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Supplemental information

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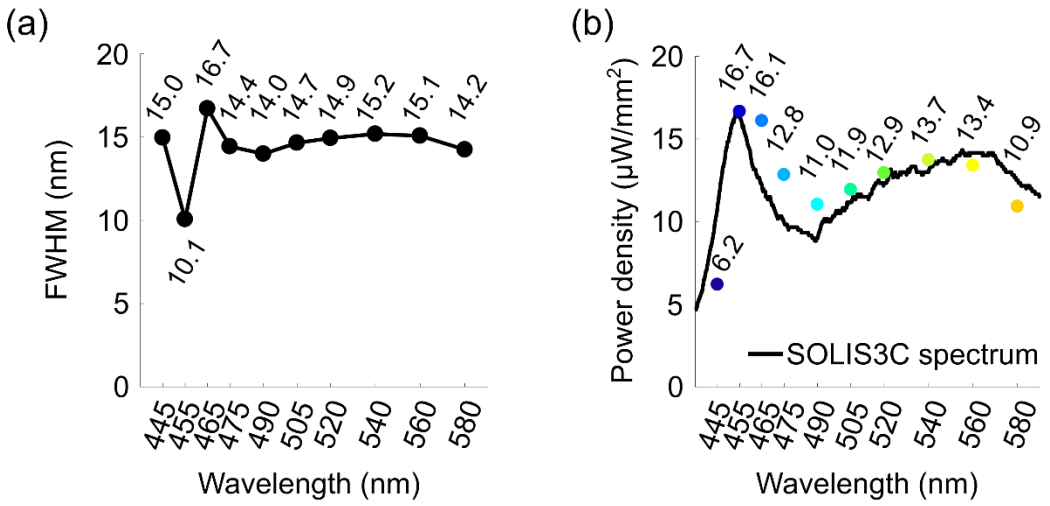
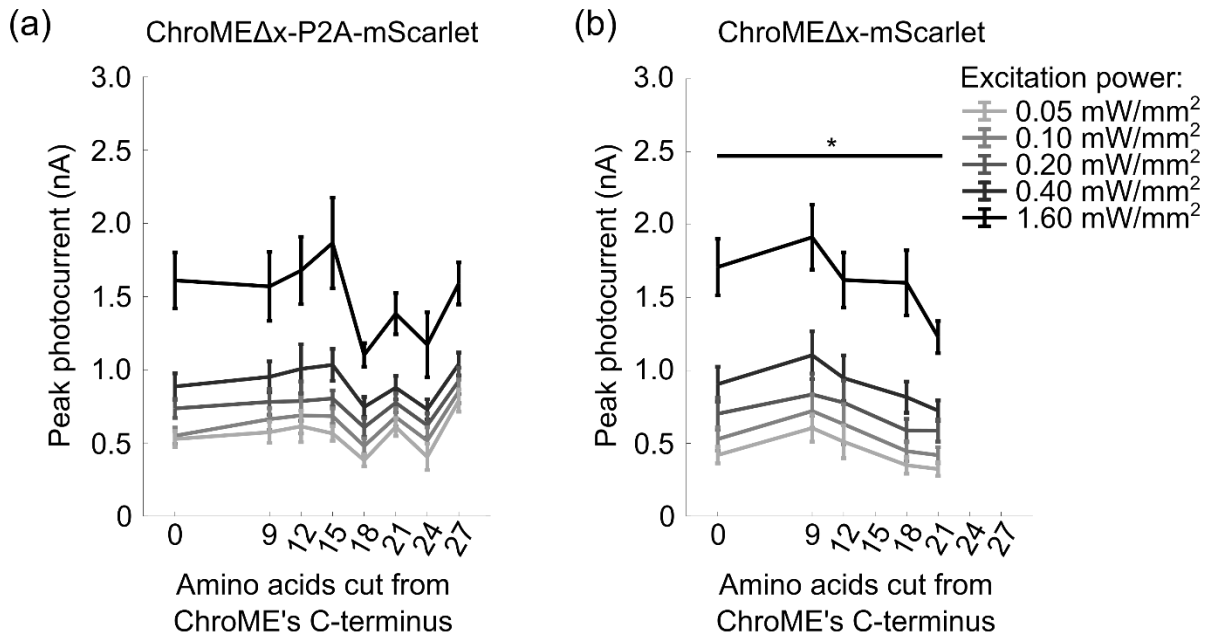


Supplemental Figure 1. Our custom-built monochromator delivered spectrally narrow pulses of light at sufficient excitation power levels.

(a) The average full width at half maximum for pulses centered around wavelengths ranging from 445 nm to 580 nm was 14.4 nm.

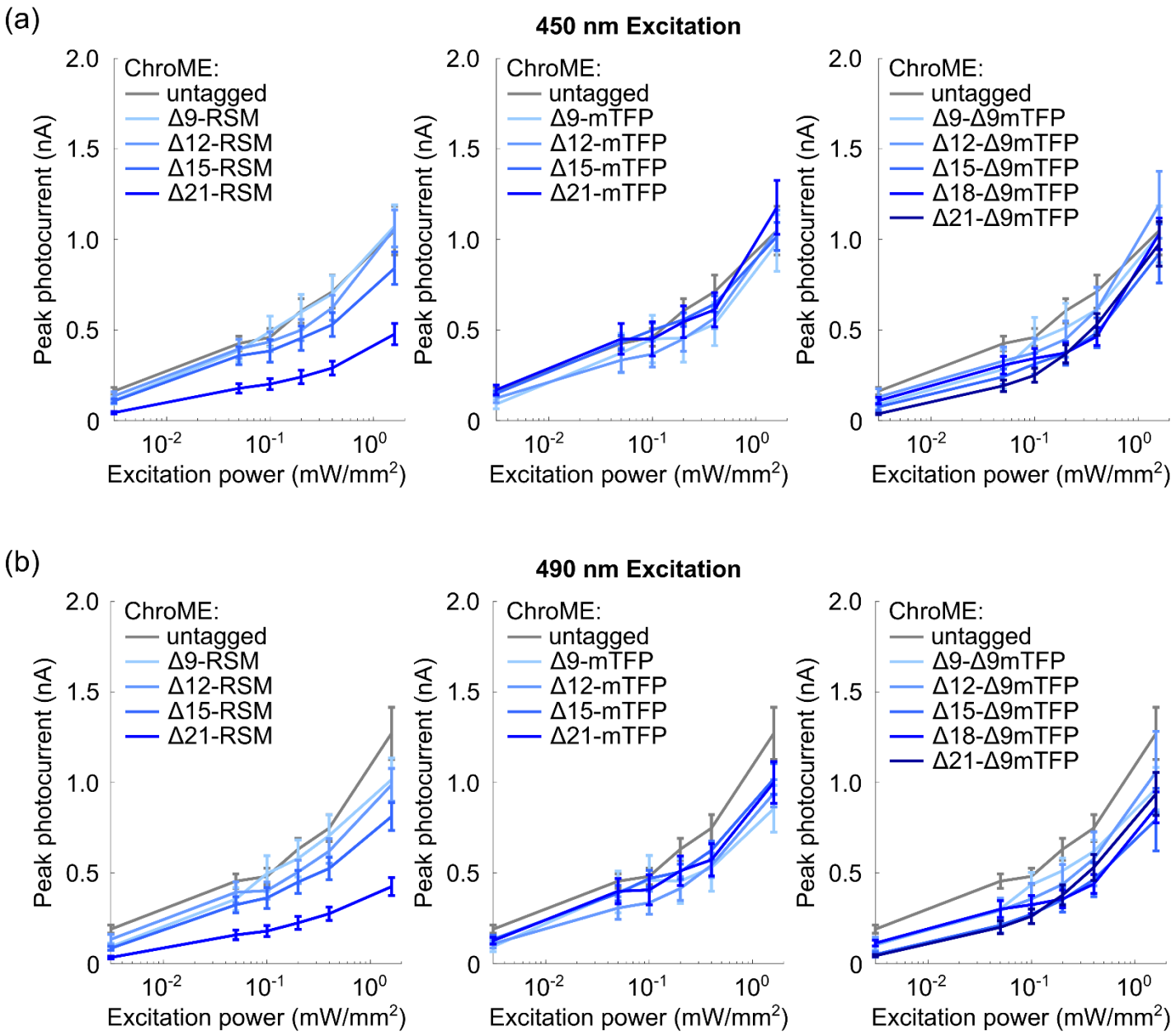
(b) Illumination in the 15 nm spectral windows ranging from 455 nm to 580 nm (*color points,* in $\mu W/mm^2$) delivered a maximum power density greater than 10 $\mu W/mm^2$ at the sample. The relative power in each spectral window matched the SOLIS LED illumination spectrum (*solid line*).



Supplemental Figure 2. Modest truncations to ChroME's unstructured C-terminal amino acids do not adversely affect peak photocurrent.

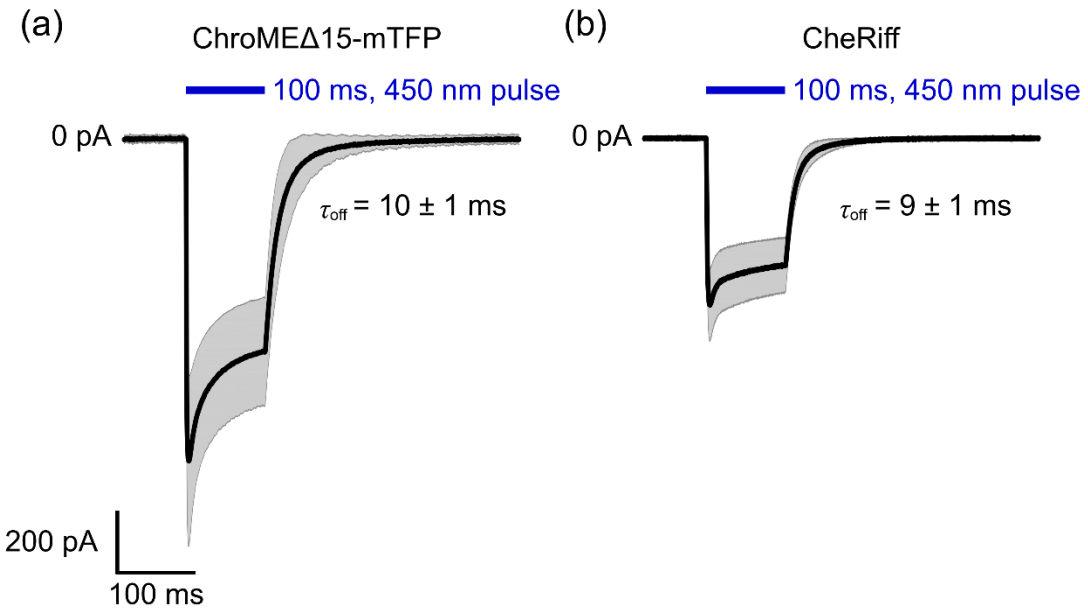
(a) Peak photocurrent in HEK293T cells expressing a bicistronic construct encoding ChroME with C-terminal truncations ranging from 0 to 27 amino acids and mScarlet. Photocurrent was elicited by 100 ms pulses of 490 nm light at excitation powers ranging from 0.05 mW/mm^2 to 1.6 mW/mm^2. Error bars represent mean ± s.e.m.

(b) Peak photocurrent in HEK293T cells expressing a ChroME-mScarlet fusion protein with C-terminal truncations of ChroME ranging from 0 to 21 amino acids. Unlike in the bicistronic construct, truncations greater than 21 amino acids abolished expression of the rhodopsin (* $p <$ 0.05, two-sided Wilcoxon rank-sum test, $n \geq 10$ cells per variant). Error bars represent mean ± s.e.m.



Supplemental Figure 3. The power densities used to measure ChroMEΔX- variants' activation at 450 nm and 490 nm excitation were below saturation.

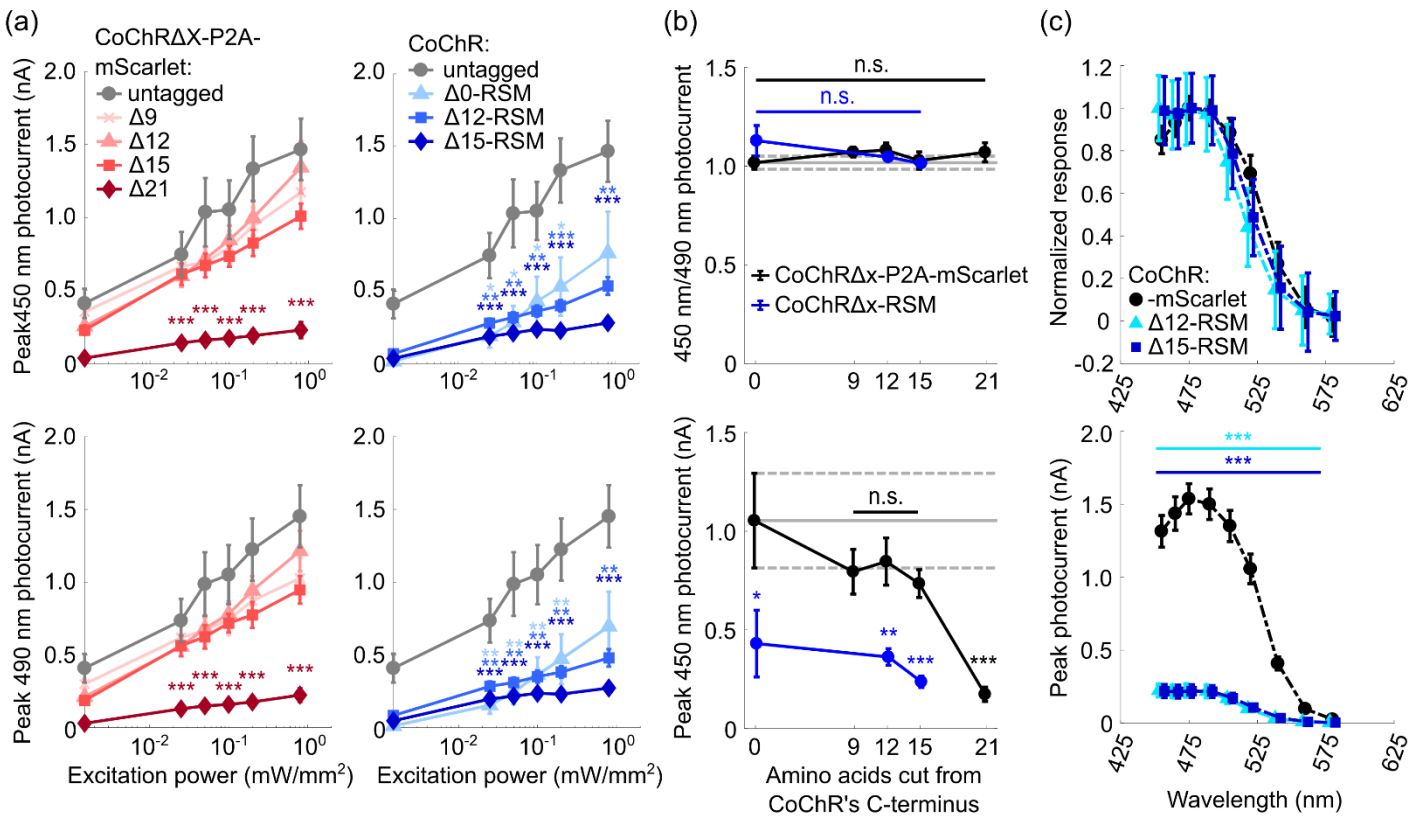
(a) Peak photocurrent as a function of excitation power for ChroMEΔX-RSM (*left*), ChroMEΔX-mTFP (*middle*), and ChroMEΔX-Δ9mTFP (*right*) in response to 100 ms pulses of 450 nm light. **(b)** Same as in (a) but for 100 ms pulses of 490 nm light. In all panels, the response of untagged, parental ChroME is included for comparison. $n \geq 6$ cells per variant; error bars represent mean ± s.e.m.



Supplemental Figure 4. ChroMEΔ15-mTFP has higher photocurrent and comparable kinetics to the blue-light-activated rhodopsin CheRiff.

(a) Photocurrent response of ChroMEΔ15-mTFP in response to 100 ms pulses of 450 nm light at 1.5 mW/mm^2. The average τ_{off} ± standard deviation is labeled on the figure.

(b) Same as in (a) but for CheRiff. Shaded regions represent mean ± s.d. While ChroMEΔ15-mTFP drove significantly higher photocurrent ($p = 10^{-4}$), the kinetics were statistically comparable (p = 0.9, two-sided Wilcoxon rank-sum test, $n \geq 6$ cells per construct).



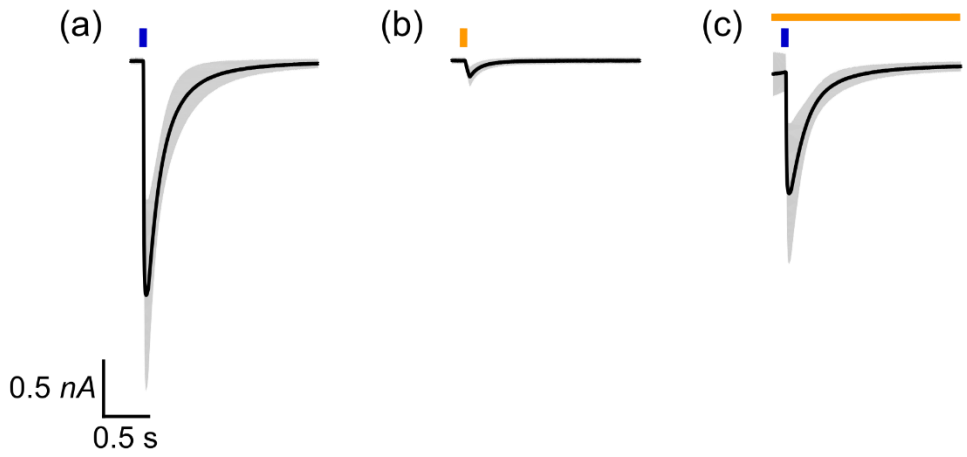
Supplemental Figure 5. A fusion of CoChR and Rosmarinus has a smaller activation spectrum enhancement than the enhancement of ChroME-TFP constructs.

(a) Photocurrent response of CoChRΔX-P2A-mScarlet (*Left*) and CoChRΔX-RSM (*Right*) in response to 100 ms pulses of 450 nm light at excitation power ranging from 15 to 800 μW/mm^2 (*Top*) or identical, 490 nm pulses (*Bottom*). CoChRΔ21-P2A-mScarlet had significantly lower photocurrent than untagged CoChR at all excitation power (*** $p < 10^{-3}$, two-sided Wilcoxon rank-sum test, $n \geq 8$ cells per construct). Similarly, all CoChRΔX-RSM constructs drove significantly lower photocurrent than untagged CoChR besides CoChRΔ0-RSM at 800 μW/mm^2 (* $p < 0.05$, ** $p < 0.01$, *** $p < 10^{-3}$, two-sided Wilcoxon rank-sum test, $n \geq 6$ cells per construct). Error bars represent mean ± s.e.m.

(b) *Top:* 450 nm/490 nm photocurrent ratio for CoChRΔX-P2A-mScarlet (*black*) and CoChRΔX-RSM (*blue*) with C-terminal truncations ranging from 0 to 21 amino acids. In contrast to ChroMEΔX-RSM/mTFP constructs, we did not observe a dependence of the 450 nm/490 nm photocurrent ratio on the linker length with. The average ratio for untagged CoChR ± s.e.m. is represented by the solid and dashed gray lines. *Bottom*: Peak photocurrent for CoChRΔX-P2A-mScarlet (*black*) and CoChRΔX-RSM (*blue*) in response to 100 ms pulses of 450 nm light at 0.1 mW/mm^2. The average photocurrent of untagged CoChR ± s.e.m. is represented by the solid

and dashed gray lines. CoChRΔ21-P2A-mScarlet and all CoChRΔX-RSM constructs have significantly lower photocurrent than untagged CoChR (* $p < 0.05$, ** $p < 0.01$, *** $p < 10^{-3}$, n.s.: not significant; two-sided Wilcoxon rank-sum test, $n \geq 6$ cells per variant). Error bars represent mean ± s.e.m.

(c) *Top*: Normalized response of CoChR-P2A-mScarlet, CoChRΔ0-RSM, and CoChRΔ12-RSM in response to 500 ms pulses of 0.1 mW/mm^2 light at wavelengths ranging from 455 nm to 580 nm scaled for the excitation photon flux at each wavelength (*Methods*). We observed a statistically significant enhancement of 455 nm photocurrent's contribution toward the normalized response of CoChRΔ0-RSM and CoChRΔ12-RSM compared to CoChR-P2A-mScarlet ($p < 0.01$, two-sided Wilcoxon rank-sum test, $n = 8$ cells per construct). *Bottom*: Peak photocurrent scaled for excitation photon flux at each wavelength (*Methods*) for CoChR-P2A-mScarlet, CoChRΔ0-RSM, and CoChRΔ12-RSM. As in (b), CoChRΔ0-RSM and CoChRΔ12-RSM had significantly lower peak photocurrent than CoChR-P2A-mScarlet ($p < 10^{-3}$ at all wavelengths, two-sided Wilcoxon rank-sum test, $n = 8$ cells per construct). Error bars represent mean ± s.e.m.

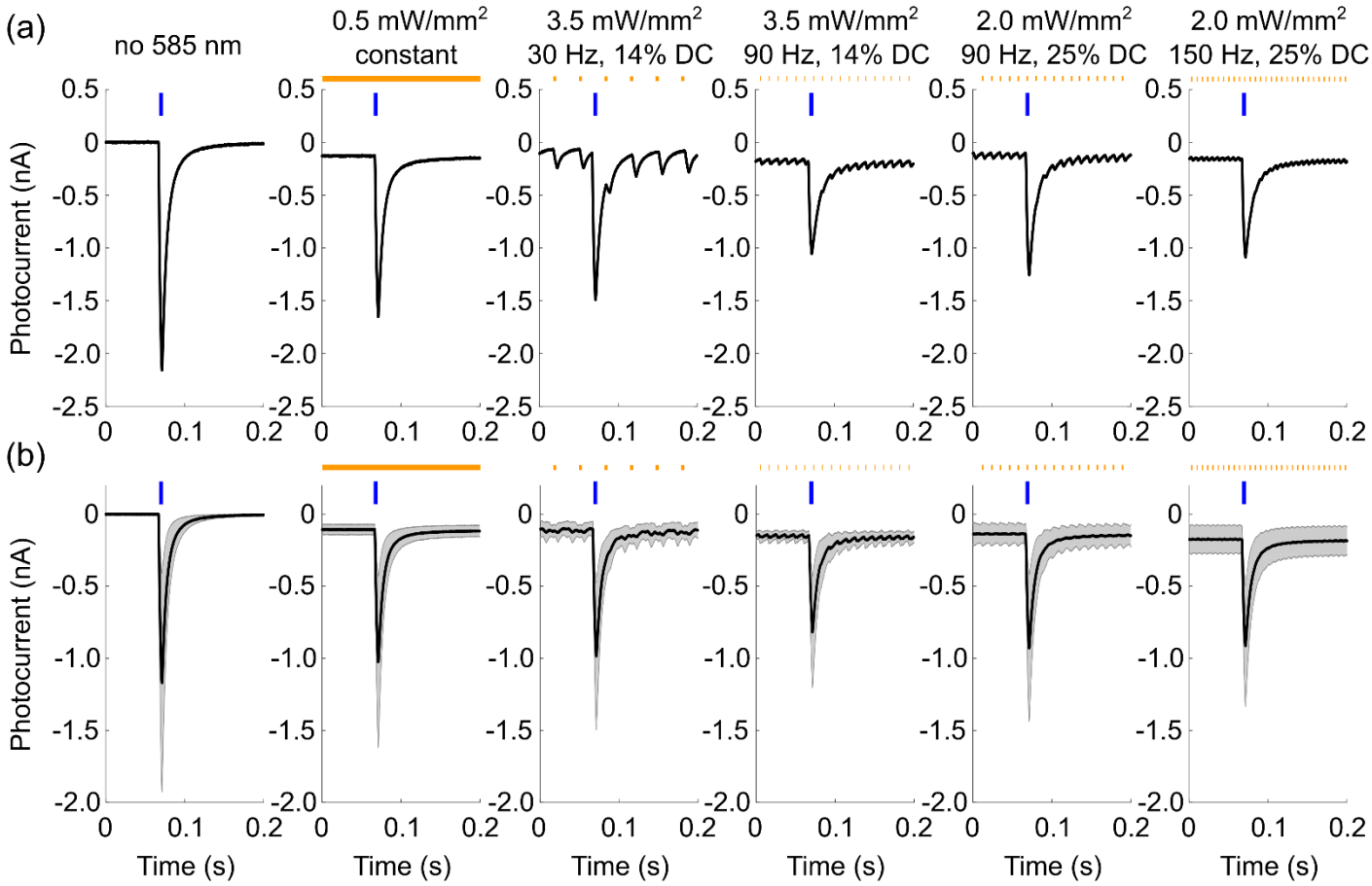


Supplemental Figure 6. ChroME's broad spectrum led to significant peak photocurrent reduction under yellow light excitation.

(a) Average photocurrent dynamics in HEK293T cells expressing ChroMEΔ15-mTFP in response to 5 ms pulses of 450 nm light at 1.6 mW/mm^2 (n = 6 cells). Shaded area represents mean ± s.d.

(b) Average photocurrent dynamics in HEK293T cells expressing ChroMEΔ15-mTFP in response to 5 ms pulses of 585 nm light at 0.5 mW/mm^2.

(c) Average photocurrent dynamics in HEK293T cells expressing ChroMEΔ15-mTFP in response to 5 ms pulses of 1.6 mW/mm^2, 450 nm light delivered during constant 0.5 mW/mm^2, 585 nm illumination. The peak photocurrent amplitude was significantly dampened compared to peak photocurrent elicited by blue light pulses alone in panel (a) (p = 2× 10^{-3}, two-sided Wilcoxon rank-sum test, n = 6 cells).



Supplemental Figure 7. Pulsed yellow imaging light did not reduce photocurrent crosstalk or improve peak photocurrent at frequencies at or above video rate.

(a) Photocurrent in response to a 5 ms, 1.5 mW/mm^2, 450 nm pulse (blue dash) in a HEK293T cell expressing ChroMEΔ15-mTFP under a variety of pulsed and non-pulsed 585 nm imaging conditions with the same average power per 30 Hz "frame" (orange dashes). From left to right, the imaging LED conditions are: 1) no 585 nm imaging LED, 2) 0.5 mW/mm^2 constant 585 nm illumination, 3) 3.5 mW/mm^2, 585 nm pulses at 30 Hz with a duty cycle of 14.3%, 4) 3.5 mW/mm^2, 585 nm pulses at 90 Hz with a duty cycle of 14.3%, 5) 2.0 mW/mm^2, 585 nm pulses at 90 Hz with a duty cycle of 25%, and 6) 2.0 mW/mm^2, 585 nm pulses at 1500 Hz with a duty cycle of 25%.

(b) Same as in (a) but showing the average for 8 cells. Note that the average offset in the baseline photocurrent is approximately equal for each imaging LED pulse condition. Shaded regions represent mean ± s.d.

Supplemental Table 1: Genetic sequences for ChroME-mTFP constructs

Nucleotide and amino acid sequences of parental ChroME and the developed constructs, including start and stop codons, are below:

ChroME

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ChroMEΔ15-mTFP

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ChroMEΔ15-Δ9mTFP

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