of Tween 20 to 500 mL of PBS. Store at room temperature for up to 1 month.

NIH/3T3 growth medium

Add 50 mL of calf serum to 450 mL of DMEM, high glucose. Add 5 mL of penicillin–streptomycin. Store at 4 °C for up to 1 month.

HEK 293T growth medium

Add 50 mL of FBS to 450 mL of DMEM, high glucose. Add 5 mL of penicillin–streptomycin. Store at 4 °C for up to 1 month.

Fluorescence protein bleaching buffer

Fluorescence protein bleaching buffer is 3% (vol/vol) H_2O_2, 20 mM HCl, pH 2.5. Add 20 mL of 30% (vol/vol) H_2O_2 to 180 mL of deionized water. Add 333 μL of 12 M HCl. Store at room temperature for up to 1 year.

Fibronectin coating solution
Fibronectin coating solution is 10 μg/mL fibronectin in PBS. Dilute fibronectin stock (1 mg/mL) 1:100 (vol/vol) in sterile PBS. Prepare fresh before each use.

Phycocyanobilin reconstitution
Phycocyanobilin should be HPLC-purified because impurities in the commercial preparation can stimulate mammalian signaling pathways. A detailed protocol for phycocyanobilin purification has been described[19]. Purified phycocyanobilin should be reconstituted in DMSO to a 5 mM concentration.

Lentiviral packaging of optogenetic constructs
Package pHR lentiviral backbone plasmids by transiently transfecting the transfer plasmid, pCMV-dR8.2, and pMD2.G helper plasmids into Lenti-X 293T cells using the FuGene HD transfection reagent. The cell supernatant should be harvested at 48 and 72 h post transfection, pooled, and sterile-filtered through a 0.45-μm filter. Store the viral supernatant at -80 °C for up to 1 year.

Stable cell-line generation
Plate 100,000 cells into one well of a 6-well plate and add 100–1,000 mL of viral supernatant. Supernatant volume will depend on viral titer, infectivity of cells, and desired copy number of the construct. Pure populations of transduced cells can be obtained through either drug selection (if a drug-selection cassette is included in the integrated construct) or through cell sorting. ▲ **CRITICAL** We recommend generating stable lines through cell sorting because it allows for tight control over expression levels of the optogenetic probes. This can be especially critical for multi-component systems, in which different expression levels of each component may be optimal.

Equipment setup

PCB printing and stencil manufacture
optoPlate-96 illuminator circuit boards can be ordered through a prototype PCB manufacturer such as PCB Unlimited (https://www.PCBUnlimited.com). To order PCBs, submit the optoPlate-96 Gerber files and drill file (http://bit.ly/OP96gerbers). When ordering PCBs, also order a laser-cut PCB stencil for solder paste deposition. Thin stencils (0.004 inch thick) allow thinner layers of solder paste, result in less bridging during reflow soldering and thus require less touch-up. The Gerber file that specifies the solder paste pattern (optoPlate96_3col_v4H-F.Paste.gbr) should be submitted to obtain the correct stencil pattern. If ordering from PCB Unlimited, also buy the stencil kit, which includes L-brackets (Supplementary Fig. 8) and a solder paste spreader, used to deposit solder paste onto the PCB.

3D printing
3D-print the microwell plate adaptor, microwell plate lid, and heat sink pedestals according to the designs provided in the online repository (http://bit.ly/OP963dPrint). The adaptor and lid are ideally printed in black to minimize cross-well bleed-through. If a local 3D printer is unavailable, 3D-printed parts can be ordered online through a vendor such as Protolabs (https://www.protolabs.com).

Laser cutting
The optoPlate baseplate, the thermal pad, and the diffuser film squares must be laser-cut according to the designs provided (http://bit.ly/OP96lasercut). The baseplate should be cut from 1/4-inch acrylic. If a local laser cutter is unavailable, laser-cut components can be ordered through a prototype laser cutting service.

Water-jet cutting
A 1/8-inch aluminum plate must be cut according to the designs provided (http://bit.ly/OP96wjcut). Custom water-jet-cut aluminum plates can be ordered from http://www.bigbluesaw.com.

Solder paste
Warm the solder paste to room temperature before using.

Procedure

optoPlate-96 assembly ● Timing 4 h

1 *Placement of components (Steps 1–12)*. Secure the illuminator PCB to a workbench to prevent lateral movement. For example, secure the PCB between two L-brackets fixed to the workbench (Supplementary Fig. 8). Make sure that the PCB is oriented with the shiny surface mount device (SMD) pads facing up.

2 Carefully align the solder paste stencil atop the PCB, such that the stencil holes align with the SMD pads. Ensure good alignment with the thinnest pads, which are the pads that correspond to the pins of the LED driver chips. Fix the stencil in this position by taping it to the L-brackets.

3 Deposit solder paste liberally across several locations on the stencil.

4 Use the solder paste spreader to spread a thin layer of solder paste evenly across the stencil, making sure that solder paste fills all openings in the stencil. Use pressure to make sure that the stencil stays flat on the PCB and there is no lateral movement between the PCB and the stencil. Lateral movement will smear the solder paste between the pads.

5 Holding the solder paste spreader vertically and applying downward pressure, scrape off excess solder paste to ensure that the solder paste filling the stencil openings is only as thick as the stencil. After this step, there should be minimal paste on top of the stencil.
▲ **CRITICAL STEP** It is important to ensure that there is only a thin layer of solder paste on the PCB. Excessive solder paste increases the risk of bridged pins/pads, which may be challenging to resolve later.

6 Carefully remove the tape holding the stencil to the PCB. Holding one end of the stencil down with your fingers, carefully lift the stencil from the opposing edge. The goal is to remove the stencil while not disturbing the solder paste patterns that you have deposited on the PCB.
▲ **CRITICAL STEP** Extra care should be taken to minimize smearing through careful lifting of the stencil. Overly smeared solder paste may lead to solder bridging and will interfere with efficient soldering.

7 Using tweezers, begin to place components onto the appropriate SMD pads that are now covered in solder paste. Begin with the LED driver chips.
▲ **CRITICAL STEP** Proper chip orientation is critical. Pin 1 is marked by a circle mark on the top of the plastic case. Make sure that this mark is oriented properly, as shown in Supplementary Fig. 9.

8 Place the LED emitters.
▲ **CRITICAL STEP** Proper LED orientation is essential. LED polarity is indicated on the LED housing, but whether the mark indicates the anode or cathode varies by LED product number. Refer to Supplementary Fig. 5 for the proper LED orientation for the desired optoPlate-96 configuration. The LED product sheet can also be referenced to determine polarity.

9 Place voltage regulators as indicated in Supplementary Fig. 9.

10 Place capacitors on the PCB, following the guide in Supplementary Fig. 10. Capacitors are nonpolar and can be placed in either orientation.

11 Place resistors on the PCB, following the guide in Supplementary Fig. 11. Resistors are nonpolar and can be placed in either orientation.
▲ **CRITICAL STEP** Note that each LED driver is associated with a resistor, whose value changes between different optoPlate-96 illuminator configurations (one, two, or three-color). All other resistor values are constant between configurations.

12 Place the PNP transistor on the board as indicated in Supplementary Fig. 11.

13 *Reflow soldering (Steps 13–19)*. Carefully place assembled PCB on a rack in the toaster oven, using the middle rack position. Slide the PCB all the way toward the back of the oven.
! **CAUTION** Reflow soldering will produce fumes. If available, the reflow toaster oven can be operated in a fume hood to minimize exposure to fumes.

14 Place oven thermometer into the oven directly in front of the PCB.

15 Turn on the oven and set the temperature to ~120 °C. Observe the thermometer in the oven until oven heat registers ~100–110 °C. Wait for 20 s.

16 Turn the oven dial to 175 °C and continue to observe the thermometer and board carefully. As the oven temperature increases, components will sequentially solder. Solder paste will liquefy and melt, turning from a matted gray to a shiny silver. This will be seen in small components first (LEDs, resistors, capacitors) and later in the larger components (LED drivers, voltage regulators). Use a

flashlight to observe the changes in solder paste appearance. Continue heating until you have seen the solder paste turn shiny silver on all the large components.

▲ **CRITICAL STEP** Overheating can destroy the PCB and/or components. Care must be taken to ensure that all components are soldered without overheating/burning the board. As a reference, we find that all components are typically soldered by the time the oven thermometer reaches ~175 °C. We recommend practicing reflow soldering one time with few components before assembling an entire illuminator.

17 When soldering is finished, immediately turn the oven off and open the door to allow cooling to begin. Using tweezers, slide the PCB-carrying rack out to speed cooling.

18 Using tweezers or heat-resistant gloves, lift the PCB and carry it to the bench for further cooling and subsequent hand soldering of through-hole components.

! **CAUTION** The soldered PCB may still be very hot.

19 Inspect the quality of reflow soldering with a magnifying glass. All pins/pads should appear soldered.

? TROUBLESHOOTING

■ **PAUSE POINT** The reflow-soldered illuminator can be stored at room temperature indefinitely.

20 *Through-hole soldering (Steps 20–24).* Insert the barrel jack terminals into the PCB, with the pins facing down. Invert the PCB and solder the terminals to the pads. A tutorial on soldering technique can be found on Sparkfun.com (http://bit.ly/solderTutorial).

? TROUBLESHOOTING

21 Insert one 17-pin female header into the board, with the pins facing down. Pressing the header against the PCB, invert the PCB and header so that the header pins are pointing up. Rest the PCB on the header. Solder one header pin to the PCB. To ensure that the header is soldered perpendicular to the PCB, hold the PCB and header (with one pin soldered) in one hand, and touch the soldered pin with the hot soldering iron. The solder will briefly liquefy, allowing you to reposition the header as needed. Hold the header flush and perpendicular to the board until the solder has re-solidified.

22 Repeat this process with the second 17-pin female header.

23 To verify that the headers are positioned properly, take the Arduino Micro and gently fit its pins into the headers. If the pins align to the headers, set aside the Arduino and solder the remaining header pins. If the Arduino pins do not align, repeat the procedure in Steps 21 and 22.

24 Insert the 4-pin male header into the 4-pin through-holes next to barrel jacks. Ensure that the header is inserted from the bottom of the PCB, and that the short side of the header is inserted into the through-holes (the long side is pointing away from the PCB). As in Step 21, solder one pin, adjust its position, and then solder the remaining three pins.

■ **PAUSE POINT** The fully soldered illuminator can be stored at room temperature indefinitely.

25 *Quality control (Steps 25–32).* Plug the Arduino into the headers. Upload the 'QC_blueOddRows.ino' script to the Arduino, using the USB cable. All QC scripts can be found at http://bit.ly/OP96qcScripts.

? TROUBLESHOOTING

26 Plug in the 7–12 V power supply and the 4–5 V power supply into the barrel jacks labeled '7–12 V' and 'Blue/red power', respectively. Ensure that (i) all blue LEDs in odd rows (1, 3, 5, 7) are illuminated and (ii) no LEDs are illuminated in the even rows (2, 4, 6, 8). If either of these events occurs, note the coordinates of the affected LED.

27 Unplug the optoPlate and upload the script labeled 'QC_blueEvenRows.ino'.

28 Repeat Step 26 and observe the illumination pattern. This time, all blue LEDs in even rows, and none in the odd rows, should be illuminated. Note any blue LED that does not follow this pattern.

29 Repeat Step 25–28 for the red and far-red channels. The test scripts for these are named 'QC_redEvenRows.ino', 'QC_redOddRows.ino', 'QC_farredEvenCols.ino', and 'QC_farredOddCols.ino'. For far-red LEDs, plug in the 4–5 V power supply into the barrel jack labeled 'FR power' instead of 'Blue/Red power'. Note that the QC scripts for red LEDs will illuminate alternating rows of LEDs (as for the blue LEDs) and the QC scripts for far-red LEDs will illuminate alternating columns of LEDs. Also note that because most of the far-red spectrum is beyond human perception, the far-red LEDs will appear very dim. If all LEDs illuminated properly, you have successfully assembled the optoPlate illuminator. Skip to Step 31. In the case that any LEDs are illuminated incorrectly, proceed to Step 30.

30 (Optional) Use Supplementary Fig. 7 to map the LED driver and pins responsible for any incorrect LED illumination. If you identified LEDs that did not turn on, it is likely that the LED driver pin was not properly soldered to the PCB. Depending on severity, you may need only to supply extra solder to make a good connection or you may need to resolder an entire LED driver chip (e.g., to rotate or readjust).
? TROUBLESHOOTING

31 Plug the fan heat sink into the 4-pin male header on the underside of the optoPlate illuminator. Ensure that the black wire matches with the pin labeled 'GND'.

32 Plug in a 12-V power supply to the barrel jack labeled '+12 V (fan)'. The fan should automatically begin spinning. Once you have verified that it works, unplug the fan from the illuminator.
! CAUTION Ensure that your fingers are clear of the fan before powering the fan.

33 *Full assembly of the optoPlate-96 (Steps 33–42)*. On the flat metal surface of the heat sink, wipe off and discard the manufacturer-deposited thermal grease. Deposit a thin layer of thermal grease across the entire raised portion of the flat heat sink surface.

34 Turn the optoPlate illuminator PCB upside down (LEDs facing down). Align the laser-cut thermal pad to the underside of the PCB (the underside should be facing up). Ensure that the pad is flat, avoiding air bubbles at the interface. Next, align the custom aluminum plate on top of the thermal pad. Finally, align the heat sink and insert the heat sink bolts through the appropriate four holes in the aluminum–thermal pad–PCB assembly.

35 Hold the assembly together and turn it right side up. Use hex nuts to fix the heat sink and thermal components to the optoPlate illuminator. Use pressure to ensure good thermal coupling between the heat sink and aluminum plate/PCB, but be aware that overtightening may damage the illuminator PCB.

36 Insert four press-fit inserts (2-56) into the four corners of the microwell adaptor bottom. Make sure that the rough end of the insert is inserted first. Strong pressure or force (e.g., a hammer) can be used to ensure that the insert opening is flush with the adaptor bottom.

37 Align the adaptor with the top side of the illuminator so that the press-fit inserts are facing down, each adaptor square fits over one of the 96 LED positions, and the clipped corners on the top of the adaptor are facing the side of the illuminator that houses the Arduino. In this configuration, the press-fit inserts should be aligned with holes in the illuminator, thermal pad, and aluminum plate. Invert the entire assembly and confirm alignment, and then secure the adaptor–PCB–thermal pad–aluminum plate assembly with four 2-56 screws.

38 Attach six 2-inch standoffs to the top side of the acrylic baseplate, using 4-40 screws. Position the six standoffs according to the model in Fig. 1b or the interactive 3D Sketchup model (http://bit.ly/OP96assembled).

39 Insert four press-fit inserts (6-32) into the bottom of the heat sink pedestals. Make sure to insert the rough end of the insert first. Secure the heat sink pedestals to the top side of the baseplate, using four 6-32 screws.

40 Place the illuminator–heat sink–adaptor assembly over the baseplate such that the heat sink fan rests on the heat sink pedestals, and the holes of the illuminator align with the 2-inch aluminum standoffs. Secure the baseplate and illuminator with six 4-40 screws through the top of the illuminator.

41 Plug the fan into the underside of the illuminator, ensuring that the black wire is in the position labeled 'GND' on the top of the illuminator.

42 To ensure even illumination across a sample, insert diffuser film squares into the 96 adaptor openings. Using tweezers, carefully place these squares on the nubbins positioned on each wall of each opening. Note that diffuser squares also reduce the effective photon flux to the sample and can be removed when the photon flux needs to be optimized. Illumination evenness is particularly a concern with 384-well experiments because of the asymmetrical positioning of various LED colors and because of dim illumination at the corners of the adaptor. For these experiments, diffusers are essential.

Performing optoPlate-96 experiments ● Timing 4.5 h of preparation; timing of experiments is variable

43 *Programming the optoPlate (Steps 43–47)*. Open the appropriate script to run the optoPlate-96 configuration that was assembled (one, two, or three-color). These scripts are provided on the online GitHub repository, together with a user manual (http://bit.ly/OP96arduinoScripts).

44 Define the LED positions to be used, the intensity of each color, the pulsing profile, and the timing of activation. Details for use are provided in the user manual and within the annotated scripts.

45 To confirm that the Arduino was programmed correctly, designate the program to run in test mode (in the script, set the variable *test* = 1). This will allow you to observe a sped-up version of your program to determine its validity. Set the *factor* variable to define a factor by which all time variables will be divided (e.g., setting *factor* = 60 speeds the illumination by a factor of 60. Here, each second represents 1 min of the experiment). Note that in test mode, LED illumination is by default constant, not pulsed.

46 Upload the script to the on-board Arduino Micro using a micro USB cable.

47 Plug in the LED and Arduino power cables. Observe the illumination in test mode. If incorrect, modify the illumination parameters in Step 44, and repeat Steps 45–47. If correct, set *test* = 0 to exit test mode, and upload the script to the Arduino as in Step 46.

48 *Seeding an experiment (Steps 48–59).* Coat a microwell plate with fibronectin coating solution. Using a multichannel pipette, add fibronectin coating solution to the wells.
- For wells in a 96-well plate, coat with 100 µL/well.
- For wells in a 384-well plate, coat with 25 µL/well.

49 Enzymatically dissociate cells from the tissue culture plate. For cells in a T75 flask, remove the cell culture medium and wash in 5 mL of PBS. Remove the PBS and add 1.5 mL of trypsin.

50 Place the flask in a 37 °C cell culture incubator for 3–5 min to speed enzymatic digestion.

51 Resuspend the dissociated cells in 4.5 mL of growth medium. Wash the flask with medium 2–3 times to ensure efficient recovery of the cells. Gently triturate (pipette the suspended cells up and down) to help dissociate cell clumps.

52 Centrifuge digested cells in a 15-mL conical tube for 3 min at room temperature at 200*g*.

53 Remove the supernatant and resuspend the cells in 4 mL of growth medium. Triturate as in Step 51 to eliminate cell clumps and ensure a single-cell suspension.

54 Determine the cell concentration using a hemocytometer.

55 Dilute the cells to the appropriate concentration. For 50–70% confluency the following day, we recommend preparing the following dilutions:

	96-well plates	384-well plates
Number of NIH/3T3 cells	5,000 cells/well	1,000 cells/well
Number of HEK 293T cells	20,000 cells/well	4,000 cells/well
Total volume per well	200 µL	100 µL

▲ **CRITICAL STEP** The optimal seeding density depends on the particular cell line, experiment, and downstream application. Overconfluence of cells can affect their biological function and can impair downstream assays. For example, for immunofluorescence, overconfluent cells will be difficult to segment and analyze. Thus, optimization of seeding density may be required.

56 Remove the fibronectin coating solution from the microwell plate.

57 Seed the cells in microwell plates, using a multichannel pipette.

58 Immediately after seeding, spin the plate for 1 min at 100*g* at room temperature. This promotes an even distribution of cells across the well bottom.

59 Place the cells in a tissue culture incubator and allow cells to attach and spread overnight. If serum starvation is not needed, skip to Step 65. If using blue-light-sensitive optogenetic probes, cover the plate in aluminum foil. If exogenous factors are not needed (e.g., phycocyanobilin for PhyB activation), skip to Step 69.

60 *(Optional) Serum starvation (Steps 60–64).* If experiments are performed that require serum starvation, warm starvation medium (i.e., growth medium without serum) to 37 °C.

61 Replace the growth medium with starvation medium. When adding starvation medium, use a multichannel pipette and ensure that the medium is added to the sides of the wells to minimize disruption of the cell layer. To prevent cell detachment during starvation, minimize the number of full washes that the cells experience.
- For 96-well experiments, perform one full medium exchange (i.e., 200 µL) followed by 3 × 75% medium (i.e., 150 µL) exchanges.
- For 384-well plates, even a single full medium exchange can cause substantial cell loss. Starve cells with successive (~7×) 70–80% medium (70–80 µL) exchanges.

62 After the final medium exchange, remove half of the starvation medium for a final volume of 100 µL (96-well plate) or 50 µL (384-well plate).

63 Briefly observe the cells under a microscope and note any cell loss as a result of starvation medium exchanges.

64 Incubate the cells in a cell culture incubator for ~3 h to allow signal activation to decay to baseline.
▲ **CRITICAL STEP** If using blue-light optogenetic tools, wrap the plate in aluminum foil before placing it in the incubator. Skip to Step 69.
▲ **CRITICAL STEP** If an exogenous chromophore is required (e.g., phycocyanobilin for PhyB activation), incubate the plate at 37 °C for 2.5 h and proceed to Step 65.

65 *(Optional) Phycocyanobilin addition (Steps 65–68).* If using PhyB optogenetic probes, phycocyanobilin should be added to the cells 30 min before the start of the optogenetics experiment. Dilute phycocyanobilin to a 2× stock in warm starvation medium (use a 2× stock concentration of 10 µM for NIH/3T3 cells and 20 µM for HEK 293Ts and most other cells).

66 Remove 50% of the starvation medium from each well.
- For 96-well plates, remove 50 µL.
- For 384-well plates, remove 25 µL.

67 Add 1 volume of the 2× phycocyanobilin solution to each well for a final concentration of 1× phycocyanobilin.
- For 96-well plates, add 50 µL for a final volume of 100 µL.
- For 384-well plates, add 25 µL for a final volume of 50 µL.

68 Wrap the plate in aluminum foil and place it in an incubator for the remaining 30 min.

69 *Running an experiment (Steps 69–73).* Place the programmed optoPlate-96 in the incubator. The optoPlate-96 can be disinfected with 70% (vol/vol) ethanol spray before being placed in the incubator. Disinfect the optoPlate lid with liberal amounts of 70% (vol/vol) ethanol. Wipe off the excess and allow the remainder of the ethanol to evaporate in the incubator.

70 In dim light and working quickly, remove the aluminum foil from microwell plate and place the plate onto the optoPlate adaptor so that the plate lip is flush with the adaptor top. Make sure that the microwell plate is properly oriented in relation to the orientation of the adaptor.
▲ **CRITICAL STEP** Ambient light can activate optogenetic probes. Take precautions to shield samples from unintended illumination. Alternatively, a 'safe', wavelength of light could be used to aid visibility during the experiment. For blue-light-sensitive tools, this would be any light with a wavelength >500 nm (e.g., green or red). Note that for the red-light-sensitive probe PhyB, any light with a wavelength <750 nm can stimulate the probe. If PhyB-expressing cells incubated with phycocyanobilin were inadvertently exposed to light before the start of the experiment, simply illuminate the cells with inactivating far-red light for 2–3 min, and then incubate in the dark until the start of the experiment.

71 Replace the plastic lid of the microwell plate with a 3D-printed lid. Make sure that the protrusions on the underside of the lid fit into each well.

72 Set a timer for the total duration of the experiment.

73 Plug in the power cords in the following order: fan, LEDs, and Arduino. At the same time as you plug in the Arduino power, start the timer. The experiment has begun.

Immunostaining and analysis ● Timing 10–15 h

▲ **CRITICAL** This section describes the procedures for analysis of the optogenetics experiment using immunostaining and fluorescence microscopy. Alternatively, the cells can be prepared for flow cytometry, In-Cell Western, Western blot, or any other downstream assay compatible with microwell plates.

74 *Fixing, permeabilizing, and bleaching cells (Steps 74–82).* 15 min before the end of the experiment, prepare 16% (wt/vol) PFA in a reservoir. Calculate the amount of PFA solution needed using the following volumes:
- For 96-well plates, use 34 µL of 16% (wt/vol) PFA for each well.
- For 384 well plates, use 17 µL of 16% (wt/vol) PFA for each well.

Prepare a reservoir with at least the necessary volume (plus ~10% reserve volume) of 16% (wt/vol) PFA. Set a multichannel pipette (preferably an electronic multichannel pipette) to the appropriate dispense volume, using the multi-dispense feature.

75 At the end of the experiment—working quickly and in dim light—unplug the 7.5-V (Arduino) power supply and move the microwell plate to the prepared PFA and pipette. Rapidly add the appropriate amount of PFA to each well to a final concentration of 4% (wt/vol) PFA, as described in Step 74.

■ PAUSE POINT Unused 16% (wt/vol) PFA can be transferred to a 15-mL conical tube and stored at -20 °C for up to 1 year.

76 Cover the plate and incubate it at room temperature for 10 min. After this incubation, samples are fixed and are no longer light sensitive, and can be manipulated under normal light conditions.

▲ CRITICAL STEP Excessive incubation in fixative can destroy antibody epitopes and impair downstream immunostaining. Limit fixation to 10–15 min.

77 Using a manual multichannel pipette, carefully remove all PFA solution from the microwells and deposit it into a reservoir. Transfer the PFA waste solution from the reservoir to a conical tube and dispose of it appropriately.

78 Permeabilize the cells by adding 100 µL of 0.5% (vol/vol) Triton X-100 in PBS and incubating the plate for 10 min at room temperature.

- For 96-well plates, add 100 µL to each well.
- For 384-well plates, add 50 µL to each well.

▲ CRITICAL STEP Certain antibodies may have specific permeabilization conditions for optimal performance. Always consult the antibody specification sheet before use.

79 Remove the Triton X-100 solution from the cells. Add ice-cold methanol to each well and incubate the plate at -20 °C for 10 min.

- For 96-well plates, add 100 µL to each well.
- For 384-well plates, add 50 µL to each well.

80 Remove the methanol from the cells.

81 (Optional) If cells express FPs that are not desired for imaging, FP fluorescence can be permanently bleached. Add FP bleaching buffer to each well and incubate the plate under direct light (e.g., a lamp) for 1 h at room temperature.

- For 96-well plates, add 100 µL to each well.
- For 384-well plates, add 50 µL to each well.

Discard the FP bleaching buffer and wash the plate once in PBS-T. Discard the PBS-T.

82 Incubate the plate in Odyssey blocking buffer for 30 min at room temperature.

- For 96-well plates, add 50 µL to each well.
- For 384-well plates, add 25 µL to each well.

■ PAUSE POINT Plates can be stored in blocking buffer at 4 °C for several days. Wrap the plate edges in Parafilm to prevent dehydration of the samples.

83 *Immunostaining (Steps 83–91).* Dilute primary antibodies according to manufacturer's specifications in Odyssey blocking buffer.

- For 96-well plates, prepare 50 µL of antibody solution per well.
- For 384-well plates, prepare 25 µL of antibody solution per well.

84 Remove the Odyssey blocking buffer and add primary antibody solution to the wells, using a multichannel pipette.

85 Incubate the cells in antibody solution for either 2 h at room temperature or overnight at 4 °C. If incubating overnight, wrap the plate in Parafilm to prevent dehydration.

■ PAUSE POINT Samples can be incubated in primary antibody at 4 °C for several days.

86 Using a multichannel pipette or plate washer, wash the wells 5× with PBS-T.

- For 96-well plates, use 100 µL per wash per well.
- For 384-well plates, use 50 µL per wash per well.

87 Prepare dilutions of fluorescently labeled secondary antibody according to the antibody manufacturer's instructions. Add DAPI for a final concentration of 300 nM for nuclear counterstaining. Antibody solution volumes are the same as in Step 83.

88 Remove the PBS-T wash buffer and add secondary antibody solution to the wells, using a multichannel pipette. Cover the plate with aluminum foil.

89 Incubate the cells in secondary antibody solution for 1 h at room temperature.

90 Using a multichannel pipette or a plate washer, wash the wells 5× each with PBS-T. Wash volumes are the same as in Step 86. At the end of this step, each well of a 96-well plate should have 100 µL of PBS-T and each well of a 384-well plate should have 50 µL of PBS-T.

91 Seal the edges of the plate with Parafilm, cover with aluminum foil, and store at 4 °C until imaging. ■ **PAUSE POINT** Although imaging should be performed shortly after immunostaining, immunostained plates can be stored at 4 °C—covered in aluminum foil—for 1 week. Loss of fluorescence intensity may occur over time.

92 *Imaging (Steps 92 and 93)*. Clean the bottom of the microwell plate with a Kimwipe soaked with 70% (vol/vol) ethanol.

93 Image immunofluorescence under a 10× or 20× objective, using an epifluorescence microscope with an automated stage. Ensure that fluorophore excitation and camera exposure conditions are constant for all images, and that focus is maintained for all images across the well plate. Also ensure that background illumination is flat across the image. Systematic flatness corrections can be applied computationally. Uncorrected nonuniformities in background fluorescence will introduce noise into single-cell quantitation.

94 *Image analysis (Steps 94 and 95)*. Quantitate single-cell fluorescence using CellProfiler[18]. A template CellProfiler pipeline is provided in the online optoPlate-96 repository (http://bit.ly/OP96code). This pipeline subtracts background pixel intensity from all images, identifies the nucleus of each cell, identifies the cytoplasm by forming a five-pixel ring around the nucleus, and then quantifies the nuclear and cytoplasmic intensities of each channel. Fluorescence values for each cell are exported in a .csv format.

95 Fluorescence quantitation of each cell from each well can be analyzed and visualized using several platforms, for example, R, Python, or CellProfiler Analyst[20].

Troubleshooting

Step 19: Identification of solder bridges that form between pins during surface mount soldering

Because of the small size and close proximity of pins on the LED drivers, solder bridges can form between driver pins during reflow soldering. Bridged pins are the most common cause of incorrect illumination patterns during QC testing. Although some bridges will be visible to the eye, others will not. Therefore, it is best to complete soldering of all components and then touch up the illuminator to fix bridging after Step 29, when the QC scripts will definitively indicate which pins are bridged.

Step 20: Trouble with through-hole soldering and lead-free solder

A good tutorial for soldering through-hole components can be found on Sparkfun.com (http://bit.ly/solderTutorial). Our protocol suggests the use of lead-free solder, but leaded solder can also be used, if preferred. Note that lead-free solder has a higher melting temperature than leaded solder and thus requires higher soldering iron temperatures for effective soldering.

Step 25: Trouble uploading Arduino scripts

Ensure that the Arduino Micro is connected by USB to the computer and that the proper port and proper Arduino board are specified (these are selected under the 'Tools' menu in the Arduino IDE). If trouble persists, try the following:

1 Press and hold the 'RESET' button on the Arduino Micro.

2 With the 'RESET' button depressed, begin upload of the Arduino script.

3 After the status bar has changed from 'Compiling sketch' to 'Uploading...', release the 'RESET' button.

If the above steps do not fix the problem, try uploading to a new Arduino Micro unit.

Step 30: Reworking the assembled PCB to fix solder bridges

If the QC scripts produce unexpected illumination patterns, this indicates an error in assembly. Most errors will result from solder bridges formed between pins of the LED driver chip and will be obvious because an LED that should be OFF will instead be ON because of a short circuit to a neighboring LED channel. Other types of errors may be caused by bridges between the control pins on the LED driver chips (pins 1–4, 29–32). These can be more difficult to diagnose but may be indicated by entire columns or rows of LEDs (mis)behaving the same way. The specification sheet for the TLC5947 LED driver chips (http://bit.ly/tlc5947) may provide more insight into the identity and function of each pin, and thus may help in troubleshooting.

Solder bridges can almost always be fixed using a combination of a soldering iron, solder braid wick, a flux pen, and a hot air rework station. Tutorials for fixing solder bridges can be found on youtube.com (http://bit.ly/SMsolder).

The first step is to identify and locate the solder bridges that underlie the improper illumination, using Supplementary Fig. 7 as a guide. Solder bridges can often—although not always—be visualized by eye or with the help of a magnifying glass. Once localized, the first step is to locally touch up the pins in question by removing excess solder and/or reflowing the existing solder with the help of solder flux. Test whether the problem is solved by re-running the QC script that revealed the problem. If incorrect illumination persists and the cause of bridging is unclear, it may be necessary to desolder the entire LED driver chip (using hot air) and to resolder it manually. Tutorials for touch-up and hand soldering of integrated chips can be found on youtube.com (http://bit.ly/SMsolder) and Sparkfun.com (http://bit.ly/SMsolder2).

Timing

Steps 1–12, optoPlate-96 assembly, placing components: 1.5–2 h
Steps 13–19, optoPlate-96 assembly, reflow soldering: 15 min
Steps 20–24, optoPlate-96 assembly, hand soldering: 15–30 min
Steps 25–32, optoPlate-96 assembly, illuminator testing and QC: 30 min
Steps 33–42, full assembly of the optoPlate-96: 45 min
Steps 43–47, performing optoPlate-96 experiments, programming the optoPlate: 20 mins
Steps 48–59, performing optoPlate-96 experiments, seeding an experiment: 30 min
Steps 60–64, performing optoPlate-96 experiments, serum starvation: 3 h
Steps 65–68, performing optoPlate-96 experiments, phycocyanobilin addition: 30 min
Steps 69–73, running an experiment: variable (minutes to weeks)
Steps 74–82, immunostaining and analysis, fixing, permeabilizing, and bleaching cells: 30–90 min
Steps 83–91, immunostaining and analysis, immunostaining: 4 h
Steps 92 and 93, immunostaining and analysis, imaging: 1–3 h
Steps 94 and 95, immunostaining and analysis, image analysis: 4–6 h

Anticipated results

After reflow and hand-soldering, the optoPlate illuminator will look as depicted in Fig. 2a and Supplementary Fig. 2. After the illuminator has passed QC, the entire otpoPlate-96 can be assembled (Fig. 2b,c). A model of the assembled optoPlate-96 can be examined through interactive 3D Sketchup models (exploded (http://bit.ly/OP96exploded) and assembled (http://bit.ly/OP96assembled)).

Fluorescence imaging of optoPlate experiments provides single-cell analytical resolution. Figure 3 depicts an experiment performed in 96-well format in which the relationship between blue-light exposure and phototoxicity was systematically examined. Serum-starved NIH/3T3 fibroblasts were illuminated with blue light with square wave pulse trains of variable ON- and OFF-pulse widths for

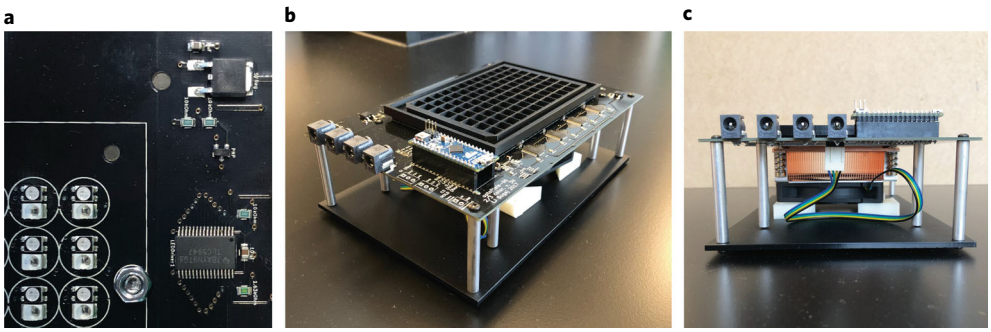

Fig. 2 | optoPlate-96 assembly. a, Zoom-in of LEDs, LED driver, voltage regulator, resistors, and capacitors soldered onto the optoPlate illuminator PCB. An image of the entire soldered illuminator can be found in Supplementary Fig. 2. **b**, A fully assembled optoPlate-96. **c**, Front view of **b**.

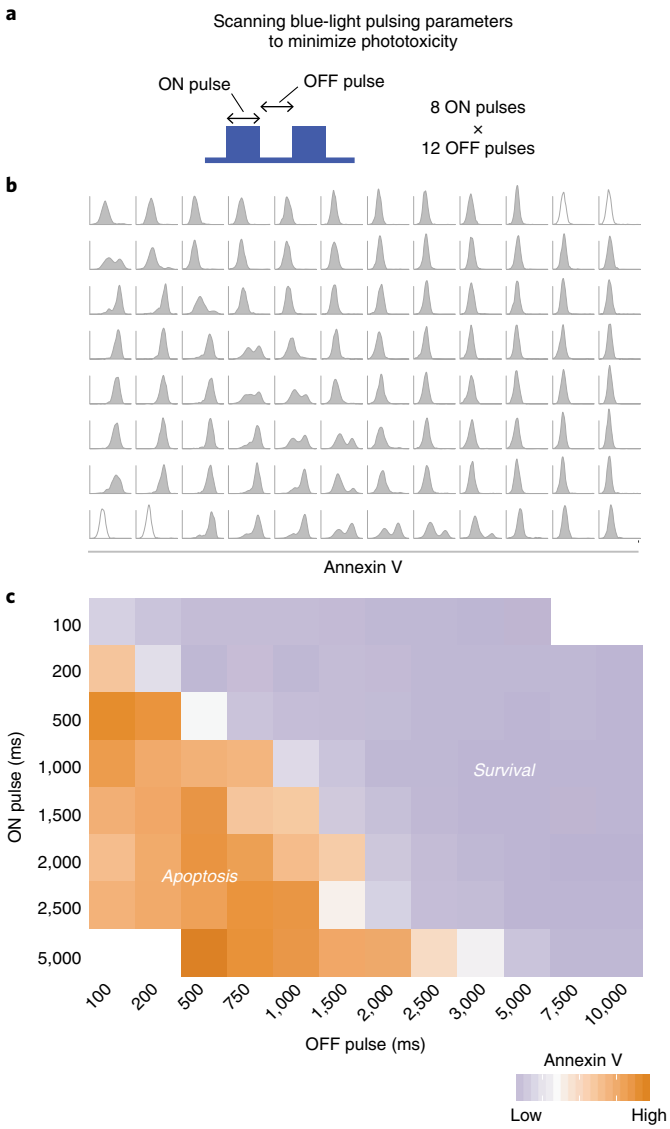

Fig. 3 | The optoPlate-96 allows systematic assessment of phototoxicity. a, Serum-starved NIH/3T3 cells were seeded in 96-well plates and exposed to pulsatile blue-light stimulation over 5 h. ON and OFF pulse times were varied to determine which pulsing parameters resulted in phototoxicity. After 5 h of stimulation, cells were stained for annexin V to measure apoptosis. Stained cells were fixed and imaged using fluorescence microscopy. **b**, Density plots of single-cell annexin V staining for each well. Unshaded traces in the bottom left and top right represent control wells that received no light. Cells in these wells stained negative for annexin V (apoptosis). Because these control wells were in regions of the plate with high LED usage (bottom left) and low LED usage (top right), this suggests that positive annexin V staining was due specifically to light illumination, and not to any systematic difference (e.g., heating) between different regions of the plate. **c**, Mean annexin V staining reveals the range of illumination parameters that are toxic to cells. Control wells are not depicted.

5 h, after which the cells were stained for markers of apoptosis and then fixed (Fig. 3a). Single-cell distributions (Fig. 3b) and their quantification (Fig. 3c) depict the parameter space of blue-light stimuli that is harmful to cells and thus should not be used for optogenetic probing. Note that the illumination parameters that induce phototoxcity depend on light intensity, cell type, and culture conditions, and thus should be determined empirically for each experimental system.

Figure 4 depicts an optimization experiment using the Ras–Erk activator optoSOS in 293T cells. OptoSOS relies on PhyB/PIF dimerization and thus on phycocyanobilin addition. This experiment simultaneously examined the effects of PhyB/PIF expression levels and phycocyanobilin concentration on the dynamic range and kinetics of Ras–Erk pathway activation. Further analysis is provided in Supplementary Fig. 12.

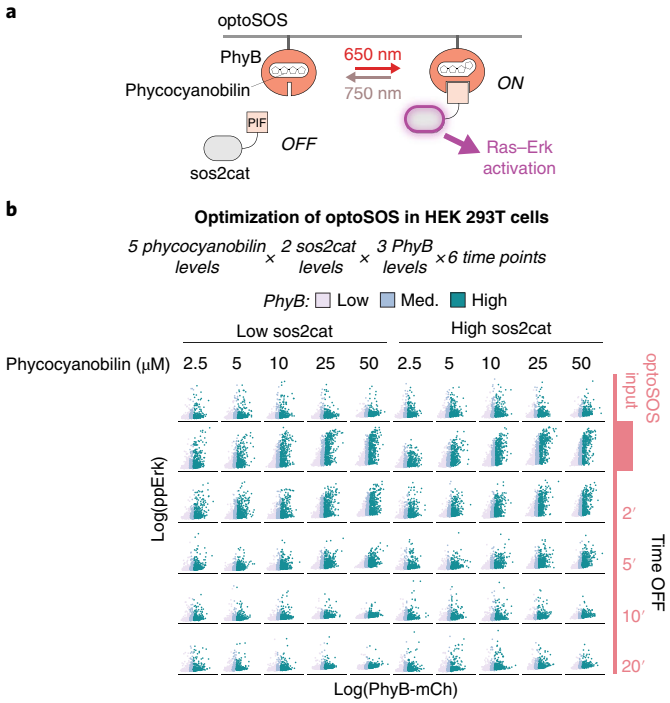

Fig. 4 | Rapid determination of optoSOS experimental parameters using the optoPlate-96. a, Schematic illustration of optogenetic control of the optoSOS system. **b**, Optimization experiment using five concentrations of phycocyanobilin, two levels of YFP-PIF-sos2cat (sos2cat), three PhyB-mCh-CAAX (PhyB) levels (binned computationally), and six time points in one experiment. Cells that stably express PhyB-mCh-CAAX and YFP-PIF-sos2cat were generated via lentiviral transduction. YFP-PIF-sos2cat levels were obtained by sorting populations of optoSOS-expressing cells (low YFP-PIF-sos2cat: ≤25th percentile; high YFP-PIF-sos2cat: 25th–75th percentile). This experiment was performed with the two-color red/far-red optoPlate configuration. All cells were illuminated with 500-ms pulses of light every 5 s. Unstimulated cells (first row) received only far-red light (Arduino intensity setting: 4,095), whereas stimulated cells (all other rows) received 30 min of the maximum amount of red light (Arduino intensity setting: 4,095), followed by the indicated amount of far-red illumination. During transitions (either from red to far-red or from far-red to red), light was held constant for 1 min to ensure a step-change in the optoSOS state. This is further described in the annotated scripts and user manual. After performing the optoPlate-96 experiment, cells were fixed and immunostained for ppErk. High-content fluorescence microscopy was used to quantitate levels of ppErk (Alexa Fluor 647), YFP-PIF-sos2cat (GFP), and PhyB-mCh-CAAX (PhyB-mCh; mCherry).

Finally, Fig. 5 demonstrates how orthogonal stimulation of red- and blue-sensitive optogenetic probes can be achieved by preventing blue-light cross-activation of PhyB. OptoSOS (controlled by the red/far-red-inducible PhyB/PIF system) and optoPI3K (controlled by the blue-inducible iLid/sspB system) were expressed in single cells to regulate Ras–Erk and PI3K–Akt signaling, respectively. OptoPI3K recruits the inter-SH2 domain of the p85 subunit to the membrane, which recruits the p110α subunit and stimulates PI3K/Akt signaling, as described previously[21,22]. Blue-light stimulation alone activated both iLid and PhyB, as indicated by phosphorylation of both Akt and Erk. However, sequential pulsing of blue and far-red light effectively counteracted cross-activation of PhyB by blue light.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data described in the article will be made available upon reasonable request to the corresponding author.

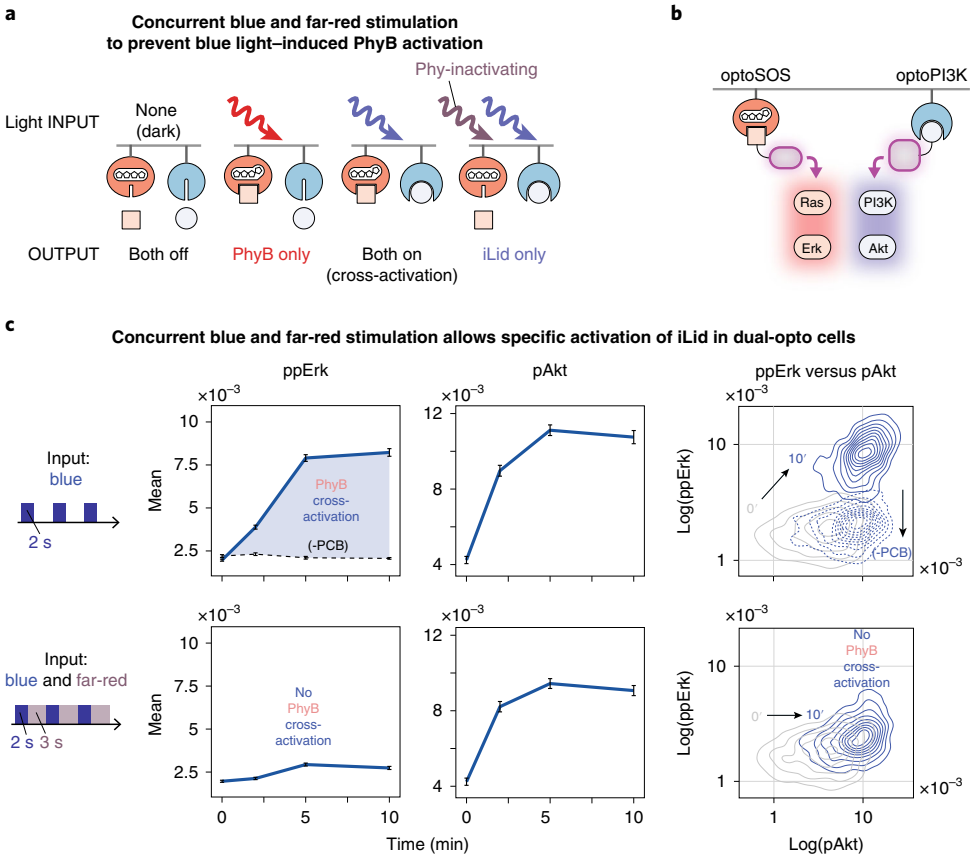

Fig. 5 | Simultaneous multicolor stimulation prevents blue light cross-activation of PhyB. a, PhyB/PIF and iLid/sspB are blue- and red-light-responsive heterodimers, respectively. Although red light will not cross-activate iLid, blue light can cross-activate PhyB. To achieve orthogonal control of PhyB and iLid, far-red light can be applied concurrently with blue light to avoid PhyB cross-activation. **b**, This strategy was tested in NIH/3T3 cells using PhyB- and iLid-based tools to control Ras and PI3K signaling (optoSOS and optoPI3K), respectively. **c**, Blue light alone strongly activated ppErk signaling because of cross-activation of PhyB. Sequential pulsing of blue and far-red light blocked PhyB cross-activation. In this experiment, cells were stimulated with pulse trains of light consisting of 2 s of blue light (Arduino intensity setting: 1,000), separated by 3 s of either darkness (top row) or far-red LED illumination (bottom row; Arduino ntensity setting: 4,095). Contour plots represent single-cell quantification of immunostaining for ppErk and pAkt as measured by high-content immunofluorescence microscopy. Line plots represent means ± 95% confidence intervals of single-cell distributions. Single-cell distributions of the 0- and 10-min time points are depicted in the right-most panels. Data are representative of 3–6 independent experiments.

Code availability

All design files and code needed for optoPlate construction and operation are provided within the public GitHub repository at the following link: http://bit.ly/optoPlate96. Design files, code, and updates can also be accessed through a dedicated optoPlate-96 webpage found at http://www.bugajlab.com/optoplate-96.

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Author contributions

L.J.B. designed, built, tested, and characterized the optoPlate-96. L.J.B. wrote the code to run and analyze optoPlate-96 experiments. L.J.B. performed and analyzed all optoPlate-96 experiments. L.J.B. wrote the manuscript, with editing from W.A.L. L.J.B and W.A.L made figures. W.A.L. oversaw the development of the optoPlate-96.

Competing interests

L.J.B. and W.A.L. have submitted a patent application describing the optoPlate-96 (no. 15/651628).

Additional information

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☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Data collection from optoPlate experiments required Arduino code to run the optoPlate illumination profile.
Data analysis	The CellProfiler image analysis platform was used to analyze immunofluorescence imaging. R was used to present the data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data described in the manuscript will be made available upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculations were performed. Sample sizes (number of cells per well) were chosen to obtain measurements on 300-1000 cells per condition.
Data exclusions	No data were excluded from analysis
Replication	Each experiment was performed between 2-5 times.
Randomization	This is not relevant because our experiments examined genetically modified cell lines in culture. Cells were sorted to minimize expression level variation of modified components. Any remaining heterogeneity was mitigated because cells were randomly seeded into microwell plates for experiments.
Blinding	Investigators were effectively blinded during analysis, because acquisition and analysis were both automated.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	phospho-Erk (Thr202/Tyr204): Cell Signaling Technologies, #4370, clone D13.14.4E. phospho-Akt (Ser473): Cell Signaling Technologies, #4060, clone D9E.
Validation	The Cell Signaling Technologies website states "we perform extensive testing on every antibody we develop to determine if it is highly sensitive and specific. Wherever possible, we use multiple cell types, multiple methods, and specific controls to verify that the product will generate biologically relevant results." The product pages for the indicated antibodies show convincing data on the appropriateness of these antibodies for immunofluorescence imaging.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	ATCC
Authentication	Cell lines were obtained from ATCC, which authenticates the cells.
Mycoplasma contamination	Cells tested negative for mycoplasma contamination
Commonly misidentified lines (See ICLAC register)	HEK 293T cells were used for their ease of transient transfection and rapid growth rate.