Relationship between simultaneously recorded spiking activity and fluorescence signal in GCaMP6 transgenic mice

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Keywords

calcium imaging, genetically encoded calcium indicator, action potential, excitatory neurons, calibration

ABSTRACT

Fluorescent calcium indicators are often used to investigate neural dynamics, but the relationship between fluorescence and action potentials (APs) remains unclear. Most APs can be detected when the soma almost fills the microscope's field of view, but calcium indicators are often used to image populations of neurons, necessitating a large field of view, generating fewer photons per neuron, and compromising AP detection. Here we characterized the AP-fluorescence transfer function *in vivo* for 48 layer 2/3 pyramidal neurons in primary visual cortex, with simultaneous calcium imaging and cell-attached recordings from transgenic mice expressing GCaMP6s or GCaMP6f. While most APs were detected under optimal conditions, under conditions typical of population imaging studies only a minority of 1AP and 2AP events were detected (often <10% and ~20-30%, respectively), emphasizing the limits of AP detection under more realistic imaging conditions.

INTRODUCTION

Genetically encoded calcium indicators (GECIs) are widely used with 2-photon laser scanning microscopy to report neuronal activity within local populations *in vivo* (Luo et al., 2018). This optical approach is minimally invasive and enables simultaneous measurement of activity from hundreds or even thousands of neurons at single-cell resolution, over multiple sessions. Using a contemporary GECI such as GCaMP6s, fluorescence changes associated with isolated spikes (action potentials, APs) *in vivo* can be detected when imaged at sufficiently high spatiotemporal resolution (Chen et al., 2013) (<http://dx.doi.org/10.6080/K02R3PMN>). Yet undetected APs are common in population imaging experiments (Theis et al., 2016; Berens et al., 2018).

Inferring the underlying AP train or firing rate from calcium imaging remains challenging for several reasons. Firstly, population imaging studies necessarily employ a large field of view containing many neurons. In contrast, the AP to calcium-dependent fluorescence transfer function is typically characterized with a soma filling the field of view of the microscope, to maximize photon flux from the soma and thereby signal-to-noise ratio. Secondly, there is no ground truth spiking information available for most neurons in a population. Spiking information, often from a cell-attached recording, can be used to refine the spike inference model and thereby optimize AP detection. Thirdly, the AP to calcium-dependent fluorescence transfer function may be different for each neuron due to various intrinsic and extrinsic factors, such as neuron-to-neuron differences in indicator expression.

Compared to viral expression, transgenic mouse lines offer convenience (e.g. bypassing virus injection and associated procedures) and achieve more uniform GECI expression in genetically defined neuronal populations (Madisen et al., 2015; Daigle et al., 2018). Using our intersectional transgenic mouse lines that enable Cre recombinase-dependent expression of GCaMP6s or GCaMP6f, we simultaneously characterized the spiking activity and fluorescence of individual GECI-expressing pyramidal neurons in layer 2/3 of mouse primary visual cortex (V1). We then tested the performance of several spike inference models, detecting APs under optimal conditions (models refined using the spiking information, with the soma filling the field of view) and under the less optimal conditions typical of population imaging experiments. Our results provide insight into the relationship between spiking activity *in vivo* and fluorescence signals and will aid the interpretation of existing and future calcium imaging datasets.

RESULTS

To characterize the single-cell transfer function between observed fluorescence signals and underlying APs *in vivo*, we performed simultaneous calcium imaging and cell-attached recordings in V1 L2/3 excitatory pyramidal neurons in anesthetized mice (Figure 1A,B). To directly compare our results to virally-expressed GCaMP6f and GCaMP6s (Chen et al., 2013) (<http://dx.doi.org/10.6080/K02R3PMN>), we used a small field of view (19.3-27.3 x 19.3-21.5 μm ; scanning rate 141.3-158.3 frames per second, fps). 2-10 minute recordings were obtained from 213 neurons, all with fluorescence excluded from the nucleus. Quality control code was deployed to exclude from further analysis recordings with artifacts such as motion, photobleaching, somatic dye from the recording pipette, electrophysiological or fluorescence baseline instability, and abrupt changes in AP waveform (see Methods). The dataset for further analysis was from 48 neurons from mice of 4 transgenic lines, two expressing GCaMP6s and two GCaMP6f in excitatory neurons in layer 2/3 and deeper layers of cortex (Table 1).

We analyzed events with fluorescence transients separated from those of adjacent events, containing a total of 5427 APs (28% of APs; Figure 1 – figure supplement 1C). An event was defined as one or more APs within 250 ms, with no APs in the preceding or subsequent 300 ms for GCaMP6f or 1 s and 500 ms for GCaMP6s (Figure 1C).

Calcium transients differ across mouse lines

Fluorescence measured from the soma is contaminated with fluorescence from the surrounding neuropil, due to the extended nature of the microscope point spread function. Neuropil contamination is often removed by subtracting a scaled version of the neuropil fluorescence from the somatic fluorescence, with the scale factor referred to as the r value (Kerlin et al., 2010; Akerboom et al., 2012). The r value can affect AP detection, with under-subtraction of neuropil leading to false positives (events detected when there was activity in the neuropil but not the soma) and over-subtraction to false negatives (failure to detect somatic activity). We examined the effects of r on detection of 1AP events, with electrical recordings providing ground truth (Figure 2 and Figure 2 – figure supplement 1). For many GCaMP6s neurons, the receiver operating characteristic (ROC) curve changed little with r (Figure 2A), indicating that APs were detected with few false positives with little effect of neuropil subtraction. Neuropil subtraction exerted a stronger influence on event detection in GCaMP6f neurons, where the ROC curve changed with r (Figure 2A), permitting identification of the optimal r as that which maximized the area under the ROC curve and, thereby, the true/false event detection ratio. Optimal r for Emx1-f and Cux2-f neurons was approximately normally distributed with mean $\pm$ sem of 0.82 ± 0.07 (20 neurons, Figure 2B). Our results indicate that the value of r has a modest effect on event detection in GCaMP6f neurons in mouse V1. The effect of neuropil subtraction may be greater during coordinated activity across the whole network, such as during strong sensory stimuli.

After neuropil subtraction (see Methods), we averaged trials by number of APs, fit a sum of exponentials to estimate rise and decay time constants and calculated peak $\Delta F/F$ (mean fluorescence over 100 ms around the maximum within 300 ms for GCaMP6f and 500 ms for GCaMP6s) for events with 1-5 APs (Figure 3). 28-55% of detected APs were in events with 1-5 APs (Figure 1 - figure supplement 1) and >70% of these analyzed APs were in multi-AP events. As expected, peak $\Delta F/F$ increased approximately linearly with 1-5 APs, and peak $\Delta F/F$ and decay time constant were greater with GCaMP6s than GCaMP6f (Figure 3C). Peak $\Delta F/F$ was comparable to or slightly greater than in previous studies with GCaMP6s and GCaMP6f, possibly because we subtracted more of the neuropil fluorescence with a slightly greater r value ($r=0.8$ vs 0.7 ; Chen et al., 2013).

As expected, photon shot noise was the dominant noise source in images from all mouse lines. The pixelwise slope of the least squares fit between the variance and mean of the photon flux was 1.04 ± 0.01 (mean $\pm$ sem), consistent with the noise following a Poisson process (intercept -0.08 ± 0.2 , 48 neurons). Trial-to-trial variability in the amplitude of the 1AP-evoked fluorescence

was substantial and exceeded photon shot noise in most neurons (Figure 3 - figure supplement 1). The sources of non-Poisson variability in our results are unclear, but negligible motion was visible in the movies after motion correction. Likely the variability results primarily from trial-to-trial differences in the AP-evoked calcium concentration and GCaMP6f and GCaMP6s are expressed at sufficient concentrations to report resting changes in calcium concentration in all four mouse lines.

Increasing field of view reduces optimized event detection

GCaMP6 indicators have been widely adopted because they exhibit greater AP-evoked $\Delta F/F$ than previous GCaMP indicators, but still some APs may go undetected (Chen et al., 2013). Under ideal conditions, almost all APs can be detected (with probability close to 1 at a false positive probability of 1%; Chen et al., 2013). However, many imaging experiments are performed with a field of view of hundreds of micrometers and this large field of view limits the dwell time per soma and thereby the photon flux per soma and signal-to-noise ratio. What event detection rate might be expected when imaging a large field of view, sufficient to include hundreds of somata? How much does field of view affect event detection?

We calculated detection probability for 1AP and 2AP events, using AP times from electrophysiology recordings to optimize event detection for each neuron (Chen et al., 2013). In high spatial and temporal resolution images, the probability of 1AP event detected spanned a wide range (probability 0.07-1 and 0.11-0.95 for GCaMP6s and GCaMP6f, at 1% false positive probability, Figure 5A-C). As expected (Dana et al., 2014; Wei et al., 2019), most 1AP events were detected in GCaMP6s and GCaMP6f neurons, but with lower average probability in GCaMP6f neurons (1AP detection probability 0.70 ± 0.06 for 18 Emx1-s neurons, 0.80 ± 0.03 for 3 tetO-s neurons, 0.40 ± 0.08 for 9 Cux2-f neurons, 0.60 ± 0.08 for 11 Emx1-f neurons, mean $\pm$ sem at 1% false positive probability). 2AP events were reliably detected in all four mouse lines (Figure 5C; detection probability 0.90 ± 0.06 for Emx1-s, 1.0 ± 0.0 for tetO-s, 0.66 ± 0.07 for Cux2-f, 0.80 ± 0.05 for Emx1-f, at 1% false positive probability). In high spatial and temporal resolution images, in all four mouse lines it was possible to detect most but by no means all 1AP and 2AP events with a false positive probability of only 1%.

In these transgenic mice, 1AP detection probabilities were lower than in previously reported neurons with virally-expressed GCaMP6s and GCaMP6f (0.99 and 0.84 at 1% false positive probability in Chen et al., 2013). There are several possible reasons for this difference. In the

transgenic mice used here, GCaMP expression is widespread throughout neocortex, which may result in labeling of greater numbers of axons and dendrites that contribute to the neuropil signal. Furthermore, GCaMP6 expression may be weaker in the four TIGRE1.0 mouse line crosses examined here than with strong promoter-driven adeno-associated virus (AAV) vectors as used in Chen et al. (2013). The newer TIGRE2.0 reporter lines drive GCaMP expression that's comparable to that from strong promoter-driven AAVs (Daigle et al., 2018), likely enabling 1AP and 2AP detection rates in transgenic mice that are comparable to those achieved with viral expression of GCaMP6.

Our recordings were obtained with a small field of view, at a high frame rate and centered on the soma ($\sim 19.3 \times 19.3 \mu\text{m}$, $\sim 158 \text{ Hz}$, Figure 4A,B). In an attempt to simulate commonly used imaging conditions, we downsampled our images in space and time to mimic imaging with a $412 \times 412 \mu\text{m}$ field of view at 30.3 Hz , as used in the Allen Brain Observatory (Figure 4C,D). The baseline fluorescence noise from downsampled images was comparable to that in the Allen Brain Observatory (Figure 4E) and is presumably comparable to images in many 2-photon datasets with populations of hundreds of neurons.

As expected, event detection probabilities were lower for downsampled images than for the original, high resolution images (Figure 5D-F). 1AP and 2AP event detection probabilities were 0.32 ± 0.05 and 0.55 ± 0.08 for 18 Emx1-s neurons, 0.43 ± 0.07 and 0.89 ± 0.06 for 3 tetO-s neurons, 0.16 ± 0.04 and 0.24 ± 0.04 for 9 Cux2-f neurons, 0.21 ± 0.04 and 0.42 ± 0.09 for 11 Emx1-f neurons (mean $\pm$ sem at 1% false positive probability). Even for 2AP events, detection probability is <0.5 when imaging with GCaMP6f and a field of view of several hundred micrometers.

In summary, 1AP and 2AP events were detected with high probability when images were acquired with high spatial and temporal resolution and when analysis was performed with an event detection algorithm optimized for each neuron using known AP times. Even with known AP times to optimize detection for each neuron, event detection was severely impaired by a reduction in spatial and temporal resolution to mimic a typical 2-photon population imaging experiment.

Modest effect of field of view on event detection under typical imaging conditions

195 In a typical 2-photon population imaging experiment, no electrophysiology recording is available
196 to optimize event detection. Often, the shape of the calcium transient is estimated from
197 published indicator rise and decay times or derived from a representative sample of
198 fluorescence transients. What are typical event detection and false positive probabilities under
199 these sub-optimal conditions, blind to the underlying AP times? Is performance degraded
200 equally for high and for low resolution images?

201
202 We compared event detection from high resolution and downsampled images using three spike
203 inference algorithms: unconstrained non-negative deconvolution (NND; Friedrich et al., 2017),
204 non-negative convolution with an L0 constraint to enforce event sparseness (Exact L0; Jewell et
205 al., 2020) and a biophysical model that explicitly accounts for intracellular calcium dynamics
206 (MLspike; Deneux et al., 2016). These three algorithms are among the highest performing spike
207 inference algorithms (Berens et al., 2018).

208
209 For each neuron, the algorithms were deployed to estimate the number of APs in each image of
210 the movie. All three algorithms estimated AP numbers that approximately recapitulated the
211 number of APs measured with electrophysiology, but the number of APs per frame was typically
212 not an integer due to imperfect spike inference (Figure 6A,D). We characterized performance
213 using the Pearson correlation coefficient and the Matthews correlation coefficient, which
214 compare measured and estimated AP number at each time point, and the presence or absence
215 of an event at each time point, respectively. Pearson correlation coefficients were ~0.4 when
216 calculated with 33 ms time bins, increasing towards 0.7 as bin size was increased to 500 ms
217 (Figure 6 - figure supplement 1), comparable to published results (Berens et al., 2018). Mean
218 Pearson and Matthews correlation coefficients were similar across inference algorithms and
219 mouse lines and differed little between high resolution and downsampled images (Figure 6B,E).

220
221 We plot ROC curves to more directly examine the relationship between detected events and
222 false positives. Since spike inference is generally useful only where false positive rates are low,
223 we focused on false positive probabilities in the range of 0-0.05. Performance differed greatly
224 between neurons, but mean ROC curves were similar across mouse lines, with only modest
225 differences between algorithms, between GCaMP6s and GCaMP6f lines or between high
226 resolution and downsampled conditions (Figure 6C,F).

Naturally, detection probability increased with the number of APs per event. At a false positive probability of 0.01, detection probability was commonly <0.1 for 1AP events, increasing approximately linearly with AP number, often to ~ 0.8 for 5AP events (Figure 7A,B). With 1AP events being the most common event type in all four mouse lines (Figure 7C), it was possible to detect only a minority of events with a low false positive probability. Using these spike inference algorithms, although detection probabilities were commonly slightly lower for downsampled than for high resolution images, the difference was modest, indicating that the decreased SNR of population imaging had little effect on event detection in our dataset.

Using our dataset, we compared event detection with three algorithms: non-negative deconvolution (NND), non-negative deconvolution with an exact L0 constraint (Exact L0) and the biophysically inspired MLspike model. For NND, performance was poor at 30 Hz and considerably improved by upsampling to 150 Hz (Figure 7 – figure supplement 1). Upsampling of low frame rate data, often to 100 Hz, is a common practice in the field (Theis et al., 2016; Berens et al., 2018; Pachitariu et al., 2018). For MLspike, performance was poor without use of the autocalibration procedure to optimize the model for each neuron (Figure 7 – figure supplement 2). MLspike thus contrasted with deconvolution-based algorithms, for which fixed parameters are more effective (Pachitariu et al., 2018). For Exact L0, neither upsampling nor optimization for each neuron was necessary for optimal mean performance across neurons.

Pachitariu et al. (2018) found that non-negative deconvolution (NND) matched and often exceeded the performance of more constrained algorithms such as NND with an approximate L0 constraint. Consistent with the conclusions of Pachitariu et al. (2018), Exact L0 often lagged the performance of NND (Figure 7). The performance of MLspike was broadly equivalent to that of NND, but the loss of performance due to downsampling was less with MLspike, resulting in outperformance of MLspike on downsampled images. Our results point to MLspike as a compelling choice for spike inference in population imaging experiments. Our results also suggest there's ample room for improvement of spike inference models since event detection by the three spike inference models falls far short of the performance of the ground truth optimized approach employed in figure 5 (Figure 7 – figure supplement 3).

In summary, relative to small field of view imaging, population imaging conditions decreased the probability of spike event detection with an event detector optimized to each individual neuron using ground truth AP information (Figure 5). With blind spike inference, many events went

undetected even under near-ideal imaging conditions with a small field of view, and event detection was not substantially worse under population imaging conditions (Figure 6, Figure 7, Figure 7 – figure supplement 3). The results of Figures 6 and 7 are likely representative of event detection in many GCaMP6 imaging experiments, where ground truth AP information is not available and blind spike inference is employed. Our results indicate that even though GCaMP6 indicators are bright and sensitive enough to enable the detection of most 1AP events in superficial cortical pyramidal neurons *in vivo* if the detection procedure is optimized using ground truth AP information, most events containing 1, 2 and sometimes greater numbers of APs go undetected in our and likely many other imaging experiments with GCaMP6.

DISCUSSION

Calcium imaging is widely used to report neuronal spiking activity *in vivo*. However, accurate spike inference from calcium imaging remains a challenge, and there are relatively few ground truth datasets with simultaneous calcium imaging and electrophysiology to aid the development of more accurate spike inference algorithms. In a recent challenge (Spike Finder; <http://spikefinder.codeneuro.org/>) (Berens et al., 2018), ~40 algorithms were trained and tested on datasets consisting of 37 GCaMP6-expressing neurons, underscoring the need for additional GCaMP6 calibration data (Berens et al., 2018). In addition to supporting efforts toward spike inference, an improved understanding of the relationship between spiking and observed fluorescence signals is necessary to further broaden the utility and impact of calcium imaging. To these ends, we contribute a ground truth dataset consisting of 48 V1 L2/3 excitatory neurons recorded at single-cell resolution (available at <https://portal.brain-map.org/explore/circuits/oephys>), and characterized their AP-to-calcium fluorescence transfer function. Complementing existing datasets with viral GECI expression (Chen et al., 2013; Theis et al., 2016; Dana et al., 2016), our work facilitates interpretation of existing and future calcium imaging studies using mainstream transgenic mouse lines, such as the Allen Institute's Brain Observatory Visual Coding dataset (<http://observatory.brain-map.org/visualcoding>) (de Vries et al., 2020).

Previous studies have established that most APs can be detected with GCaMP6 indicators under near-optimal conditions (Chen et al., 2013). Yet undetected APs are common in population imaging experiments (Theis et al., 2016; Berens et al., 2018). To investigate why APs are often missed during population imaging, we compared event detection in 250 ms time-

296 windows with a neuronal soma occupying most of the image, near-optimal conditions for AP
297 event detection, and event detection when the soma occupies just a small percentage of the
298 field of view, less ideal conditions that are common in population imaging studies. Importantly,
299 we downsampled images to simulate population imaging conditions, enabling comparison for
300 the same APs under different imaging conditions.

301
302 Our results indicate that, in GCaMP6 transgenic mice, most APs can be detected under near-
303 optimal conditions, while detection is less effective during population imaging. These
304 conclusions are similar to those of previous studies with viral GCaMP6 expression, but our
305 results also reveal two reasons for the difference in detection. Unsurprisingly, the reduced
306 signal-to-noise ratio of population imaging, relative to single soma imaging, results in less
307 effective event detection. However, a high signal-to-noise ratio, achieved by imaging one soma,
308 was no guarantee of effective event detection. Effective detection also required optimization of
309 detection for the neuron of interest, using known AP times to identify events with different AP
310 numbers and so generate kernels of the appropriate amplitude and time course. Parameter
311 tuning in the absence of known AP times, with the MLspike autocalibration routine, improved
312 event detection but not to the high standard of ground truth-optimized detection. Unfortunately,
313 measuring AP times for every neuron with electrophysiology is rarely feasible, severely limiting
314 the percentage of events one might reasonably expect to detect with GCaMP6 in most imaging
315 experiments.

316
317 Our results point to several practices that might be adopted to maximize spike detection. Firstly,
318 minimize the field of view, maximizing photon flux per neuron. Secondly, tune the spike
319 inference model for each neuron independently, where possible. Thirdly, compare the results of
320 several spike inference models. The 3 models employed here produced similar AP detection
321 rates, whether applied to high resolution or to downsampled images. Similarly, Pachitariu *et al.*
322 (2018) observed that the L0 constraint failed to improve performance of the NND model.
323 Nonetheless, each model has strengths and weaknesses. For example, a model may detect
324 more APs than another but at the cost of a greater false positive rate. As a result, model
325 performance may diverge for some AP rates and patterns. In the worst case, comparing models
326 provides some protection from errors in implementation. Fourthly, ensure that traces are
327 sampled (or upsampled) at a sufficiently high rate when employing NND and use autocalibration
328 with MLspike; both make a substantial difference to model performance. Finally, exercise

caution when interpreting the inferred spike rates. Commonly, many APs are not detected using even the most accurate spike inference models.

In summary, in this study we present a ground truth dataset from anesthetized mice with simultaneous electrophysiology and calcium imaging. Analysis of this dataset revealed that only a small fraction of isolated action potentials were detected under typical population imaging conditions and with existing spike inference algorithms. By making our data freely available, we hope that it will serve the community as a further resource to better understand the quantitative link between calcium-evoked fluorescent imaging signals and spiking activity.

MATERIALS AND METHODS

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (Mus musculus)	B6.129S2-Emx1tm1(cre)Krl/J, Emx1-IRES-Cre	Jackson Laboratory	RRID:IMSR_JA X:005628 RRID:MGI:2684610	
Genetic reagent (Mus musculus)	B6(Cg)-Cux2tm3.1(cre/ERT2)Mull/Mm mh, Cux2-CreERT2	MMRRC	RRID:MMRRC_032779-MU RRID:MGI:5014172	
Genetic reagent (Mus musculus)	B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ, Camk2a-tTA	Jackson Laboratory	RRID:IMSR_JA X:007004 RRID:MGI:2179066	
Genetic reagent (Mus musculus)	B6;DBA-Tg(tetO-GCaMP6s)2Niell/J, tetO-GCaMP6s	Jackson Laboratory	RRID:IMSR_JA X:024742 RRID:MGI:5553332	
Genetic reagent (Mus musculus)	B6;129S6-Igs7tm93.1(tetO-GCaMP6f)Hze/J, Ai93(TITL-GCaMP6f)	Jackson Laboratory	RRID:IMSR_JA X:024103 RRID:MGI:5558086	

Genetic reagent (Mus musculus)	B6.Cg-lgs7tm94.1(tetO-GCaMP6s)Hze/J, Ai94(TITL-GCaMP6s)	Jackson Laboratory	RRID:IMSR_JAX:024104 RRID:MGI:5607576	
Software, algorithm	MATLAB R2016b	http://www.mathworks.com/products/matlab/	RRID:SCR_001622	
Software, algorithm	Python 3.7.4	http://www.python.org/	RRID:SCR_008394	
Software, algorithm	LabVIEW 2015	http://www.ni.com/labview/	RRID:SCR_014325	

Experimental procedures were conducted in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the Allen Institute for Brain Science under protocol number 1509.

Mice. 2-photon-targeted electrophysiology and 2-photon calcium imaging was conducted in 2-5 month-old male and female transgenic mice: five *Emx1-IRES-Cre;Camk2a-tTA;Ai94* (Emx1-s) mice, one *Camk2a-tTA;tetO-GCaMP6s* (tetO-s) mouse, three *Emx1-IRES-Cre;Camk2a-tTA;Ai93* (Emx1-f) mice, and four *Cux2-CreERT2;Camk2a-tTA;Ai93* (Cux2-f) mice. All four lines drive GCaMP expression primarily in excitatory neurons. In Cux2-CreERT2 mice, Cre and GCaMP expression are enriched in layer 2/3 (Franco *et al.*, 2012; Harris *et al.*, 2014). In Emx1-IRES-Cre and Camk2a-tTA mice, GCaMP is expressed throughout cortical layers (Gorski *et al.*, 2002; Wechselblatt *et al.*, 2016). Images showing the pattern of Cre and GCaMP expression in these mouse lines are available via the Transgenic Characterization pages of the Allen Mouse Brain Connectivity Atlas and Allen Brain Observatory: <https://connectivity.brain-map.org/transgenic>, <http://observatory.brain-map.org/visualcoding/transgenic>.

Mice of some of the genotypes used here, most notably Emx1-f, can exhibit epileptiform activity (Steinmetz *et al.*, 2017), including overt seizures. Mice with seizures were excluded from the study. However, the spiking patterns of neurons from GCaMP6s and -f lines commonly differed, suggesting that one or more transgenes affected cell or circuit activity (figure 1 – figure supplement 1C).

Surgery. Mice were anesthetized with either isoflurane (0.75-1.5% in O₂) or urethane (1.5 g/kg, 30% aqueous solution, intraperitoneal injection), then implanted with a metal head-post. A circular craniotomy was performed with skull thinning over the left V1 centering on 1.3 mm anterior and 2.6 mm lateral to the Lambda. During surgery, the craniotomy was filled with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.25, MgCl₂ 1, NaHCO₃ 26, glucose 10, CaCl₂ 2, in ddH₂O; 290 mOsm; pH was adjusted to 7.3 with NaOH to keep the exposed V1 region from overheating or drying. Durotomy was performed to expose V1 regions of interest that were free of major blood vessels to facilitate the penetration of recording micropipettes. A thin layer of low melting-point agarose (1-1.3% in ACSF, Sigma-Aldrich) was then applied to the craniotomy to control brain motion. The mouse body temperature was maintained at 37°C with a feedback-controlled animal heating pad (Harvard Apparatus).

Calcium imaging. Individual GCaMP6+ neurons ~100-300 µm below the pial surface of cortex were visualized under adequate anesthesia (Stage III-3) using a Bruker (Prairie) 2-photon microscope and Chameleon Ultra II Ti:sapphire laser (Coherent). Fluorescence excited at 920 nm wavelength, with <70 mW laser power measured after the objective, was collected in two spectral channels using green (510/42 nm) and red (641/75 nm) emission filters (Semrock) to visualize GCaMP6 and the Alexa Fluor 594-containing micropipette, respectively. Fluorescence images of 96-136 x 96-107 pixels and a 19.3-27.3 x 19.3-21.5 µm field of view were acquired at 141.3-158 frames per second through a 16x water-immersion objective lens (Nikon, NA 0.8). Recordings included periods with and without visual stimuli. Mean ± sem number of pixels per neuron was 1136 ± 46.

Electrophysiology. 2-photon targeted cell-attached recording was performed following established protocols (Margrie et al., 2003; Kitamura et al., 2008; Knoblich et al., 2019). Long-shank borosilicate (KG-33, King Precision Glass) micropipettes (5-10 MΩ) were pulled with a P-97 puller (Sutter) and filled with ACSF and Alexa Fluor 594 to perform cell-attached recordings on GCaMP6+ neurons. Micropipettes were installed on a MultiClamp 700B headstage (Molecular Devices), which was mounted onto a Patchstar micromanipulator (Scientifica) with an approaching angle of 31 degrees from horizontal plane. Minimal seal resistance was 20 MΩ. Data were acquired under “I = 0” mode (zero current injection) with a Multiclamp 700B, recorded at 40 kHz using Multifunction I/O Devices (National Instruments) and custom software written in

LabVIEW (National Instruments) and MATLAB (MathWorks). Isoflurane level was intentionally adjusted during recording sessions to keep the anesthesia depth as light as possible, resulting in fluctuation of the firing rates of recorded neurons.

Visual stimulation. Whole-screen sinusoidal static and drifting gratings were presented on a calibrated LCD monitor spanning 60° in elevation and 130° in azimuth to the contralateral eye. The mouse's eye was positioned ~22 cm away from the center of the monitor. For static gratings, the stimulus consisted of 4 orientations (45° increment), 4 spatial frequencies (0.02, 0.04, 0.08, and 0.16 cycles per degree), and 4 phases (0, 0.25, 0.5, 0.75) at 80% contrast in a random sequence with 10 repetitions. Each static grating was presented for 0.25 seconds, with no inter-stimulus interval. A gray screen at mean illuminance was presented randomly a total of 60 times. For drifting gratings, the stimulus consisted of 8 orientations (45° increment), 4 spatial frequency (0.02, 0.04, 0.08, and 0.16 cycle per degree) and 1 temporal frequency (2 Hz), at 80% contrast in a random sequence with up to 5 repetitions. Each drifting grating lasted for 2 seconds with an inter-stimulus interval of 2 seconds. A gray screen at mean illuminance was presented randomly for up to 15 times.

Neuron selection. We obtained recordings from 213 neurons and developed a numerical routine to exclude neurons with questionable electrophysiology or fluorescence movies, such as abrupt changes in baseline voltage or AP waveform or image artifacts such as those due to motion, photobleaching or other slow baseline changes. Neurons were accepted for analysis if they passed both electrophysiology and image quality control criteria. Electrophysiology quality control is described in the next section and imaging quality control in the 'image downsampling' section. 145 and 10 neurons were eliminated in the electrophysiology and image quality control steps, leaving 58 neurons. Of these, 10 were excluded from further analysis: red indicator had entered the soma from the pipette in 7 instances; 2 neurons segmented poorly during image analysis; and 1 had a truncated electrophysiology recording. The final data set consisted of 48 neurons.

Electrophysiology quality control. Electrophysiology traces were first baseline-subtracted to remove slow drift (3rd order Savitzky-Golay filter over 20,001 samples using Matlab sgolayfilt). Action potentials were detected as peaks of amplitude more than ten times the Quiroga threshold (QT), the median(|V(t)|/0.6745).

To develop a numerical routine, a group of human annotators identified 48 ‘high quality’ electrophysiology recordings. We then compiled a large set of descriptive statistics, listed below, and calculated the distribution of each of these statistics in the reference data set, thereby defining an acceptable range expected of high quality recordings. Each descriptive statistic was subsequently computed for recordings from all 213 neurons. Each recording was passed for further analysis if for all metrics it fell within the range spanned by the manually selected data set of 48 recordings.

For each electrophysiology recording, we calculated 35 descriptive statistics.

Metrics computed on continuous electrophysiological data:

- (1) Median relative deviation of the membrane potential (MRDM), the ratio between the median absolute deviation (MAD) and the median: $MRDM = MAD(V_m) / \text{median}(V_m)$
- (2) Mean of the Baseline (BL).
- (3) Coefficient of variation of the baseline: $\text{std}(BL) / \text{mean}(BL)$
- (4) Mean of the baseline noise, approximated by the Quiroga threshold (QT) [15]
- (5) Stability of the QT: 1000 10s-intervals were uniformly sampled from each recording, and the QT was computed on each sample. Quiroga Noise Stability (QNS) was defined as the coefficient of variation over the 1000 QT samples.
- (6) r^2 of linear regression (Matlab regression function) of the 1000 QT samples against the start times of the 10s segments on which the QT was computed.
- (7) Slope of linear regression (Matlab regression function) of the 1000 QT samples against the start times of the 10s segments on which the QT was computed.
- (8) r^2 of linear regression (Matlab regression function) of the baseline against time.
- (9) Slope of linear regression (Matlab's regression function) of the baseline against time.
- (10) The number of samples for which the baseline-subtracted trace exceeds the Quiroga threshold divided by the number of samples for which it dips below the negative of the Quiroga threshold.

Metrics computed on the AP time series. Only recordings with > 3 APs were included:

- (11) Number of APs
- (12) Maximum likelihood inter-AP interval (Matlab lognfit function).
- (13) Mean AP amplitude.
- (14) AP amplitude coefficient of variation.
- (15) AP amplitude median relative deviation.

466 (16) Relative AP amplitude range: $(\max(\text{amplitude}) - \min(\text{amplitude})) / \text{median}(\text{amplitude})$.

467 (17) AP amplitude max/min ratio: $\max(\text{amplitude}) / \min(\text{amplitude})$.

468 (18) Signal-to-noise ratio (SNR), $\text{median}(\text{amplitude}) / \text{QT}$.

469

470 Metrics computed on 2 ms-long AP waveforms, AP time ± 1 ms smoothed with Matlab smooth

471 function with sgolay option:

472 (19) 'left' width-half-max (LWHM), the mean width at half the amplitude before the detected AP

473 time.

474 (20) 'right' width-half-max (RWHM), the mean width at half the amplitude after the detected AP

475 time.

476 (21) full width at half amplitude (FWHM). $\text{FWHM} = \text{LWHM} + \text{RWHM}$.

477 (22) Coefficient of variation of LWHM.

478 (23) Coefficient of Variation of RWHM.

479 (24) Coefficient of Variation of FWHM.

480 (25) r^2 of linear regression (Matlab regression function) of AP amplitude against AP time.

481 (26) Slope of linear regression (Matlab regression function) of AP amplitude against AP time.

482 (27) r^2 of linear regression (Matlab regression function) of AP FWHM against AP time.

483 (28) Slope of linear regression (Matlab regression function) of AP FWHM against AP time.

484

485 Firing-rate based metrics. Firing rate was estimated by convolution of the AP train with a 1s-long

486 box-car window (Matlab conv function):

487 (29) Mean firing rate (FR).

488 (30) Coefficient of variation of FR.

489 (31) r^2 of linear regression (Matlab regression function) of firing rate against time.

490 (32) Slope of linear regression (Matlab regression function) of firing rate against time.

491 (33) Pearson correlation (Matlab corrcoef function) of BL vs FR.

492 (34) Pearson correlation (Matlab corrcoef function) between the baseline at AP time points and

493 AP amplitude.

494 (35) Pearson correlation (Matlab corrcoef function) between the baseline at AP times and the

495 AP FWHM.

496

497 **Neuropil subtraction, high resolution images.** To approximate somatic fluorescence ($F_{\text{cell_true}}$)

498 without neuropil contamination, a scale version of the neuropil fluorescence (F_{neuropil}) was

499 subtracted from each somatic fluorescence trace, after (Akerboom et al., (2012): $F_{\text{cell_true}}(t) =$

$F_{\text{cell_measured}}(t) - r * F_{\text{neuropil}}(t)$. We determined the optimal scale factor (r) for neurons with GCaMP6f to be 0.82 (see results). We therefore used $r = 0.8$ as our default scale factor. For some neurons, F_{neuropil} was large enough relative to $F_{\text{cell_measured}}$ that $r = 0.8$ resulted in negative fluorescence. For these neurons, we set r to 0.7, 0.6, or 0.5. For our data set of 48 GCaMP6s and GCaMP6f neurons, we set r to 0.8 for 40 neurons, to 0.7 for 4 neurons, to 0.6 for 3 neurons, and to 0.5 for 1 neuron.

Neuropil subtraction, downsampled images. neuropil subtraction was performed as described for the Allen Brain Observatory (de Vries *et al.*, 2020).

Trace analysis. Electrophysiology and calcium imaging data were analyzed using custom MATLAB and Python scripts. For electrophysiology, V_m was filtered between 250 Hz and 5 kHz, and automated AP detection was performed using a threshold criterion ($5 \times \text{std of } V_m$). For calcium imaging, in-plane motion artifacts were corrected (Dombeck *et al.*, 2007), and neuron/ROI selection was performed using a semi-automatic algorithm (Chen *et al.*, 2013) (kindly provided by Karel Svoboda, Janelia Research Campus). Ring-shaped ROIs were used to select GCaMP6-positive excitatory neurons, with GCaMP6 expression typically excluded from the nucleus and restricted to the cytoplasm.

To construct AP-calcium fluorescence response curves, we first identified all isolated AP events. For GCaMP6s, isolated events were separated from previous and subsequent events by ≥ 1000 ms and ≥ 500 ms, respectively. For GCaMP6f, isolated events were separated from previous and subsequent events by ≥ 300 ms. One result of finding isolated events is that only a minority of APs were used to construct AP-calcium fluorescence response curves. Within each event, APs were summed over 250 ms. Fluorescence traces were aligned to the first AP in each event, with $t = 0$ preceding the AP by < 1 frame (6.3 ms at 158 Hz). For each event, $\Delta F/F = (F_{0,\text{local}} - F_{0,\text{global}})/F_{0,\text{global}}$, where $F_{0,\text{local}}$ was the mean fluorescence over 100 ms before the first AP, and $F_{0,\text{global}}$ was the minimum $F_{0,\text{local}}$ across trials. For GCaMP6s and GCaMP6f, peak $\Delta F/F$ was calculated by first finding t_{max} , the time of the maximum $\Delta F/F \leq 500$ ms and 300 ms after the first AP, respectively. Peak $\Delta F/F$ was the mean $\Delta F/F$ from $t_{\text{max}} - 50$ ms to $t_{\text{max}} + 50$ ms. Bursts of > 5 APs were excluded from analysis due to the low frequency of such events.

Fluorescence-to-photon conversion. Mean and variance of the fluorescence, calculated pixelwise for each image, were linearly related, consistent with shot noise-limited imaging.

The resulting slope and offset of the least squares fit were used to convert fluorescence to number of photons: $\text{photons} = (F - (-\text{offset}/\text{slope})) / \text{slope}$ (http://github.com/AllenInstitute/QC_2P). To account for different pixel dwell times along the resonant scanning axis, photon gain and offset were computed pixel-by-pixel along the resonant axis.

Trial-to-trial variability. For each neuron, fluorescence was summed over all somatic pixels and converted to photons. For each 1AP event, mean photon count 0.1-0 s before the AP was subtracted. t_{max} , the time of the maximum photon count, was calculated from the mean 1AP trace. Photon count in each trial was determined at t_{max} , and the 95% confidence interval was calculated as $\text{mean (across trials)} \pm 1.96 * \sqrt{\text{mean peak}}$. The percentage of trials with peak fluorescence outside the 95% confidence interval was used as a measure of trial-to-trial variability.

Ground truth-optimized event detection. We compared fluorescence traces of the response (1AP or 2AP events) to that of 0AP events (Chen et al., 2013). For each recording, the mean response trace was used as the template vector. The template vector was normalized after subtracting the mean to create the unit vector, and the scalar results of projecting the response and noise traces on the unit vector were computed: r_i and n_i for response and noise scalars, respectively. The detection threshold was defined as the x^{th} percentile of n_i values, where $1-x$ represented the false positive probability (e.g., $x = 95$ for 95th percentile or 5% false positive probability), and the detection probability (true positive probability) was the fraction of r_i values above the detection threshold.

Image downsampling. Fluorescence movies were sub-sampled by a factor of 4 in space, and 5 in time (to pixel size 0.80 μm and frame rate 30.3 Hz) to match the sampling rate and approximate number of pixels per soma of the Allen Brain Observatory (de Vries et al., 2020). To assess the effect of downsampling on subsequent processing, 20 different approaches were tried in parallel (downsampling starting with the 1st, 2nd, ... 4th pixel x 1st, 2nd, ... 5th frame, respectively). 4 x 5 internally identical blocks, one block for each downsampling strategy, were tiled for a total of 400 almost identical regions of interest (ROI) per recording. Segmentation to find somatic regions of interest, demixing of traces from nearby somata, neuropil subtraction and the calculation of $\Delta F/F$ were performed as described for the Allen Brain Observatory (de Vries et al., 2020).

In cases where only the neuron of interest was found during segmentation, trace extraction would yield a family of 400 self-similar traces. To catch cases where segmentation yielded additional objects that were not part of the neuron of interest, additional QC steps were required. The traces were first clustered using DBSCAN (Ester et al., 1996; Schubert et al., 2017), and each cluster median was compared against white noise of the same mean and standard deviation (KS-test) and rejected as artifact if it was not significantly different ($p < 0.05$). In cases where multiple clusters were significantly different from noise, this was either due to multiple neurons being present in the field-of-view, or due to residual motion artifacts resulting in multiple translated copies of the same neuron. To disambiguate these two possibilities, the top three clusters were merged: sums were computed for all six possible combinations (sampled without replacement) of (up to) three most distinct cluster medians, and the combination most significantly correlated with the measured electrophysiological AP train was selected. Correlation significance was determined by building a null distribution of correlations between the cluster medians and 1000 random Poisson trains with a rate matching that of the recorded AP train. If there was no more significant correlation between any cluster median (or sum thereof) and the measured AP train than the 0.5-th percentile of the null distribution (i.e. $p > 0.005$), the recording was failed. Finally, we eliminated from further analysis <10 neurons with an abrupt and sustained (seconds) rise in spike rate and subsequent loss spiking activity out of concern that this activity pattern might indicate a breached plasma membrane.

To compare the noise characteristics of the downsampled images to the Allen Brain Observatory, we computed the robust standard deviation (rSTD), a median-based method with outlier-removal (de Vries et al., 2020). For the Allen Brain Observatory, we analyzed fluorescence over periods in which there were no apparent AP-evoked changes in fluorescence.

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COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

The dataset is available at <https://portal.brain-map.org/explore/circuits/oephys>.

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Table 1. Dataset. Sample size for each mouse line (see also Figure 1 – figure supplement 1).

Mouse line	Acronym	GECI	Mice	Cells	Recording duration	APs per neuron
Emx1-IRES-Cre; Camk2a-tTA;Ai94	Emx1-s	GCaMP6s	5	21	241 ± 32 s	478 ± 121
Camk2a-tTA; tetO-GCaMP6s	tetO-s	GCaMP6s	1	4	347 ± 108 s	348 ± 71
Cux2-CreERT2; Camk2a-tTA;Ai93	Cux2-f	GCaMP6f	3	12	300 ± 79 s	484 ± 112
Emx1-IRES-Cre; Camk2a-tTA;Ai93	Emx1-f	GCaMP6f	4	11	201 ± 23 s	219 ± 38

FIGURE LEGENDS

Figure 1. Simultaneous calcium imaging and electrophysiology *in vivo*.

(A) Experimental design. (B) Fluorescence and Vm traces from an exemplar Emx1-s neuron. (C) 5 seconds of data from the neuron in panel B, showing a 2AP, a 1 AP and a 3 AP event. Pre- and post-AP exclusion windows, used to separate events, are illustrated for each event.

Figure 1 – figure supplement 1. Mouse age, cell depth, and firing rate.

(A) Mouse age. (B) Cell depths. (C) For each neuron, percentage of APs contained within 1-5 AP events.

Figure 2. Neuropil subtraction optimized for 1AP detection.

(A) Effect of changing neuropil subtraction on detection for exemplar GCaMP6s and GCaMP6f neurons. Upper plots: family of ROC curves. Each curve illustrates detection probability for true APs against probability of false positives as detection threshold is changed, for 1 AP events. False positives were calculated from time windows with no APs. Each ROC curve represents a different value of r . Lower plots: area under the ROC curve as a function of r . Grey symbols represent value of r for which $r * F_{\text{neuropil}}(t)$ was greater than $F_{\text{cell_measured}}(t)$, resulting in a negative F_0 and inversion of the $\Delta F/F$ trace. (B) Distribution of r values for 20 GCaMP6f neurons.

Figure 2 – figure supplement 1. Simulated effect of neuropil subtraction on event

detection. (A) Simulated cell and neuropil traces. The neuropil trace contained transients that were (1) associated with cell transients and (2) between cell transients, and amplitudes were scaled by the neuropil contamination ratio r ($r = 0.3$) relative to the cell amplitudes. (B) (Left) ROC curves for classifying cell amplitudes, where r was varied from 0 to 1 for neuropil correction. The detection threshold was defined as the x^{th} percentile of noise amplitudes (amplitudes of the neuropil trace that were between cell transients), where $1-x$ represented the false positive probability, and the detection probability (true positive probability) was the fraction of estimated cell amplitudes (amplitudes of the summed trace that were associated with cell transients) above the detection threshold. (Right) Area under ROC curves as a function of r .

Figure 3. AP-evoked calcium transients in 4 mouse lines.

(A) Example fluorescence traces ($\Delta F/F$, with $r=0.82$ neuropil subtraction) for 1, 2 and 3 AP events for an exemplar Emx1-s neuron (42 1AP events, 38 2AP events, 16 3 AP events) and an exemplar Emx1-f neuron (23 1AP events, 12 2AP events, 11 3AP events). (B) Mean

fluorescence traces and fits (sum of two exponentials) for 1-5 AP events for the two neurons in A. (C) Mean $\pm$ sem peak $\Delta F/F$, rise time constant, and decay time constant for four mouse lines. Number of neurons for 1-5 AP events were: 15, 16, 14, 9, 6 for Emx1-s; 4, 4, 4, 0, 0 for tetO-s; 10, 10, 10, 5, 4 for Emx1-f; 9, 9, 7, 4, 2 for Cux2-f. Asterisks indicate differences between mouse lines ($p < 0.05$, one-way ANOVA).

Figure 3 - figure supplement 1. Trial-to-trial variability of 1AP events

Fraction of trials in which the peak of the fluorescence transient exceeded the range expected from Poisson noise, a measure of trial-to-trial variability. (A) Example 1AP event for an Emx1-s neuron, with fluorescence in units of photons. Dashed line: mean, across trials, of peak fluorescence. Grey: 95% confidence interval for Poisson-distributed noise. (B) Amplitudes of 1AP events for 30 trials. Dashed line and grey area: mean and 95% confidence interval. (C) Mean $\pm$ SEM percentage of trials with peak ΔF outside the 95% confidence interval, for each mouse line. In all four mouse lines, peak fluorescence exceeded the 95% confidence interval for Poisson-distributed noise in $>>5\%$ of 1AP trials. The fraction of trials outside the confidence interval correlated with resting fluorescence, likely the result of different illumination intensities across experiments.

Figure 4. Downsampling mimics large field-of-view images

(A) Original, high spatial and temporal resolution GCaMP6f image of an exemplar Cux2-f neuron. (B) 1, 2 and 3AP traces for the same neuron. Thin lines, individual trials; thick line, mean. Dashed line, $\Delta F/F = 0$. (C) Downsampled image of the same neuron. (D) Traces from the downsampled neuron. (E) Normalized distribution of baseline noise (rSTD) for 48 downsampled neurons (red) and 11,816 layer 2/3 neurons from Emx1-f and Cux2-f mice in the Allen Brain Observatory (blue).

Figure 4 – figure supplement 1. Example fluorescence traces from image quality control procedures, implemented during image downsampling.

(A) Median of traces extracted from an example segmentation in which only the neuron of interest was found in each image during segmentation. The trace is significantly different from noise (KS test, $p = 1.8 \times 10^{-40}$). (B) Median of traces extracted from an example segmentation in which only the neuron of interest was found in each image during segmentation. The trace is not significantly different from noise (KS test, $p = 0.072$). (C) Medians of traces extracted from an example segmentation in which three regions of interest were identified. Each trace is

significantly different from noise (KS tests, $p = 7.7 \times 10^{-152}$, 1.6×10^{-19} , 2.2×10^{-25}). The sum of these three cluster medians had the highest correlation with the firing rate and was therefore used as the $\Delta F/F$ trace for this recording in the dataset. Scale bars apply to A-C. (D) Example fluorescence trace with an abrupt and sustained rise in fluorescence and in spike rate and subsequent loss of spiking activity. This neuron was manually removed from the dataset.

Figure 5. Downsampling affects event detection.

(A-C) Event detection from images at high spatial and temporal resolution. (A) ROC curves for 1 AP events in 42 neurons, organized by mouse line. Neuropil subtraction was performed with $r = 0.8$ where possible (see Methods). Numbers of neurons were 18 Emx1-s, 3 tetO-s, 11 Cux2-f, 9 Emx1-f. (B) Mean ROC curves for the four mouse lines. (C) Event detection probabilities for 1 and 2 AP events. Bars represent mean $\pm$ sem. (D-F) Equivalent plots for the same neurons after downsampling.

Figure 6. Performance of spike inference algorithms on high resolution and

downsampled images. (A) Results from an exemplar Cux2-f neuron, at high resolution. Fluorescence and AP rate from electrophysiology (black). Below, APs per image frame estimated with 3 spike inference algorithms: MLspike (blue), Exact L0 (purple), and non-negative deconvolution (NND, orange). (B) Pearson correlation coefficient (r) and Matthews correlation coefficient (MCC) for the three algorithms for each mouse line. 300 ms bins. (C) ROC curves, reporting probabilities of detecting true and false events in each time bin. Thin lines: individual neurons. Thick lines: mean across neurons. 300 ms bins. (D-F) Equivalent plots for downsampled images.

Figure 6 - figure supplement 1. Effect of bin duration on measures of spike inference

algorithm performance. (A) ROC curves for MLspike (blue), exact L0 (purple), and NND (orange) algorithms after binning of spike probabilities into 33, 100, 200, 300 and 500 ms bins, for downsampled images resampled to 150 Hz. (B and C) Pearson correlation coefficients (r) and Matthews correlation coefficients (MCC) for each bin duration.

Figure 7. Performance of blind spike inference algorithms for 1-5 AP events.

(A) Mean $\pm$ SEM detection probabilities at 1% false positive probability for high resolution images. (B) Mean $\pm$ SEM detection probabilities at 1% false positive probability for downsampled images. (C) Frequency of 0-5 AP events for 250 ms bins for each mouse line.

806

807 **Figure 7 - figure supplement 1. Upsampling enhances performance of unconstrained**
808 **non-negative deconvolution.** Performance of the NND algorithm when applied to fluorescence
809 traces from downsampled images (frame rate 30 Hz) and to the same fluorescence traces after
810 upsampling to 150 Hz. 100 ms bins. (A) Pearson r and Matthews correlation coefficient (MCC).
811 (B) ROC curves.

812

813 **Figure 7 - figure supplement 2. Autocalibration enhanced performance of MLspike.**
814 Performance of MLspike with and without use of the autocalibration procedure, applied to
815 downsampled images, with fluorescence traces upsampled to a frame rate of 158 Hz. 100 ms
816 bins. (A) Pearson r and Matthews correlation coefficient (MCC). (B) ROC curves.

817

818 **Figure 7 - figure supplement 3. Comparison of spike inference with blind and ground**
819 **truth AP-optimized algorithms.** (A) Mean $\pm$ SEM 1AP event detection probability at 1% false
820 positive probability for ground-truth optimized and blind algorithms (used in figure 5 and in
821 figures 6 and 7, respectively). Traces were selected as for Figure 5. (B) 1AP events detected by
822 each blind algorithm as a fraction of those detected by the ground truth-optimized algorithm, at
823 1% false positive probability.

figure 1

figure 1 supplement 1

figure 2

figure 2, figure supplement 1

figure 3

figure 3, figure supplement 1

figure 4

figure 4 - figure supplement 1

figure 5

figure 6

figure 6 supplement 1

figure 7

figure 7, figure supplement 1

figure 7, figure supplement 2

figure 7 - figure supplement 3