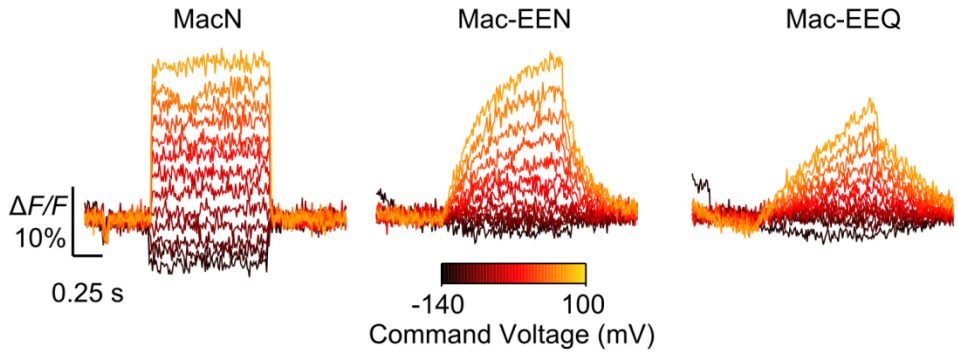
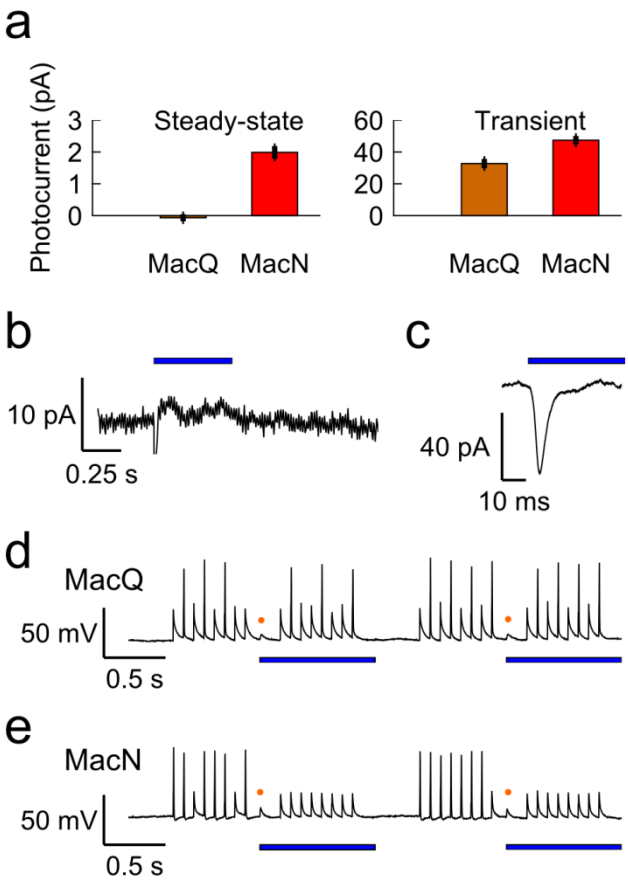
Supplementary Figure 1 | Fluorescence measurements from Mac reveal the dynamics of its voltage-sensitive domain.



By monitoring Mac's fluorescence emissions, we examined how various Mac mutants respond to the application of voltage depolarizations. These measurements involved illumination of 633 nm wavelength and 1400 mW mm^{-2} intensity at the specimen plane, and an imaging frame rate of 440 Hz. MacN exhibited a ~5 ms rise time (defined as the time to rise to $1 - 1/e$ of the steady-state response), whereas Mac-EEN and Mac-EEQ had rise times of >100 ms.

Supplementary Figure 2 | MacQ exhibits no steady-state photocurrent and a smaller transient photocurrent than MacN.



(a) MacQ exhibited scarcely any steady-state photocurrent. Both MacQ (*n* = 12 cells) and MacN (*n* = 13 cells) exhibited transient photocurrents at the onset.

(**b**) Patch-clamp electrophysiological trace from a MacN-expressing cultured neuron that exhibited the initial, transient depolarizing (downward) photocurrent at the onset of illumination (blue bar). (The peak of the depolarizing transient is cut off in the graph).

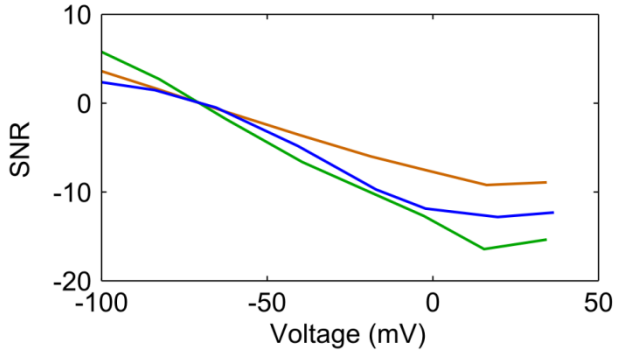
(**c**) Expanded time axis view of the transient depolarization in (**b**).

(**d**,**e**) We injected 2 ms pulses of current to initiate action potentials in neurons transfected with MacQ, **d**, and MacN, **e**. The injected current was barely sufficient to drive spikes, as evidenced

by the ~50% success in spike initiation. The blue bars denote periods of illumination. In MacQ-expressing cells, the negligible steady-state hyperpolarizing photocurrent did not visibly perturb the success rate in spike initiation. By comparison, in MacN-expressing cells, this photocurrent blocked spiking throughout the illumination periods. In both cases, we also observed depolarizing voltage transients (orange dots) at illumination onset.

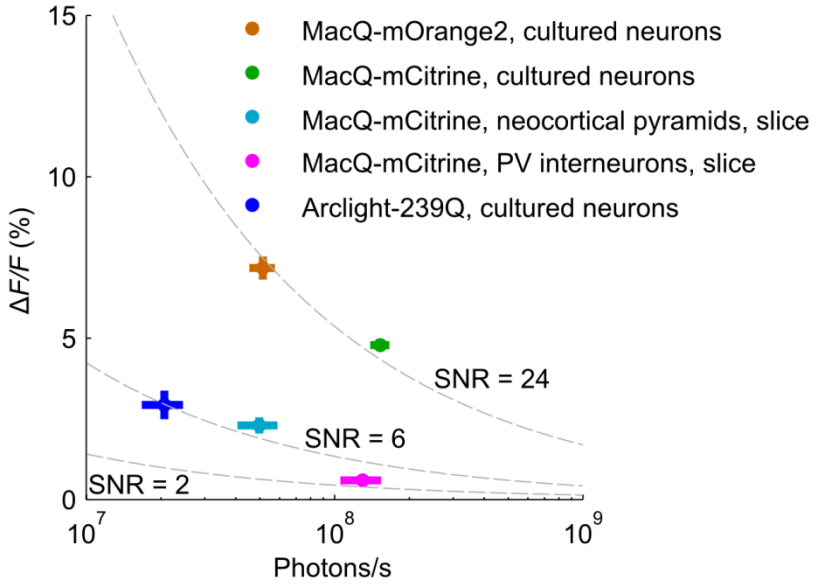
All neurons were illuminated using the same parameters as used elsewhere in the paper for imaging (15 mW mm^{-2}, λ = 530 nm). Error bars are s.e.m.

Supplementary Figure 3 | MacQ exhibits comparable or higher SNR in response to steady-state voltage depolarizations than Arclight.



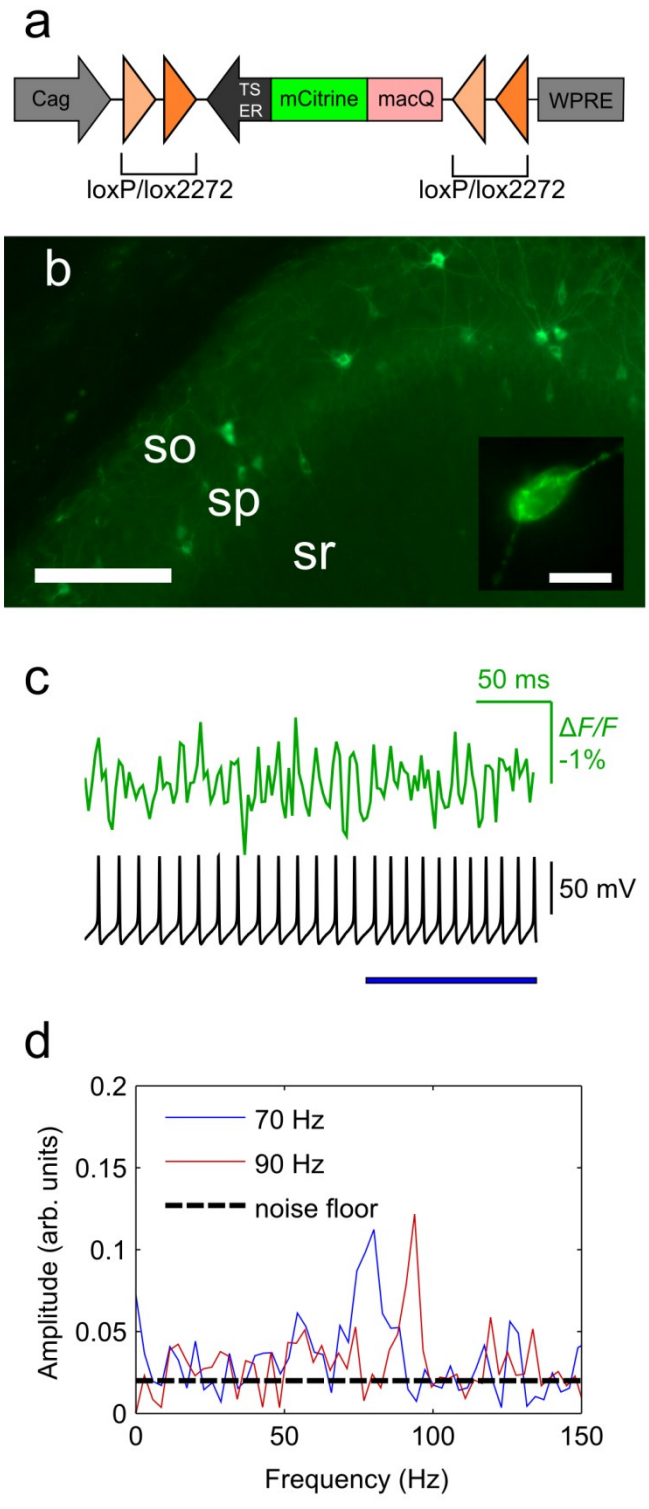
We computed the normalized SNR for steady-state depolarizations as $(\Delta F / F) \times \sqrt{F_{\text{norm}}}$, where F_{norm} is the fluorescence intensity normalized to the average intensity of the MacQ-mCitrine sensor. We used this definition of SNR, because it takes into account the magnitude of the $\Delta F/F$ response relative to the fluctuations in baseline fluorescence due to photon shot noise. (This is somewhat akin to a *z*-score in statistics). Due to MacQ-mCitrine's superior brightness, the fractional baseline fluctuations of MacQ-mCitrine were less than those of Arclight, and thus MacQ-mCitrine outperformed Arclight in steady-state SNR, despite Arclight's superior steady-state dynamic range ($\Delta F/F$).

Supplementary Figure 4 | Mac sensors outperformed Arclight in SNR when reporting single action potentials.



Characterizations of voltage sensor performance based on our experiments in cultured neurons and brain slices. For each study, the data points indicate the measured fluorescence dynamic range and the rate of photon detection. The dashed lines denote isocontours of SNR (defined as $(\Delta F/F)\times\sqrt{F/\nu}$, where F is the baseline fluorescence intensity and ν is the imaging frame rate). The data points for the various sensors and expression conditions are normalized for excitation power density. In cultured neurons, Mac-Orange2 outperformed Arclight in SNR by a factor of 3.8, whereas Mac-mCitrine outperformed Arclight by a factor of 4.4. Moreover, Mac-mCitrine achieved an SNR value in neocortical pyramidal cells in live brain slices that was higher than Arclight's SNR value in cultured neurons. All neurons were imaged under the same illumination conditions as their corresponding experiments in **Fig. 5d** and **Fig. 6d**, with the excitation wavelength matched to each sensor (**Methods**). Error bars are s.e.m.

Supplementary Figure 5 | MacQ-mCitrine reports high frequency spiking from interneurons.



(a) We expressed the MacQ-mCitrine construct in a CAG-DIO backbone, with the WPRE enhancer.

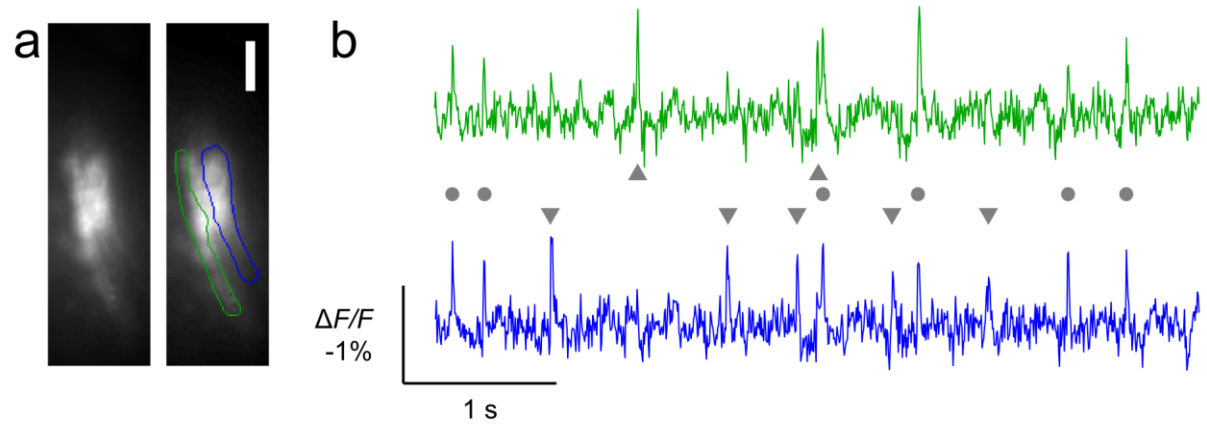
(b) We targeted MacQ-mCitrine to inhibitory interneurons by injection of virus into the hippocampus of Parv-Cre mice. Fluorescence was localized to putative interneurons located both dorsal and ventral to the pyramidal layer of the hippocampus (so – stratum oriens, sp – stratum pyramidale, sr – stratum radiatum). Scale bar is 400 μm. Inset: Magnified view of a labeled neuron. Scale bar is 20 μm.

(c) In addition to reporting isolated spikes from single interneurons, MacQ-mCitrine can report high frequency oscillatory activity. In this virally infected interneuron, we observed fast and spontaneous periodic spiking above 50 Hz with short action potentials (width < 0.6 ms). During the blue bar, we injected 30 pA of current and observed a corresponding increase in the firing rate.

(d) By taking the Fourier transform of the fluorescence traces during the current injection period (firing ~90 Hz) and the non-injection period (firing ~70 Hz), we observed that the sensor represented the electrophysiological firing rates with reasonable fidelity, as evidenced by the corresponding peaks at the two frequencies. By using experimentally collected fluorescence intensity, we estimated the shot-noise noise floor of the spectrum and found that the sensor offered at least SNR~5 during periodic spiking.

Neurons were imaged using illumination of 30 mW mm^{-2} intensity, 505 nm wavelength, and 440 Hz frame rate.

Supplementary Figure 6 | The MacQ-mCitrine voltage sensor concurrently reported dendritic voltage transients in multiple cerebellar Purkinje neurons of live mice.



(a) One-photon image of two Purkinje neuron dendritic trees expressing MacQ-mCitrine in the same field of view (*left*), along with the overlay of the regions of interest (*right*) used to compute fluorescence traces. Scale bar is 40 µm.

(b) Fluorescence traces aggregated from the regions of interest in panel (**a**) showed different temporal patterns of dendritic voltage activity. Markers denote transients that appear in only the upper trace (▲), only the lower trace (▼), and both traces (●). Neurons were imaged using illumination of 10 mW mm^{-2} intensity, 505 nm wavelength, and 190 Hz frame rate.

Supplementary Table 1 | Voltage sensor kinetics as determined from bi-exponential fits

	+ Kinetics			– Kinetics			Bleaching Rate
Sensor	τ_{fast} **(ms)**	τ_{slow} **(ms)**	P_{fast} **(%)**	τ_{fast} **(ms)**	τ_{slow} **(ms)**	P_{fast} **(%)**	**% per s**
MacQ-mOrange2	2.9 ± 0.1	115 ± 10	96 ± 1	3.4 ± 0.4	20 ± 1	79 ± 5	0.6 ± 0.1
MacQ-mCitrine	2.8 ± 0.2	71 ± 3	74 ± 2	5.4 ± 0.3	67 ± 11	77 ± 2	1.3 ± 0.1
Arclight	12 ± 1.6	91 ± 20	58 ± 5	31 ± 7	150 ± 26	71 ± 2	0.7 ± 0.1

Errors are s.e.m. n = 3 cells for all kinetics measurements. n = 10 cells for photobleaching measurements, for each construct, using illumination of 15 mW mm^{-2}.

The '+' and '–' kinetics modeled the voltage sensor's optical responses to depolarizing and hyperpolarizing voltage transients using bi-exponential fits to the experimental data, as described by Equations 1 and 2, respectively, in the Methods. In brief, $P_{\{+,-\},fast}$ is the percentage of the amplitude associated with the fast component in response to depolarizing and hyperpolarizing voltage transients, and $\tau_{\{+,-\};\{fast,slow\}}$ are the fast and slow time constants of the response to depolarizing and hyperpolarizing voltage transients.