Supplementary Figures

Temperature-responsive optogenetic probes of cell signaling

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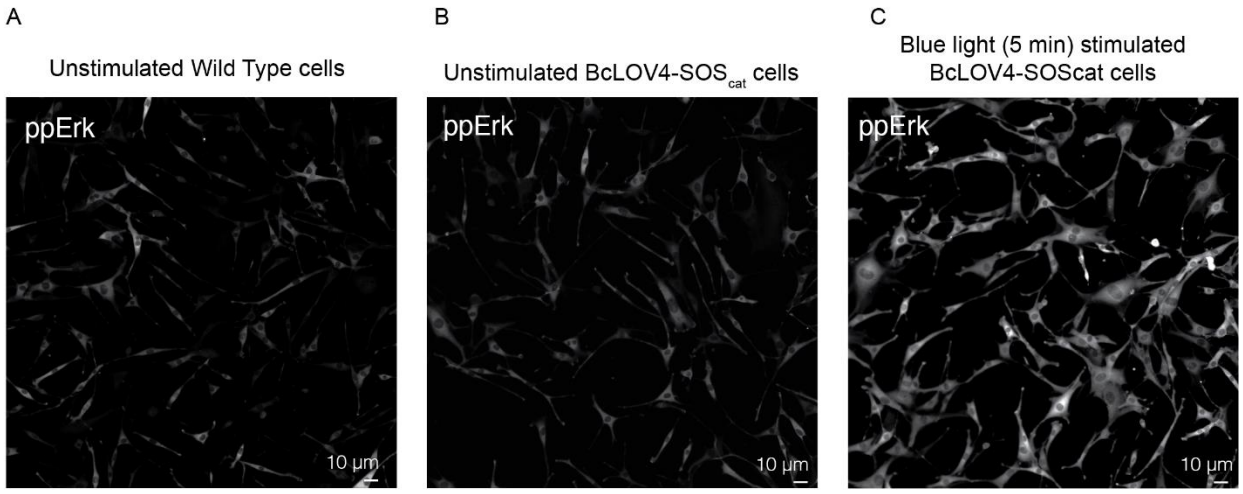
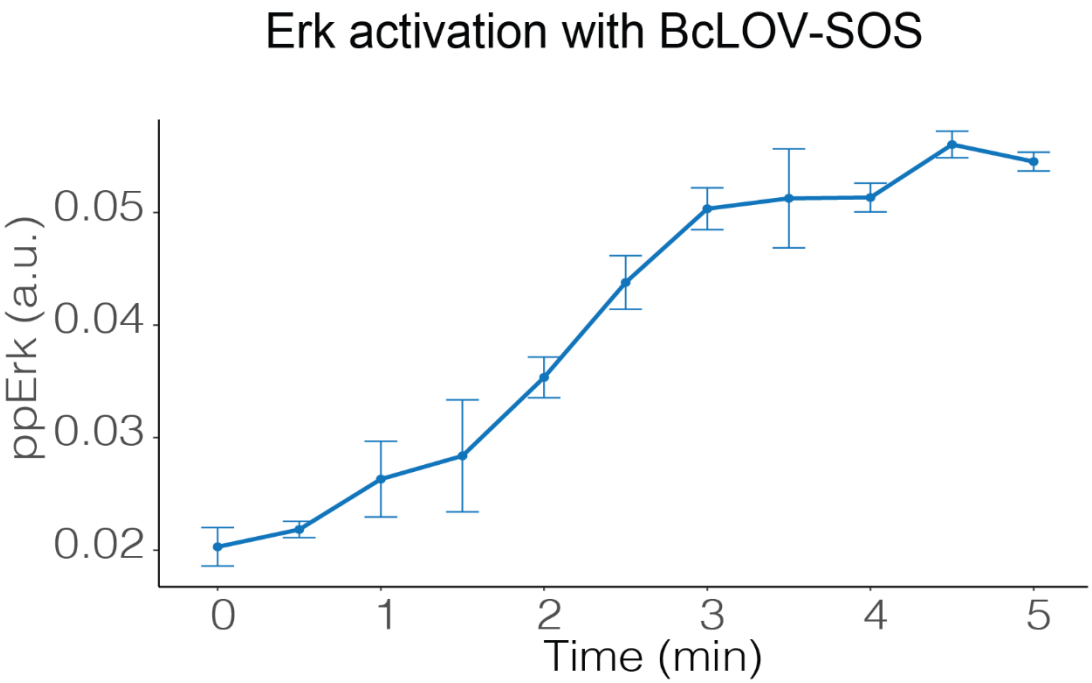
Supplementary Table 1.

Illumination and culture conditions for all experiments							
	Temp (°C)	Duty Cycle	Intensity	ON time	OFF time	humidified	CO2
1D	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
1E	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
1F	37	100.0%	variable	const.	--	Yes	5%
1G	37	100.0%	variable	const.	--	Yes	5%
2B	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
2C	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
2G	variable	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
2H	37	variable	160mW/cm2	.5s	variable	Yes	5%
3C	variable	3.3%	1.45W/cm2	1s	30s	Yes	5%
3D	37	variable	1.45W/cm2	variable	30s	Yes	5%
4C	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
4D	variable	variable	160mW/cm2	.5s	variable	Yes	5%
5A/B	25	3.3%	1.45W/cm2	1s	30s	Yes	5%
5D/E	25	3.3%	1.45W/cm2	1s	30s	Yes	5%
5F/G	25	3.3%	1.45W/cm2	1s	30s	Yes	5%
5H	25	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
6B/C	37	3.3%	1.45W/cm2	1s	30s	Yes	5%
6E Short/Long Light	30	0.3%	1.45W/cm2	0.1s	30s	Yes	5%
6E Inactive + Long Light	37	3.3%	1.45W/cm2	1s	30s	Yes	5%
S1	37	20.0%	160mW/cm4	.5s	2.5s	Yes	5%
S2	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
S3	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
S5	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
S6	37	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
S8A-D	variable	20.0%	160mW/cm2	.5s	2.5s	Yes	5%
S8E	37	20.0%	160mW/cm2	.5s	variable	Yes	5%
S9	variable	3.3%	1.45W/cm2	1s	30s	Yes	5%
S10	variable	3.3%	1.45W/cm2	1s	30s	Yes	5%
S11	variable	3.3%	1.45W/cm2	1s	30s	Yes	5%
S12	37	3.3%	1.45W/cm2	1s	30s	Yes	5%
S14	variable	variable	160mW/cm2	.5s	variable	Yes	5%
S15	37	50.0%	160mW/cm2	.5s	1s	Yes	5%

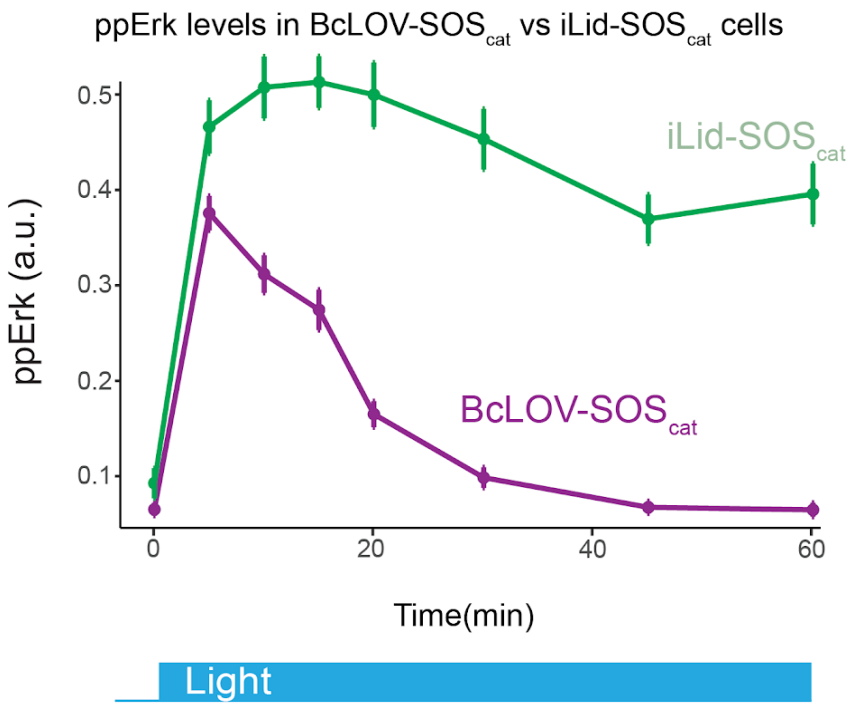
Supplementary Figures



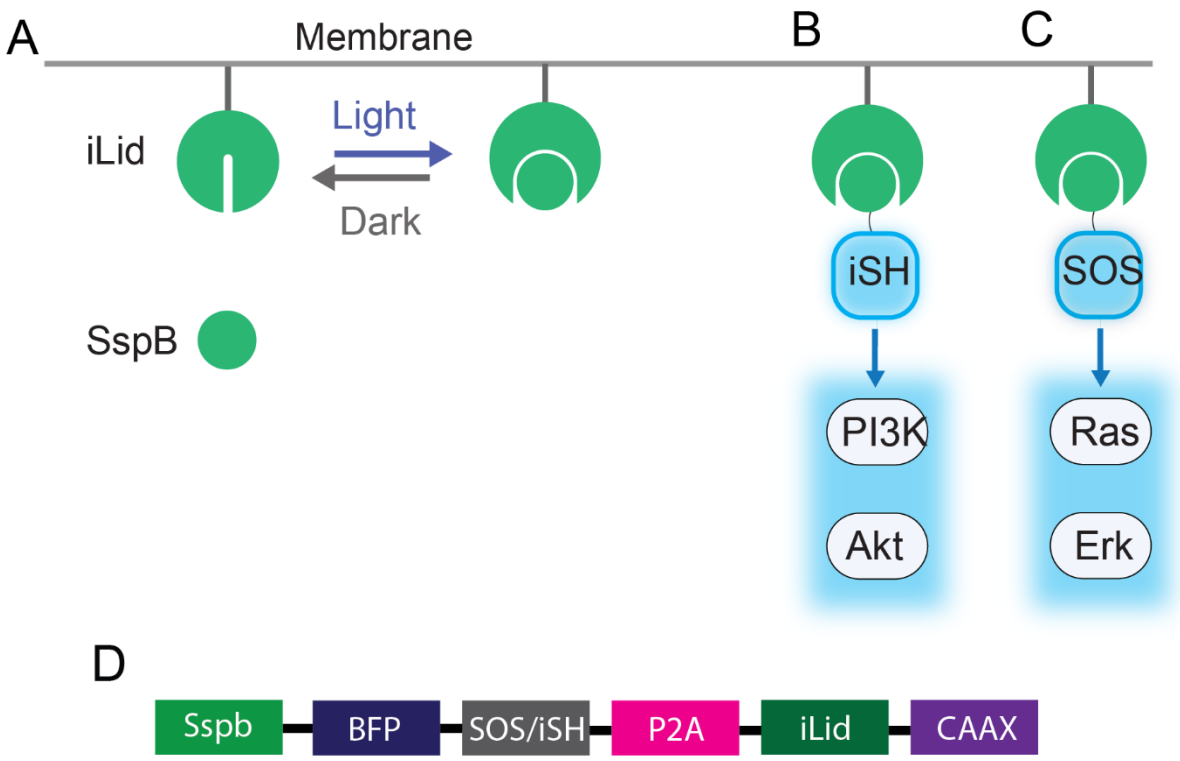
Supplementary Figure 1. Representative images of immunofluorescence staining of ppErk in BcLOV-SOS$_{cat}$ and WT cells. Immunofluorescent ppErk signal from unilluminated WT (A) vs unilluminated (B) and illuminated (C) NIH 3T3s expressing BcLOV-SOS$_{cat}$. Illuminated cells show increase in fluorescence compared to unilluminated cells. Cells are stained for phospho-Erk, with an Alexa488-conjugated secondary antibody.



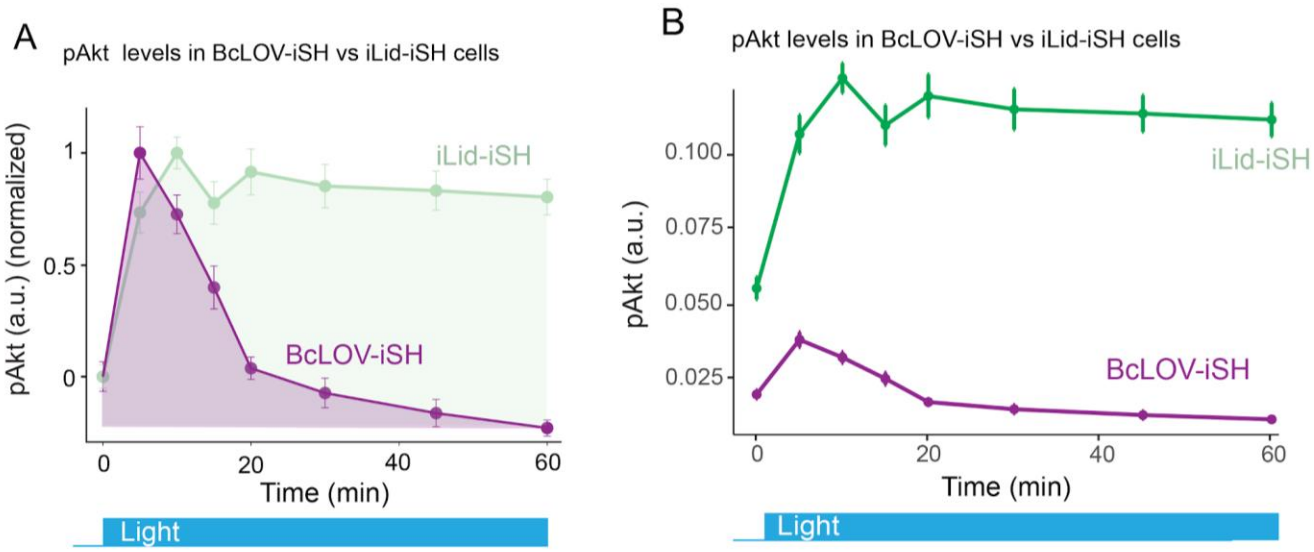
Supplementary Figure 2. Time course of early ppErk activation in BcLOV-SOS$_{cat}$ cells upon illumination. Early activation of Erk using BcLOV-SOS$_{cat}$. NIH 3T3 cells expressing BcLOV-SOS$_{cat}$ were exposed to blue light for the time shown, fixed, and stained for ppErk in order to establish the activation kinetics of Erk activation by SOS, with 30 s resolution. The peak of signaling occurs ~ 4.5 minutes. Data points represent the mean +/- 1 SD of three biologically independent replicates. Each replicate mean represents ~1000 cells.



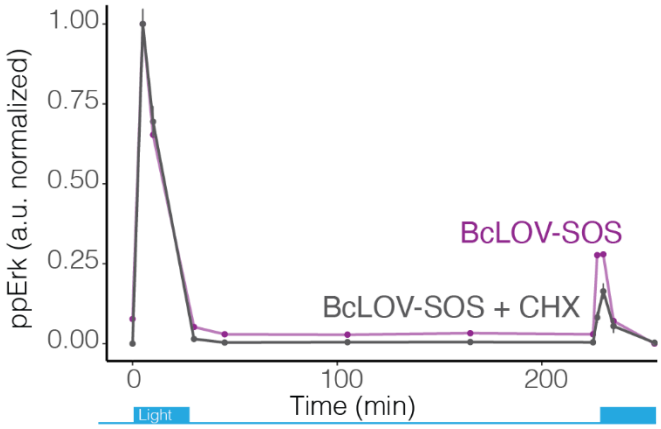
Supplementary Figure 3: Unnormalized traces of ppErk induction presented in Figure 2B. ppErk levels observed through immunofluorescence in response to BcLOV-SOS$_{cat}$ vs iLid-SOS$_{cat}$ stimulation (160 mW/cm^2 at 20% duty cycle). Data points represent mean +/- SEM of ~1000-4000 single cells.



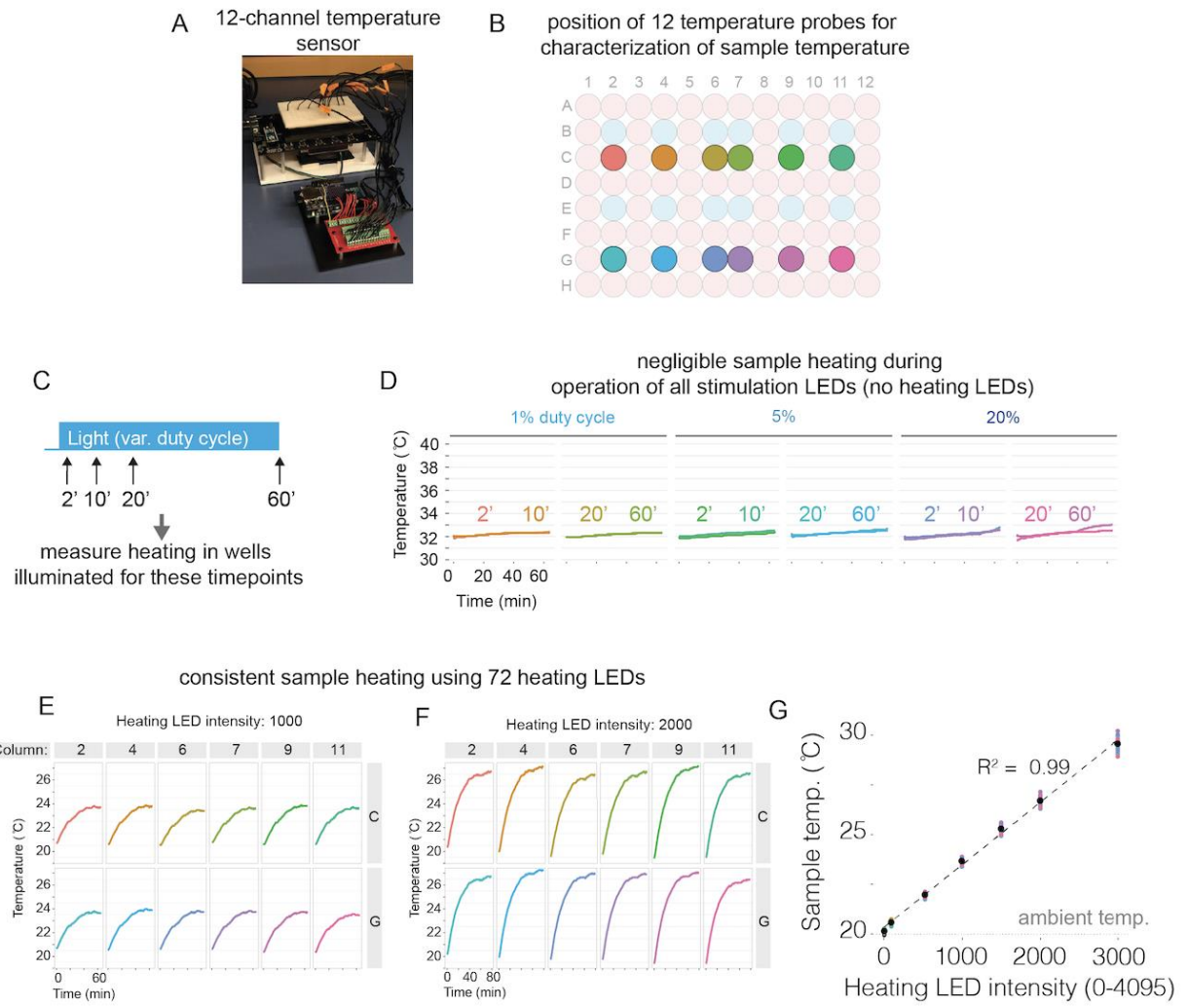
Supplementary Figure 4. Schematic of iLid-based probes of Ras/Erk and PI3K/Akt signaling. A) The iLid protein heterodimerizes with sspB when exposed to blue light. Anchoring iLid to the cell membrane allows for light inducible membrane recruitment. B) Fusing sspB to the iSH domain allows for light inducible stimulation of PI3K signaling. C) Fusing sspB to the SOS_{cat} domain allows for light inducible stimulation of Ras/Erk signaling. D) Schematic of iLid expression construct.



Supplementary Figure 5. PI3K signal decay in BcLOV-iSH cells despite constant illumination. A) pAkt activation through stimulation of BcLOV-iSH and iLid-iSH over 60 min (160 mW/cm^2 at 20% duty cycle) demonstrates sustained signal induction by iLid-iSH but transient signal induction by BcLOV-iSH. B) Unnormalized data presented in (A). Data points represent mean +/- SEM of ~2000-3000 single cells.

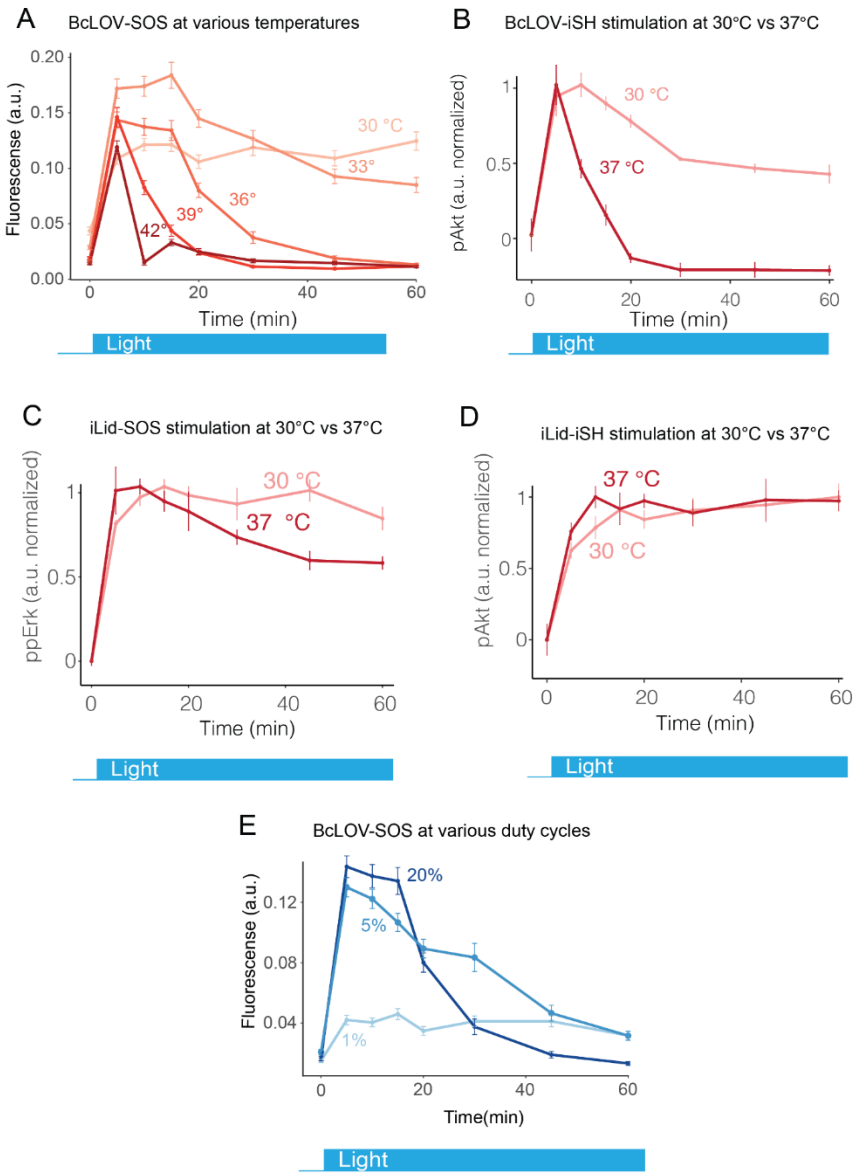


Supplementary Figure 6. Mild recovery of BcLOV-SOS$_{cat}$ is at least partially due to new protein synthesis. BcLOV-SOS$_{cat}$ cells were stimulated with blue light for 45 minutes with and without translation inhibitor cycloheximide (CHX, 20 µg/mL) at 37 °C. Cells were then allowed to recover in the dark for 3 hours and were subsequently restimulated. The modest signal recovery of BcLOV-SOS$_{cat}$ was further attenuated in the presence of CHX, suggesting that signal recovery is at least partially due to protein synthesis. Data represent mean +/- 1 SEM of ~1000-2000 cells measured for each condition. Where error bars are not visible, they are smaller than the data point.

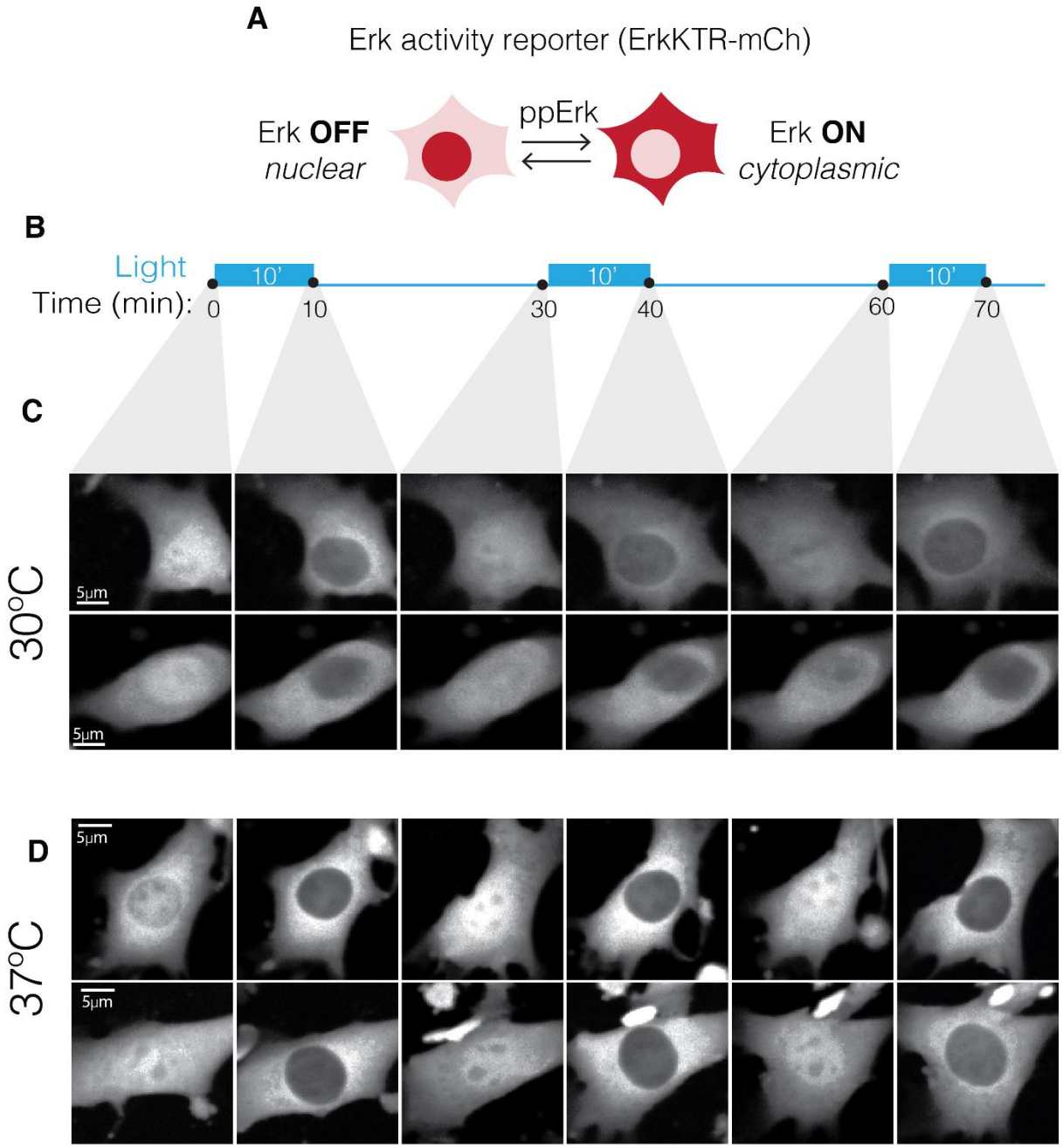


Supplementary Figure 7. Repurposing the optoPlate-96 for dual thermal and optical control of cells. A) Sample heating across a microwell plate was characterized with a custom-built 12-channel temperature probe. B) Temperatures of sample wells were measured at the indicated positions (colored circles) to verify the uniformity of sample heating across the sample plate. C) To test sample heating as a function of duty cycle, 3 simultaneous 8-point time courses were simulated (160 mW/cm^2) from 0-60 min, and heating was measured in the wells (filled with PBS) that would have received the indicated duration of light. The three time-series were performed at 1%, 5%, and 20% duty cycle (500 ms light every 50s, 10s, or 2.5 s). D) Temperature remained stable over the course of the experiment, with a ~0.5°C increase in the 60' well of the 20% duty cycle condition at the end of the experiment. These results suggest that 20% duty cycle is the most intense stimulation we can perform while avoiding heating in this experimental arrangement. Other experimental parameters (lower light intensity, taller adapter, fewer LEDs) would permit a higher duty cycle with less or no heating. E,F) Uniformity of sample heating was assessed by measuring sample well temperature under varying illumination intensities of the heating LEDs (constant light). We observed uniform temperature increases across the plate as a function of heater LED intensity (E and F). G) Experiments in (E,F) were repeated across heater intensity settings from 0-3000, and plateau temperature in each of the 12 wells was plotted for each

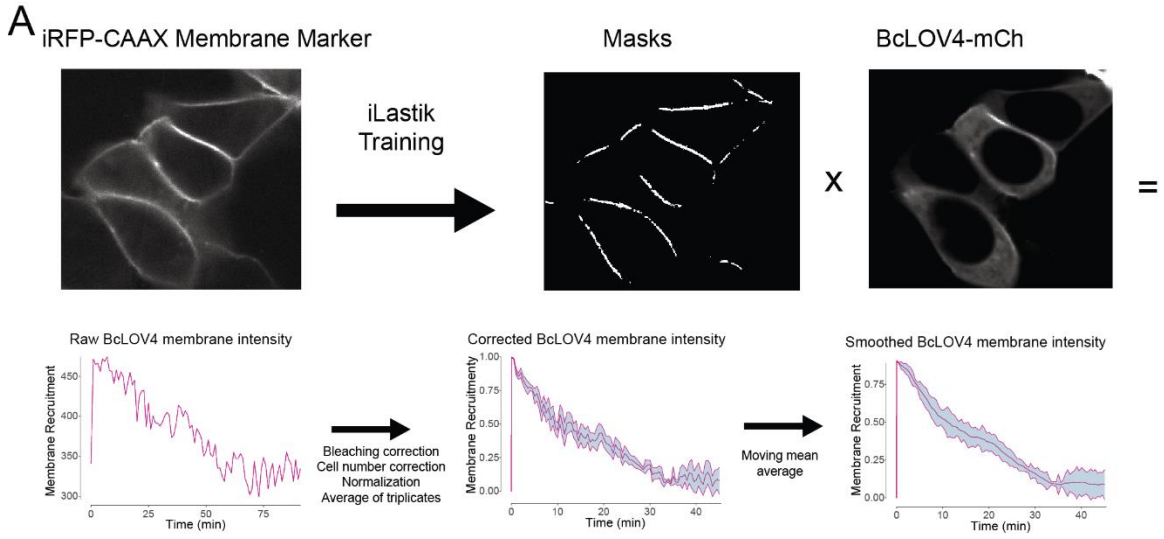
intensity setting. Black dot represents the mean temperature from each of the 12 wells, whose temperatures are individually depicted by the colored data points. Results reveal a linear relationship between heater LED intensity and plateau temperature of the sample wells. Heater LEDs allow programmatic increase of temperature above ambient. All characterizations were were performed at room temperature. See **Figure 2E** for analogous characterization performed in a cell culture incubator that was set to 32 °C, allowing linear control of temperature between 32 - 42 °C.

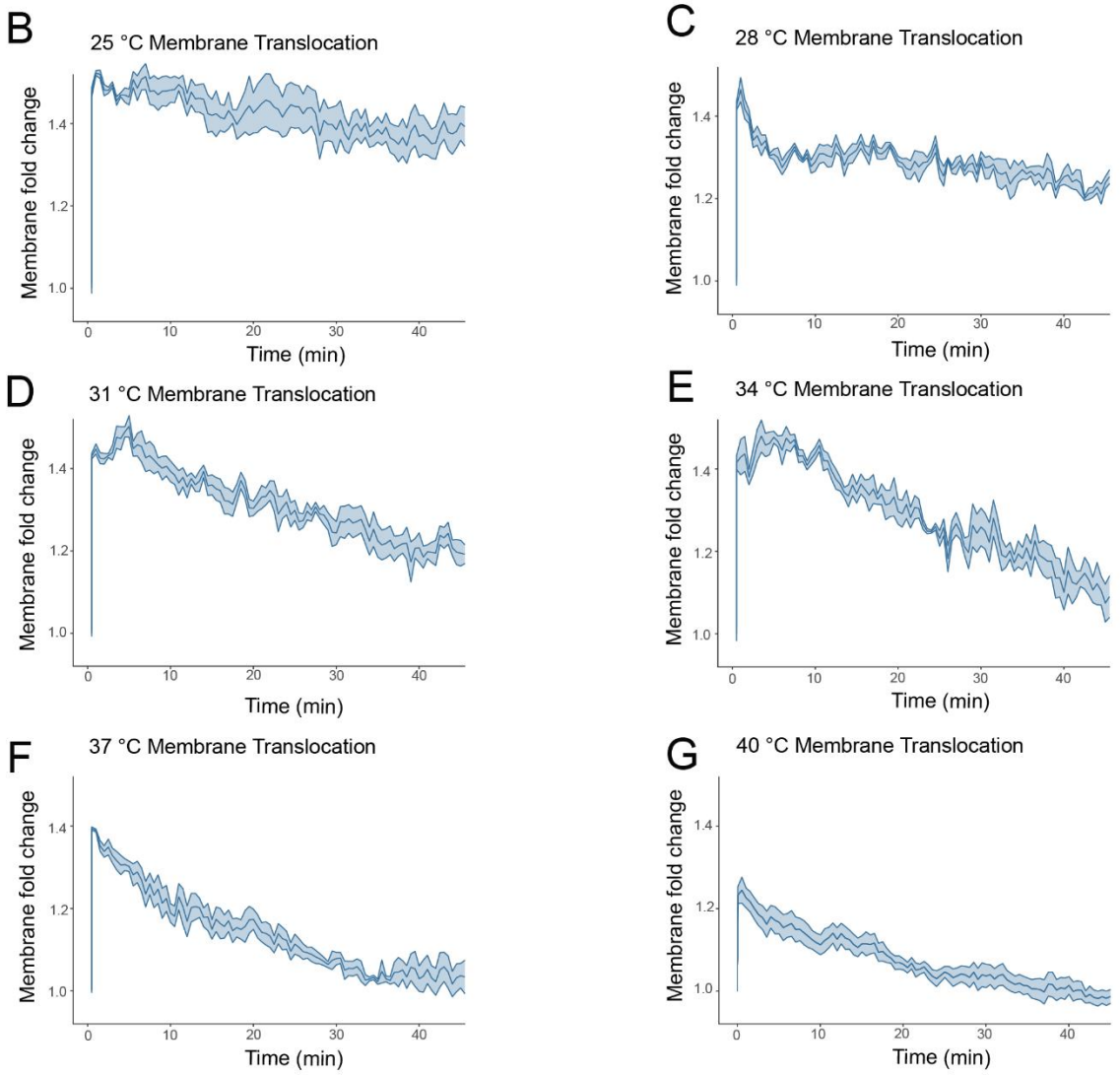


Supplementary Figure 8. Unnormalized BcLOV-SOS traces from Figure 2, BcLOV-iSH, and iLid probes at various temperatures. A) Unnormalized ppErk levels resulting from BcLOV-SOS_{cat} stimulation (160 mW/cm^2 at 20% duty cycle) at various temperatures. B) NIH 3T3 cells expressing BcLOV-iSH were stimulated with blue light at 30°C or 37°C. Similar to BcLOV-SOS_{cat}, signaling was transient at 37 °C but was stable at 30 °C. Traces were normalized at each temperature independently. C,D) NIH 3T3 cells expressing either iLid-SOS_{cat} (C) or iLid-ISH (D) were stimulated with blue light at 30°C or 37°C. In contrast to BcLOV4-induced signaling, iLid drove sustained levels of ppErk or pAkt at both low and high temperatures. Traces were normalized at each temperature independently. E) Unnormalized ppErk levels resulting from BcLOV-SOS_{cat} stimulation at various duty cycles. (36 °C and stimulated at 160 mW/cm^2). Data points in A/E represent the mean +/- SEM of ~1000-3000 cells. Data points in B/C/D represent the mean +/- 1 SD of three biologically independent replicates. Each replicate mean represents ~2000-3000 cells.

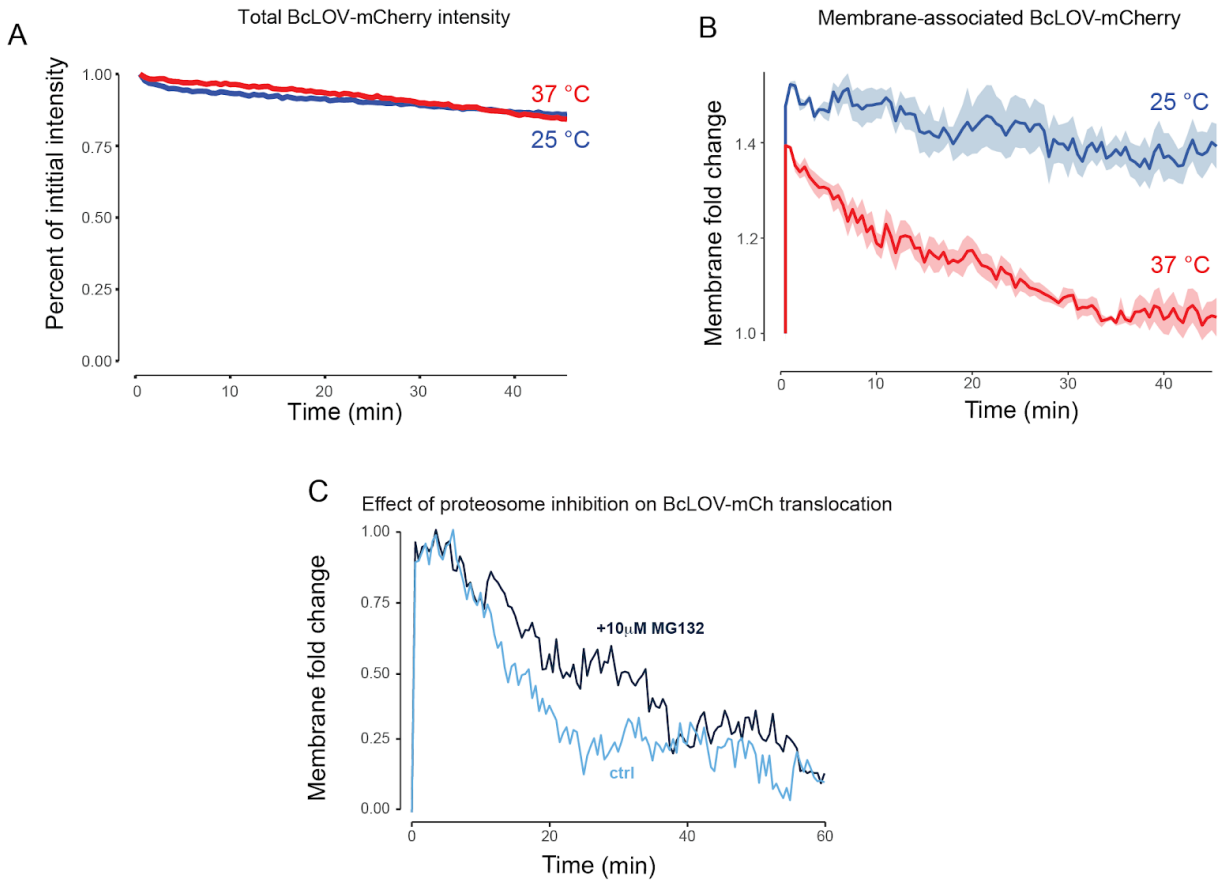


Supplementary Figure 9. Reversibility of signaling induced by BcLOV-SOS$_{cat}$. A) Schematic of ErkKTR activity. ErkKTR is nuclear when Erk signaling is off and translocates to the cytoplasm when Erk is activated. B) Cells expressing BcLOV-SOS$_{cat}$ and ErkKTR-mCh were stimulated with repeating cycles of 10 minutes of blue light followed by 20 minutes of darkness. C,D) ErkKTR imaging shows that after 10 minutes of blue light stimulation, ErkKTR has translocated out of the nucleus, and after 20 minutes of darkness, translocates back into the nucleus. This process was repeated 3 times to demonstrate the reversibility of Erk signaling using BcLOV-SOS$_{cat}$ at (C) 30 °C and (D) 37 °C.


A
iRFP-CAAX Membrane Marker
iLastik Training
Masks
x
BcLOV4-mCh
=
Raw BcLOV4 membrane intensity
Membrane Recruitment
Time (min)
Bleaching correction
Cell number correction
Normalization
Average of triplicates
Corrected BcLOV4 membrane intensity
Moving mean average
Smoothed BcLOV4 membrane intensity


B
25 °C Membrane Translocation
C
28 °C Membrane Translocation
D
31 °C Membrane Translocation
E
34 °C Membrane Translocation
F
37 °C Membrane Translocation
G
40 °C Membrane Translocation
Membrane fold change
Time (min)

Supplementary Figure 10. BcLOV quantification workflow and unnormalized fold-change of BcLOV-mCherry translocation presented in Figure 3. A) HEK 293T cells were transfected with BcLOV-mCh and an iRFP-CAAX membrane marker. Cells were stimulated with blue light. A small number (~2%) of iRFP-CAAX images were chosen at random to train the machine learning image processing software iLastik to identify iRFP-CAAX enriched cell membranes. iLastik produced masks of iRFP-CAAX membrane which were then multiplied by BcLOV-mCh images to quantify mCherry present at the membrane. The raw intensity of mCherry was then used to normalize all images for bleaching and cell number, normalized membrane intensity was normalized between 0 and 1 for each time series, and means of biological triplicate time series were calculated. Finally, a moving mean average filter was applied to smooth the high frequency noise associated with raw imaging data. B-G) Quantification of live cell imaging of BcLOV-mCh membrane recruitment at B) 26.4 °C C) 29.4 °C D) 31.8 °C E) 34.4 °C F) 36.6 °C G) 39.3 °C when exposed to 1.45 W/cm^2 at 3% duty cycle. Each trace is the mean +/- SEM of three biologically indpendent samples, each representing the mean of ~100 cells.



Supplementary Figure 11. Temperature-dependent BcLOV4 dynamics are not due to protein degradation. A) BcLOV4 was illuminated for 1 s every 30 s at 25 and 37 °C. After 45 minutes, total mCherry intensity had decreased by ~15% at both temperatures, likely due to bleaching. B) In contrast, membrane recruitment of BcLOV4 is more transient at 37 °C compared to 25 °C, demonstrating that loss of membrane fluorescence does not result from BcLOV-mCh degradation. Data in (B) is reproduced from **Supplementary Figure 10.** Each trace is the mean +/- SEM of three biologically independent replicates. Each replicate the mean measurement of ~100 cells. C) BcLOV-mCh was stimulated in HEK 293T cells in the presence or absence of proteosome inhibitor MG132 (10 μM). Proteosome inhibition did not prevent membrane dissociation, indicating that membrane dissociation does not require protein degradation. Traces represent the mean membrane localization of ~100 cells.

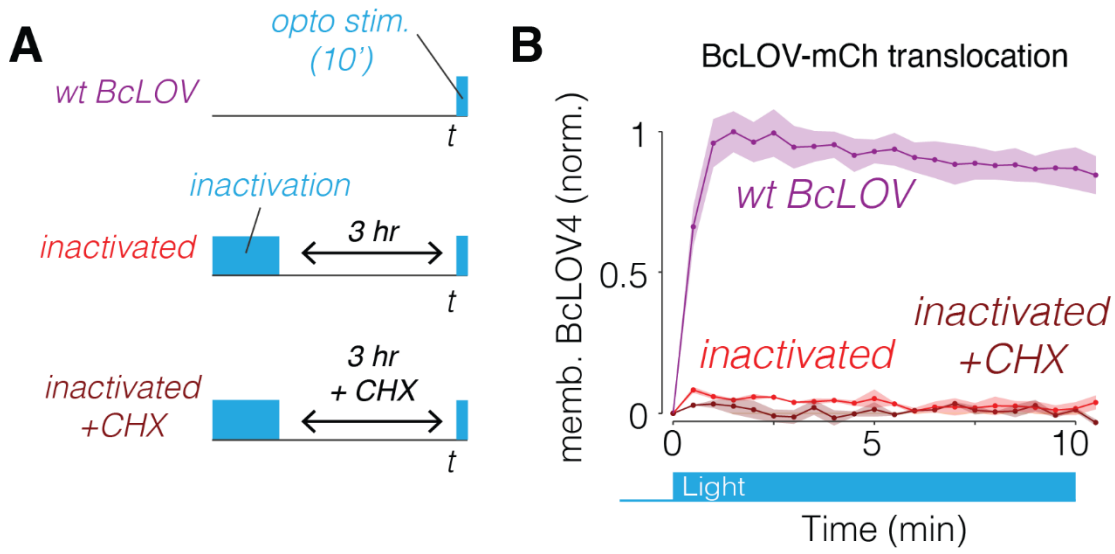
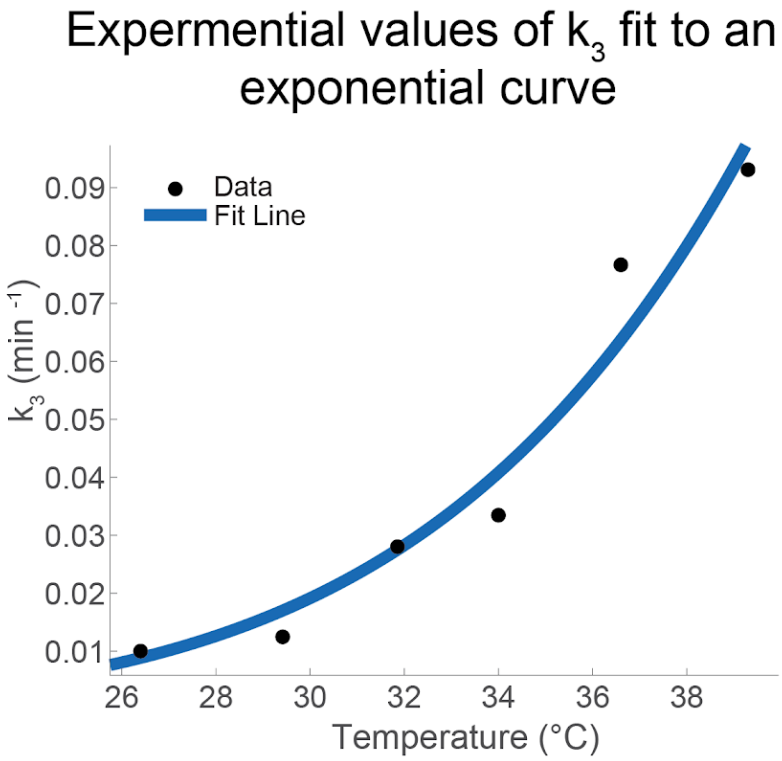
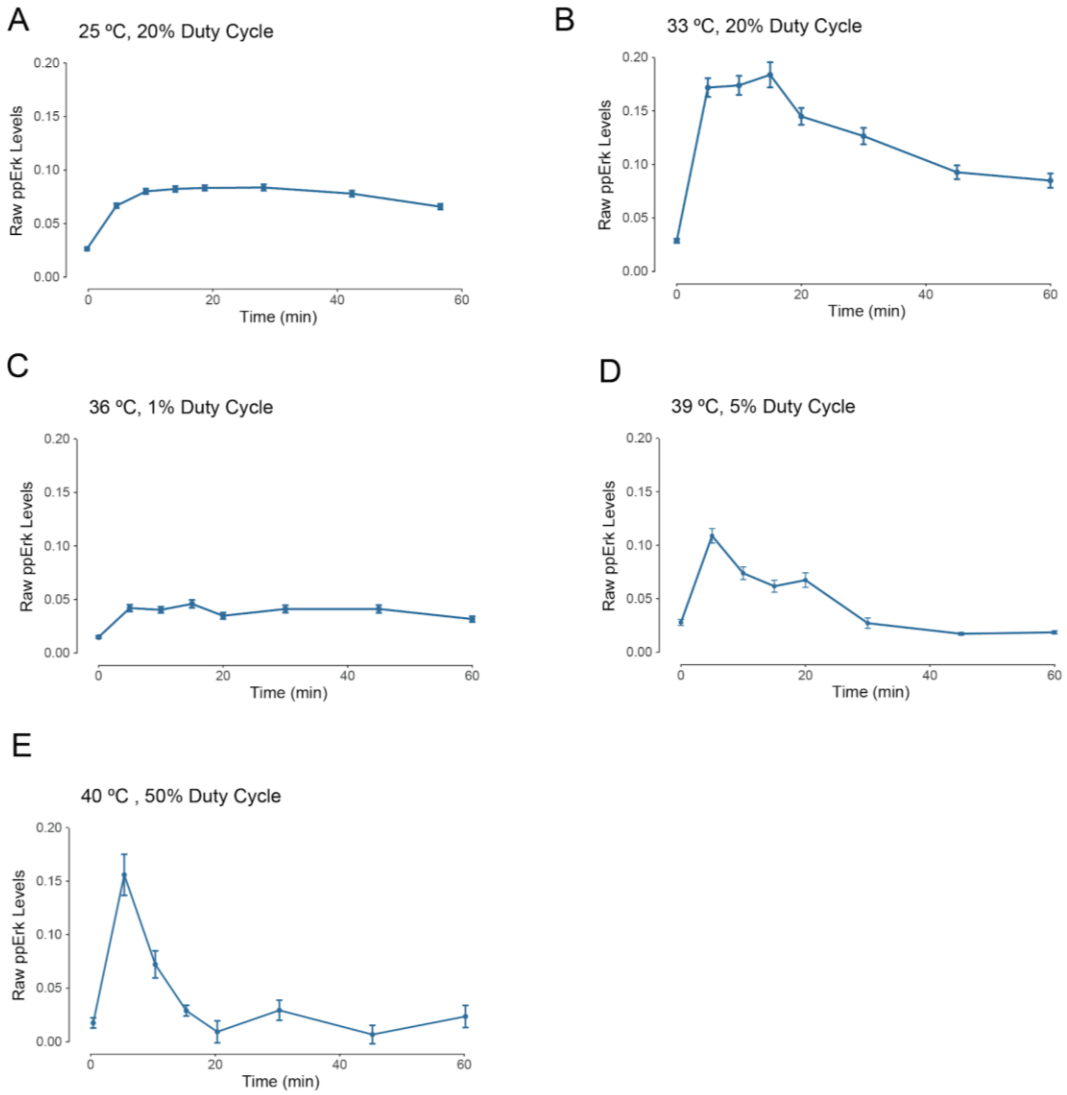


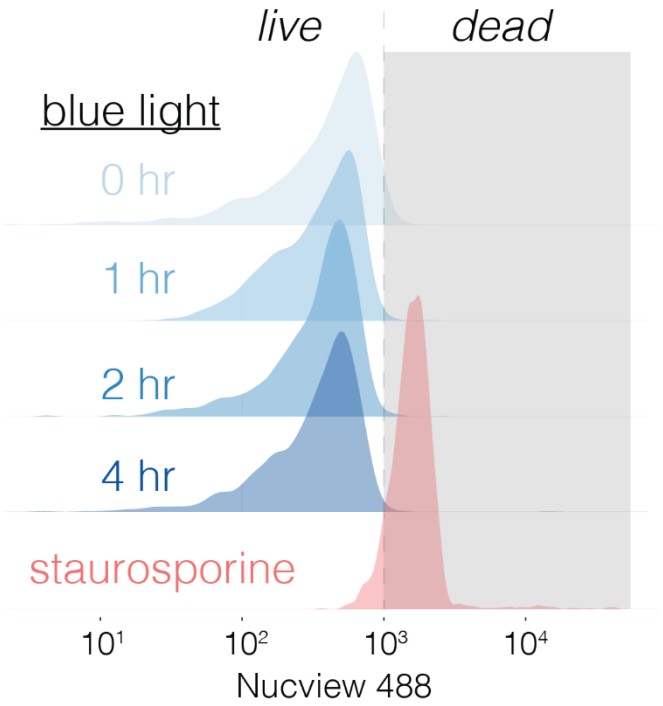
Figure 12. Inactivation of BcLOV-mCh translocation is effectively irreversible. A) HEK 293T cells expressing BcLOV-mCh were either stimulated for 10 minutes (”WT BcLOV”), inactivated by exposure to blue light for 1 hour (37 °C) and then stimulated for 10 minutes (”Inactivated”), or were inactivated and stimulated as above but in the presence of translation inhibitor cycloheximide (”Inactivated + CHX”, 20 µg/mL CHX). Cells were allowed to recover for 3 hrs after inactivation, prior to the 10 minute stimulation. B) Quantitation of membrane translocation resulting from conditions in (A), as measured through confocal microscopy. WT BcLOV-mCh showed strong membrane recruitment, whereas inactivated BcLOV-mCh exhibited only slight recruitment after 3 hrs recovery. This low-level recruitment was further abolished in the presence of CHX, suggesting that new protein synthesis accounts in part for recovery of BcLOV4 function after inactivation. Data points are the means +/- SEM of three biologically independent replicates, and each replicate represents the mean of ~100-200 cells. Ribbons represent standard error.



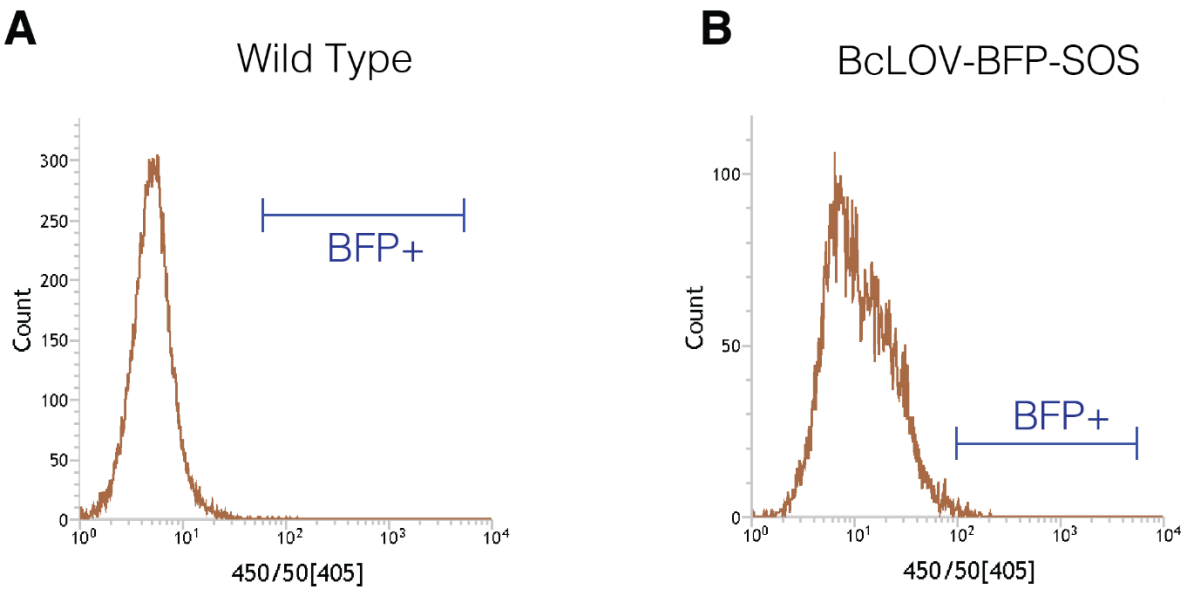
Supplementary Figure 13. Values of k_3 obtained experimentally show an exponential dependence on temperature. Values for k_3 were found for each temperature tested for BcLOV-mCh membrane recruitment (**Supplementary Figure 10**) and were fit to an exponential curve. This allowed for extrapolation of the value of k_3 at any temperature within this range. See **Methods** for complete model details.



Supplementary Figure 14. Unnormalized ppErk stimulation in BcLOV-SOS$_{cat}$ cells at various temperatures and duty cycles as presented in Figure 4. Data points in all plots above represent the mean +/- SEM of ~1000 single cells.



Supplementary Figure 15. Maximum intensity light used in this study was not toxic to cells. A) NIH 3T3 cells expressing BcLOV-SOS$_{cat}$ were exposed to the maximum light intensity used in this study (160 mW/cm^2 at 50% duty cycle) and stained with Nucview488, a fluorescent marker of caspase-3 activation. Cells were exposed from 0-4 hrs of blue light, and 20 µM staurosporine was used as a positive control of caspase activation and cell death. No measurable increases in caspase activation were observed under any light condition. Each density plot represents Nucview intensities of ~1000-2000 cells.



Supplementary Figure 16. Representative gating strategy for cell sorting. WT cells (A) were analyzed to establish the lower edge of gating. The lower bound was set to exclude > 99.9% of WT cells . BcLOV-BFP-SOS displayed a rightward shift in fluorescence, allowing ~1% of the population to be accepted.

Supplementary Movie Captions

Supplementary Movie 1. Temperature-dependent decay of BcLOV-mCh membrane localization. HEK 293T cells expressing BcLOV-mCh were stimulated with 488 nm light and imaged over 40 min. Membrane localization was sustained at lower temperatures (left), but decayed at higher temperatures (right). Time is mm:ss.

Supplementary Movie 2. BcLOV-mCh translocation in zebrafish embryos. 24 hpf zebrafish embryos expressing BcLOV-mCh were stimulated with 488 nm light and imaged with confocal microscopy. Time is mm:ss.