SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid construction for *in vitro* experiments. Plasmids were constructed by standard molecular biology methods and verified by sequencing of all cloned fragments. All ASAP variants were cloned between the NheI and HindIII sites of pcDNA3.1/Puro-CAG (Lam et al., 2012) and contain identical Kozak sequences. Expression plasmids and complete sequences for ASAP1, ASAP2f, and ASAP1 I67T Q397R can be obtained via Addgene.

HEK293A cell culture, patch clamping, and voltage imaging. HEK293A cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM, HyClone) supplemented with 5% fetal bovine serum (FBS, Life Technologies) and 2 mM glutamine (Sigma) at 37 °C in air with 5% CO_2. Cells were plated onto glass-bottom 24-well plates (In vitro Scientific) for standard imaging or onto uncoated no. 0 12-mm coverslips (Glaswarenfabrik Karl Hecht GmbH) for patch clamping experiments. Transfections were carried out using FuGene HD (Promega) according to manufacturer instructions, except that cells were transfected at ~50% confluence with lower amounts of DNA (200 ng) and transfection reagent (0.6 µl) to reduce cell toxicity.

At 2 days post-transfection, cells were patch-clamped at 22 °C using borosilicate glass electrodes with resistances of 3.5 to 5.0 MΩ attached to an Axopatch 700B amplifier (Axon Instruments). Cells were superfused in a chamber mounted on the stage of an Axiovert 100M inverted microscope with a 40X/1.3-numerical aperture (NA) oil-immersion objective (Zeiss). The extracellular solution contained 110 mM NaCl, 26 mM sucrose, 23 mM glucose, 5 mM HEPES-Na, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.3 mM $MgSO_4$, adjusted to pH 7.4. The intracellular (pipette) solution contained 115 mM potassium gluconate, 10 mM HEPES-Na, 10 mM EGTA, 10 mM glucose, 8 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$, adjusted to pH 7.4. Cells were illuminated with a high-power light-emitting diode (LED, UHP-MIC-LED-460, Pryzmatix) through a 470/30-nm filter at a power density of 3.7 to 23.7 mW/mm^2 at the sample plane. Fluorescence traces were acquired while cells were voltage clamped in whole-cell mode, using an iXon 860 electron multiplying charge coupled device camera (Andor) cooled to −80 °C and set to Frame Transfer mode. Fluorescence traces were corrected for photobleaching. Voltage and fluorescence traces were analyzed using custom software written in MATLAB (Mathworks). Voltage traces were corrected for the junction potential post hoc.

Unless otherwise indicated, step voltage depolarizations were applied to change the membrane potential from a holding voltage of −70 mV to voltages ranging from −120 mV to 50 mV for 1.0 s. For these voltage step experiments, we captured images at 200 Hz without binning, and the fluorescence response was measured from pixels at the perimeter of the cell (plasma membrane). For experiments with artificial action potential waveforms, we captured images at 1 kHz with 4 × 4 binning. The action potential waveform, derived from a recording of a hippocampal neuron action potential, had a full width at half maximum of 4.0 ms and peak amplitude of 100 mV. The fluorescence response was measured from the perimeter of the cell (plasma membrane). For experiments to determine sensor response kinetics, we increased sampling frame rate to 2.5 kHz by cropping the imaged area down to 64 × 64 pixels and increasing binning to 8 × 8 pixels. The resulting image thus contained 64 pixels (8 × 8); fluorescence was calculated by summing all 64 pixels. Command voltage steps were applied for 1 second; three identical voltage steps were measured for every cell. Models of the form $a \cdot e^{-bt} + c \cdot e^{-dt}$ were applied to the rising and falling portions of the mean fluorescence trace using MATLAB.

Quantifying voltage sensor brightness and photostability in HEK293 cells. For quantifying the brightness of ASAP variants, we used a HEK293 cell line expressing the inwardly rectifying Kir2.1 channel (Zhang et al., 2009) (HEK293-Kir2.1). This cell line, a generous gift of Gui-Rong Li, has a resting membrane potential of approximately −75mV, similar to that of primary hippocampal neurons. Cells were plated onto 60 mm tissue culture dishes at a density of 400,000 cells/dish. Cells were transfected using 3.6 µL of FuGENE HD reagent (Promega) and 1.2 µg of DNA per dish. At 2 days post-transfection, cells were collected by trypsinization using a 0.25% trypsin/EDTA solution (Gibco, Life Technologies), washed with phosphate buffered saline (PBS; HyClone, Life Technologies), and resuspended using a solution of Hank's Buffered Saline Solution (HyClone, Life Technologies) supplemented with 1% w/v bovine serum albumin and 5 mM ethylenediaminetetraacetic acid (EDTA). Cells were analyzed by flow cytometry using an LSRII.UV analyzer (BD Biosciences), using the 488-nm and 561-nm lasers to excite our GFP-based sensor and our FusionRed reference RFP, respectively. Gating conditions were set using Flowjo to analyze cells expressing ASAP-FusionRed constructs. We quantified the brightness of each cell as the ratio of green

fluorescence (from ASAP) and red fluorescence (from our FusionRed standard). The overall brightness of each construct was defined as the median brightness over all sensor-expressing cells.

For quantifying the photostability of ASAP variants, we transfected HEK293A or HEK293-Kir2.1 cells as previously described (St-Pierre et al., 2014). At 2 days post-transfection, cells were superfused with the same extracellular solution we used for electrophysiological recordings in HEK293A cells. To evaluate photostability under one-photon excitation, cells were imaged using an Axiovert 100M microscope (Zeiss) fitted with a 40X 1.3-NA oil-immersion objective (Zeiss). To collect images, we used the software HCImage (Hamamatsu) to drive an ORCA Flash4.0 V2 C11440-22CU (Hamamatsu) scientific CMOS camera set to 4 × 4 pixel binning and cooled to −10 °C. Cells were continuously illuminated with a high-power light-emitting diode (LED, UHP-MIC-LED-460, Pryzmatix) through a 470/30-nm filter. To evaluate photostability under two-photon excitation, we used an Ultima Multiphoton Microscopy System (Prairie Technologies) equipped with a 40X LUM Plan FI W/IR-2 0.8-NA objective (Olympus). Cells were illuminated using a Mai Tai HP Deep See Ti:sapphire laser (Spectra-Physics) tuned to 920 nm and set to a power level of 4.7 mW. Laser scanning was performed using using galvanometric mirrors. Each image pixel was sampled at 2.23 Hz with a dwell time of 0.4 μs. Emitted photons were filtered using a 525/50-nm filter (Chroma) and collected using a non-descanned multi-alkali photomultiplier tube (Hamamatsu). For both one-photon and two-photon photobleaching time series, fluorescence from the entire cell was used to compute optical traces.

Neuronal cell culture and transfection. Animal experiments were performed in accordance with the rules of the Stanford University Administrative Panel on Laboratory Animal Care. Primary hippocampal or cortical neurons were dissected from Sprague-Dawley rats on embryonic day 22 and digested with 0.03% trypsin (Sigma) in Dulbecco's Modified Eagle Media (DMEM, HyClone) for 20 min at 37 °C in air with 5% CO_2. Neurons were then dissociated by gentle trituration in Hanks' Balanced Salt Solution (HBSS, Life Technologies) and washed twice in HBSS. Neurons were plated at 3.5×10^4 cells cm^{-2} on 12-mm no. 0 coverslips (Glaswarenfabrik Karl Hecht GmbH) within wells of 24-well plates. Prior to plating, each coverslip was pre-coated for 24 hours with > 300-kDa poly-D-lysine (Sigma) in PBS and washed three times with distilled water. Neurons were cultured overnight at 37 °C in air with 5% CO_2 in Neurobasal with 1× B27 supplement (Life Technologies), 2 mM GlutaMAX (Life Technologies), and 10% FBS. The following day, 90% of the medium was replaced with identical medium without FBS. Cytosine β-d-arabinofuranoside (Sigma) was added to a final concentration of 2 μM when glia reached > 70% confluence, typically around 5 days *in vitro* (DIV). At 7 DIV, 50% of the media was replaced with fresh media without serum. Neurons were transfected at 7 to 9 DIV using 0.5 to 0.75 μL of Lipofectamine 2000 (Life Technologies) and 800 ng of total DNA per well of a 24-well plate. Given the strong promoter (CAG) driving indicator expression, the 800 ng of total DNA per well consisted of 400 ng of indicator expression plasmid and 400 ng of pNCS, an empty bacterial expression plasmid (Lam et al., 2012).

Confocal Imaging of Rat Cortical Neurons. Rat cortical neurons were transfected at 8 DIV and imaged 3-4 days post-transfection in HBSS supplemented with 10 mM HEPES pH 7.4, 1 × B27, 2 mM GlutaMAX, and 1 mM sodium pyruvate on an IX81 microscope with a FluoView FV1000 laser-scanning confocal unit operated using the FV10-ASW v3.01 software (Olympus). Fluorescence excitation was delivered using a 488-nm argon laser model #GLG3135 (Showa Optronics) through a 40X/1.3-NA oil-immersion objective (Olympus). Emission was passed through a 530/40-nm emission filter. Z-sections were imaged using a 1-Airy pinhole setting. A maximum-intensity projection was generated from 2-3 sections spaced 2 μm apart.

Patch clamping and voltage imaging of dissociated neurons. At 11 to 13 DIV (2 to 4 days post-transfection), cultured neurons were patch-clamped at 22 °C using borosilicate glass electrodes with resistances of 3.5 to 5.0 MΩ attached to an Axopatch 700B amplifier (Axon Instruments). Cells were superfused in a chamber mounted on the stage of an Axiovert 100M inverted microscope with a 40×/1.3-NA oil-immersion objective (Zeiss). The extracellular solution contained 110 mM NaCl, 11 mM glucose, 5 mM HEPES-Na, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.3 mM $MgSO_4$, adjusted to pH 7.4. The intracellular (pipette) solution contained 92 mM potassium gluconate, 8 mM HEPES-Na, 8 mM EGTA, 8 mM glucose, 6.4 mM KCl, 4 mM $MgCl_2$, and 0.8 mM $CaCl_2$, adjusted to pH 7.4. Cells were illuminated with a high-power light-emitting diode (UHP-MIC-LED-460, Pryzmatix) through a 470/30-nm filter at a power density of 23.7 mW/mm^2 at the sample plane. Images were captured at 1000 Hz with 4×4 binning using an iXon 860 electron multiplying charge coupled device camera (Andor) cooled to −80 °C in frame transfer mode. Fluorescence response was measured in all pixels from the cell body. Fluorescence traces were acquired while cells were current-clamped in whole-cell mode. For all experiments, fluorescence traces were corrected for photobleaching. To generate action potentials, 700 to 1100 pA of current was injected for 1 ms.

Analyzed neurons had the following characteristics: an access resistance less than 15 MΩ, a membrane resistance greater than 10 times the access resistance, and action potentials with peak height > 0 mV and width < 5 ms at −20 mV. Electrode voltages were recorded using pClamp (Axon Instruments). Voltage and fluorescence traces were analyzed using custom software written in MATLAB. Voltage traces were corrected for the junction potential post hoc.

Statistical methods for *in vitro* experiments. Results presented in the form x ± y represent the mean ± SEM (standard error of the mean), unless indicated otherwise. Statistical comparisons of pre-identified measures of interest between two data sets were performed with the Student's t-test unless otherwise indicated. Prior to performing such statistical comparisons, the Shapiro-Wilk method was used to test the null hypothesis that the data followed a Gaussian (normal) distribution. When this normality hypothesis could not be rejected, Student's t-tests were performed; otherwise, the Mann-Whitney U nonparametric test was used. Prior to performing t-tests, we also tested the null hypothesis of equal variance between the two data sets, and employed Welch's correction when the null hypothesis was rejected. Statistical tests of normality and equal variance were performed with a significance level (α) of 0.05. When analyzing the results of a specific performance test, we applied the Bonferroni correction to the significance levels if more than one pairwise comparison was calculated. Statistical tests were performed in Excel (Microsoft) and MATLAB.

Transgenic Flies. The ASAP variants were cloned into the pJFRC7-20XUAS vector (Pfeiffer et al., 2010) using standard molecular biology methods to generate the *UAS-ASAP1*, *UAS-ASAP2f*, and *UAS-ASAP1 I67T Q397R* transgenes and inserted into the attP40 phiC31 landing site by injection of fertilized embryos (Rainbow Transgenic Flies, Inc.). *UAS-ASAP2f* was additionally inserted into the VK00005 phiC31 landing site though all experiments presented here used the attP40 insertion. GCaMP6f and GCaMP6m were expressed using *UAS-GCaMP6f* and *UAS-GCaMP6m* also inserted into attP40 (Chen et al., 2013). The L1 Gal4 driver (*R48A08AD; R66A01DBD*) was from Tuthill et al., 2013. The L2 Gal4 driver (*21D-Gal4*) was from Rister et al., 2007. The Mi1, Tm3, and Tm1 Gal4 drivers (*R19F01-Gal4*, *R13E12-Gal4*, and *R74G01-Gal4*, respectively) were from the Janelia FlyLight Project (https://www.janelia.org/project-team/flylight). The Tm2 Gal4 driver (*otd-Gal4*) was a generous gift from C. Desplan. The genotypes of the imaged flies were:
L1>>ASAP2f: *w* or *+/+; UAS-ASAP2f/R48A08AD; R66A01DBD/+*
L1>>GCaMP6f: *w* or *+/+; UAS-GCaMP6f/R48A08AD; R66A01DBD/+*
L2>>ASAP1: *w/+; UAS-ASAP1/+; 21D-Gal4/+*
L2>>ASAP2f: *w* or *+/+; UAS-ASAP2f/+; 21D-Gal4/+*
L2>>ASAP1 I67T Q397R: *w* or *+/+; UAS-ASAP1 I67T Q397R/+; 21D-Gal4/+*
L2>>GCaMP6f: *+/+; UAS-GCaMP6f/+; 21D-Gal4/+*
L2>>GCaMP6m: *w/+; UAS-GCaMP6m/+; 21D-Gal4/+*
Mi1>>ASAP2f: *w/+; UAS-ASAP2f/+; R19F01-Gal4/+*
Mi1>>GCaMP6f: *+/+; UAS-GCaMP6f/+; R19F01-Gal4/+*
Tm3>>ASAP2f: *w* or *+/+; UAS-ASAP2f/+; R13E12-Gal4/+*
Tm3>>ASAP1 I67T Q397R: *+/+; UAS-ASAP1 I67T Q397R/+; R13E12-Gal4/+*
Tm3>>GCaMP6f: *+/+; UAS-GCaMP6f/+; R13E12-Gal4/+*
Tm3>>GCaMP6m: *w/+; UAS-GCaMP6m/+; R13E12-Gal4/+*
Tm1>>ASAP2f: *w* or *+/+; UAS-ASAP2f/+; R74G01-Gal4/+*
Tm1>>ASAP1 I67T Q397R: *+/+; UAS-ASAP1 I67T Q397R/+; R74G01-Gal4/+*
Tm1>>GCaMP6f: *+/+; UAS-GCaMP6f/+; R74G01-Gal4/+*
Tm1>>GCaMP6m: *w/+; UAS-GCaMP6m/+; R74G01-Gal4/+*
Tm2>>ASAP2f: *w/+; UAS-ASAP2f/GMR-Gal80; otd-Gal4/+*
Tm2>>GCaMP6f: *+/+; UAS-GCaMP6f/GMR-Gal80; otd-Gal4/+*

Fly Husbandry. All flies used for imaging were raised on standard molasses food at 25 °C on a 12/12-h light-dark cycle. Female flies of the appropriate genotypes were collected on CO_2 within 1 day of eclosion and imaged at room temperature (20°C) 5-6 days after eclosion. Tm2>>ASAP2f and Tm2>>GCaMP6f female flies were collected on ice within 1 day of eclosion and imaged the same day.

***In Vivo*, Two-Photon Imaging.** Flies were cold anaesthetized, positioned in a fly-shaped hole cut in steel foil such that their heads were tilted forward approximately 60° to expose the back of the head capsule above the foil while leaving most of the retina below the foil, and then affixed in place with UV-cured glue (NOA 68T from Norland

Products Inc.). The brain was exposed by removing the overlying cuticle and fat bodies with fine forceps, and an oxygenated saline-sugar solution (Wilson et al., 2004) was perfused over the fly. The saline composition was as follows: 103 mM NaCl, 3 mM KCl, 5 mM TES, 1 mM NaH_2PO_4, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM trehalose, 10 mM glucose, 7 mM sucrose, and 26 mM $NaHCO_3$. The pH of the saline equilibrated near 7.3 when bubbled with 95% O_2/5% CO_2. Neurons were imaged with a Leica TCS SP5 II two-photon microscope with a Leica HCX APO 20X/1.0-NA water immersion objective (Leica) and a pre-compensated Chameleon Vision II femtosecond laser (Coherent, Inc.). The excitation wavelength was 920 nm and 5-15 mW of power was applied at the sample. Emitted photons were collected with a 525/50-nm filter. All data were acquired at a constant frame rate of 38.9 Hz using a frame size of 200×20 pixels, 15X digital zoom, a line scan rate of 1400 Hz, and bidirectional scanning. Imaging time per fly never exceeded 1.5 h.

Visual stimulation. Visual stimuli were generated with custom-written software using C++ and OpenGL and presented using a digital light projector as described previously (Clark et al., 2011). The visual stimulus was projected onto a coherent fiber optic bundle that then re-projected onto a rear-projection screen positioned approximately 4 cm anterior to the fly that spanned 80° of the fly's visual field horizontally and 50° vertically. Immediately prior to being projected onto the screen, the stimulus was filtered with a 447/60-nm bandpass filter so that it could not be detected by the microscope PMTs. The stimulus was refreshed at 240 Hz and had a radiance of approximately 30 $mW \cdot sr^{-1} \cdot m^{-2}$. The imaging and the visual stimulus presentation were synchronized as described in Freifeld et al., 2013. Following this procedure, the time of stimulus onset relative to the start of imaging varied within one stimulus frame (8.33 ms). To compensate for this, the average delay was measured (6.25 ms), and all imaging data was shifted in time by this delay.
The visual stimuli used were:
300 ms full field flash (Figures 1, S2, and S6): alternating full contrast light and dark flashes, each 300 ms in duration, were presented over the entire screen. This stimulus was presented for 2400 imaging frames (62 s) per field of view except in Figure S2 where 24000 imaging frames were presented (617 s).
8 ms light and dark flashes (Figure 2): single 8.33 ms light and dark flashes, with 500 ms of gray between the flashes, were presented over the entire screen. The light and dark flashes were alternated with each presentation. The Michelson contrast of the flashes relative to the gray was 0.5. This stimulus was presented for 4400 imaging frames (113 s) per field of view.
25 ms light and dark flashes (Figures 2-7, S3-S5, and S7): for voltage imaging, this stimulus was identical to the 8 ms flashes, except the light and dark flashes were 25 ms in duration. For calcium imaging, the gray interleave was extended to 1500 ms to account for the slower kinetics, and the stimulus was instead presented for 6600 imaging frames (170 s). In Figures 6 and 7, flashes with Michelson contrasts of 0.5, 0.25, and 0.125 were presented.

***In vivo* imaging data analysis.** Raw images in each time series were aligned in x and y coordinates in ImageJ (NIH) using a macro based on the plugin Turboreg (http://bigwww.epfl.ch/thevenaz/turboreg/) and then further processed in MATLAB. Regions of interest (ROIs) around individual arbors or cell bodies were manually selected in the time series-averaged image. An example of such an image is shown in Figure 1E. For each imaging frame within the time series, intensity values for the pixels within each ROI were averaged and the mean background value (the average intensity in a region of the image without cells) was subtracted. To correct for bleaching, the time series for each ROI was fit with the sum of two exponentials, and in the calculation of $\Delta F/F = (F(t) - F_0)/F_0$, the fitted value at each time t was used as F_0. For the 300 ms full field flash stimulus, all imaging frames were used to compute the fit, thereby placing $\Delta F/F = 0$ at the mean response after correction for bleaching. For the 8 or 25 ms light and dark flashes off of gray stimulus, only imaging frames that fell in the last 25% of the gray period were used to fit the bleaching curve; this places $\Delta F/F = 0$ at the mean baseline the cell returns to after responding to the flash instead of at the mean of the entire trace, which is inaccurate when the responses to the light and dark flashes are not equal and opposite. The stimulus-locked average was computed for each ROI by reassigning the timing of each imaging frame to be relative to the stimulus transitions (dark to light or light to dark for the 300 ms full field flash, gray to light or gray to dark for the flashes off of gray) and then computing a simple moving average with a 25 ms averaging window and a shift of 8.33 ms. This effectively resampled our data from 38.9 Hz to 120 Hz with 3-point boxcar smoothing but did not otherwise distort the fluorescence signal. As the screen on which the stimulus was presented did not span the fly's entire visual field, only a subset of imaged ROIs were actually observing the stimulus (empirically, approximately 70%). For Figures 1-5, S2-S5, and S7, responding ROIs were identified as those whose responses at time-matched points during the 2 stimulus epochs (dark and light flashes for the 300 ms full field flash and light to gray and dark to gray for the flashes off of gray) were significantly different (t-test, $p < 0.01$) for at least 3 consecutive time points. All calls were examined manually and adjusted if necessary. For Figures 6 and 7, ROIs

were presented with the 300 ms full field flash stimulus in addition to 25 ms flashes off of gray of different contrasts; responding ROIs were identified solely through their 300 ms full field flash response. All traces, except the single ROI and single trial examples in Figures 1F and 1G, are presented as the mean ± 1 SEM across all of the responding ROIs of the moving average response to all trials of each ROI. In Figures 1F and 1G, bottom, the moving-average response across all trials of single example ROIs (that are approximately in the 75th percentile for response amplitude for all ROIs of that indicator) was plotted as mean ± 1 SEM, and 5 0.6-s single trial excerpts of the ΔF/F trace for the same ROIs was presented in the single cell examples (plotted at the imaging frame rate of 38.9 Hz).

The quantification metrics for each ROI were computed as follows:

For the responses to the 300 ms full field flash stimulus in Figures 1 and S6, the peak response (peak ΔF/F) during each phase (depolarization and hyperpolarization) was the absolute value of the ΔF/F value farthest from zero in the expected direction of the response. The time to peak (t_{peak}) was the time at which this peak response occurred, relative to the start of the stimulus. The decay of the response from the peak was fit with a single exponential and the time constant of this fit was τ_{decay}. The mean across all responding ROIs ± 1 SEM was plotted, except for τ_{decay}, for which the ROIs were further filtered by those whose exponential decay fits had *r* values greater than 0.5. Pairwise Student's t-tests were performed, and multiple comparisons were corrected for using the Bonferroni method.

For the responses to the 25 ms light and dark flashes off of gray stimuli in Figures 3-5, S3, and S4, the peak response to each flash (peak ΔF/F) was the ΔF/F value farthest from zero in the expected direction of the initial response (depolarization or hyperpolarization). The time to peak (t_{peak}) was the time at which this peak response occurred, relative to the start of the light or dark flash. The full width at half maximum was computed by identifying, for the rising phase of the response prior to t_{peak} and for the falling phase after t_{peak}, the two adjacent time points with ΔF/F values bracketing one-half of the peak ΔF/F value; fitting a line between two points to derive the time at which the value would be one-half of the peak ΔF/F value; and subtracting this time for the rising phase from that of the falling phase. Some traces were too noisy to identify the two adjacent time points with ΔF/F values bracketing one-half of the peak ΔF/F value; such traces were excluded. In Figures 4 and S3, pairwise Student's t-tests were performed for the peak ΔF/F and full width at half maximum metrics, and the Mann-Whitney U test was performed for the t_{peak} metric. Multiple comparisons were corrected for using the Bonferroni method. In Figures 5 and S4, one-way ANOVAs were performed for the peak ΔF/F and full width at half maximum metrics and followed by post-hoc pairwise t-tests with the Bonferroni correction for multiple comparisons; the Kruskal-Wallis test followed by post-hoc pairwise Mann-Whitney U tests with the Bonferroni correction for multiple comparisons were performed for the t_{peak} metric.

For Figure 3C, the fraction decay from M1 was computed as the mean peak ΔF/F of the indicated region divided by the mean peak ΔF/F of layer M1. The error bars were computed as $\text{mean}_{\text{fraction decay}} \times (\text{SEM}_{\text{region}}/\text{mean}_{\text{region}} + \text{SEM}_{\text{M1}}/\text{mean}_{\text{M1}})$.

For Figures 6, 7, S5, and S7, the stimulus was 25 ms light and dark flashes off of gray, and selectivity for ON and OFF was computed as: |peak light response/peak dark response| for the ON pathway and |peak dark response/peak light response| for the OFF pathway. The peak response was quantified as the maximal ΔF/F of the initial response phase minus the average ΔF/F value before the response. For Figure 7, the index was computed separately for each of the 3 pairs of contrast values. When the response is perfectly linear, this index equals 1. Selectivity was computed for each individual ROI, and a box and whisker plot across ROIs was plotted for each condition. The line indicates the median, the box indicates the interquartile range, the whiskers extend to the maximum or minimum values less than one interquartile range past the box, and outliers are omitted. Mann-Whitney U tests with the Bonferroni correction for multiple comparisons were performed.

NEURON modeling (Figures 3E-3H). The morphology of Mi1 from the Janelia Fly EM: Medulla TEM Reconstruction project (Takemura et al., 2013) was downloaded from the Chklovskii Archive in the Neuromorpho public repository. The neuron used was the central column neuron Mi1 215. The .swc morphology was loaded into NEURON 7.4 (www.neuron.yale.edu; Carnevale and Hines, 2006) using the Import 3D tool and exported to the CellBuilder tool, which converted the morphology to a multi-compartment cell geometry for the model. To complete the cell, a single-segment cell body of diameter 5 µm and length 5 µm was attached to the end of the most distal process by a process of diameter 250 nm and length 10 µm. The model neuron was given uniform passive membrane properties, estimated from the parameters in (Behnia et al., 2014; Cuntz et al., 2013; Gouwens and Wilson, 2009; Haag et al., 1996): resting membrane potential = -70 mV, axial resistance = 40 to 420 Ω•cm, specific membrane capacitance = 1 µF/cm^2, and specific membrane resistance = 1 to 21 kΩ•cm^2. 50 pA of current was injected into layer M1 for 20 ms. The duration of the simulation was 100 ms. The membrane potential responses over time were recorded in the arbors of layers M1, M5, and M10. The peak membrane potential measured during

current injection was determined and heat maps were generated displaying the fraction of this peak membrane potential relative to that achieved in layer M1. This was compared to the decays ± 1 SEM measured with ASAP2f imaging.

GCaMP6f dynamic range analysis (Figures S7G-S7I). To determine the maximal decrease in fluorescence GCaMP6f is capable of achieving *in vivo*, we imaged while first flowing in the standard oxygenated fly saline (for 2 min), then flowing in a modified oxygenated fly saline with 0 mM $CaCl_2$ and 10 mM EGTA (for 10 min), and then flowing in standard oxygenated fly saline (for 10 min). The entire extent of the medulla and lobula was imaged simultaneously. Data was acquired at 1 Hz, using an imaging window of 512×300 px, 3X digital zoom, a line scan rate of 400 Hz, and bidirectional scanning. These time series were processed identically to those measuring visually evoked responses, except as subsequently described. ROIs were manually drawn around the terminals and cell bodies, with one ROI for each layer. $\Delta F/F$ was computed using the mean fluorescence during the initial standard saline epoch as the baseline F_0. The mean $\Delta F/F$ during each solution epoch was computed by averaging $\Delta F/F$ over the frames during which the fluorescence had stabilized, chosen manually for each time series but consistent among ROIs from the same time series. The minimum $\Delta F/F$ achievable was therefore -100%.

		ASAP1	ASAP2f
Depol. (–70 to 30 mV)			
	τ_{fast} (ms)	2.9 ± 0.3	2.8 ± 0.1
	τ_{slow} (ms)	161 ± 33	135 ± 16
	% fast	74 ± 5	81 ± 2
Repol. (30 to –70 mV)			
	τ_{fast} (ms)	2.3 ± 0.4	2.4 ± 0.2
	τ_{slow} (ms)	177 ± 38	155 ± 16
	% fast	63 ± 6	71 ± 3
Hyperpol. (–70 to –100 mV)			
	τ_{fast} (ms)	11 ± 3	8.8 ± 1.0
	τ_{slow} (ms)	131 ± 16	142 ± 15
	% fast	59 ± 3	54 ± 4
Repol. (–100 to –70 mV)			
	τ_{fast} (ms)	15 ± 3	13 ± 1
	τ_{slow} (ms)	131 ± 14	154 ± 9
	% fast	52 ± 2	50 ± 4

n = 4–5 cells per sensor. Data are presented as mean ± SEM.

Table S1. Related to Figure 1. Response kinetics of ASAP indicators in HEK293A cells.

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