1

1	High-Speed Low-Light In Vivo Two-Photon Voltage Imaging of Large Neuronal Populations
2	
3	Jelena Platisa ^{1,2,3,9} , Xin Ye ^{4,5,9} , Allison M. Ahrens ⁶ , Chang Liu ⁷ , Ichun Anderson Chen ⁵ , Ian G.
4	Davison ^{5,6,8} , Lei Tian ^{4,5,7} , Vincent A. Pieribone ^{1,2, 3*} , Jerry L. Chen ^{4,5,6,8*}
5	
6	¹ Department of Cellular and Molecular Physiology, Yale University, New Haven CT 06510
7	² Department of Neuroscience, Yale University, New Haven CT 06510
8	³ The John B. Pierce Laboratory, New Haven CT 06519
9	⁴ Department of Biomedical Engineering, Boston University, Boston MA 02215
10	⁵ Center for Neurophotonics, Boston University, Boston MA 02215
11	⁶ Department of Biology, Boston University, Boston MA 02215
12	⁷ Department of Electrical and Computer Engineering, Boston University, Boston MA 02215
13	⁸ Center for Systems Neuroscience, Boston University, Boston MA 02215
14	⁹ These authors contributed equally to this work.
15 16 17	*Correspondence: vpieribo@jbpierce.org (V.A.P.), jerry@chen-lab.org (J.L.C.)
18 19 20	SUMMARY
21	Monitoring spiking activity across large neuronal populations at behaviorally relevant timescales is critical
22	for understanding neural circuit function. Unlike calcium imaging, voltage imaging requires kilohertz
23 24	sampling rates which reduces fluorescence detection to near shot noise levels. High-photon flux excitation can overcome photon-limited shot noise but photo-bleaching and photo-damage restricts the number and
24 25	duration of simultaneously imaged neurons. We investigated an alternative approach aimed at low two-
26	photon flux, voltage imaging below the shot noise limit. This framework involved developing: a positive-
27	going voltage indigator with improved spike detection (SpikeyGi); an ultra-fast two-photon microscope for

kilohertz frame-rate imaging across a 0.4x0.4mm² field of view, and; a self-supervised denoising algorithm

(DeepVID) for inferring fluorescence from shot-noise limited signals. Through these combined advances,

we achieved simultaneous high-speed, deep-tissue imaging of more than one hundred densely-labeled

neurons over one hour in awake behaving mice. This demonstrates a scalable approach for voltage imaging

28 29

30

31

32

across increasing neuronal populations.

2

37 **INTRODUCTION**

To understand the nervous system, experiments are needed in which activity from large numbers of neurons 38 39 can be measured in a detailed and comprehensive manner across multiple timescales during behavior. 40 Current approaches to image neuronal activity at cellular resolution occupy two ends of a spectrum; recording large populations of neurons at very slow sampling rates (i.e. calcium imaging) or very high 41 speed voltage imaging of small numbers of cells. However, calcium imaging is a poor proxy for action 42 43 potential activity (Huang et al., 2021). To measure spiking accurately using voltage indicators, sampling 44 must be at least an order of magnitude faster (~400 Hz) (Sjulson and Miesenbock, 2007; Wilt et al., 2013) 45 than calcium imaging (<15 Hz). To date, ultra-fast voltage imaging (1kHz) has only been achieved, simultaneously, in a small number (~10) of neurons (Abdelfattah et al., 2019; Piatkevich et al., 2019; 46 47 Villette et al., 2019; Wu et al., 2020). The goal of the present studies are to achieve high speed recording 48 of a large number of neurons in vivo.

49 To optically resolve individual action potential in numerous neurons in vivo faces two critical 50 limitations. The first limit is determined by photon shot noise. Under the assumption of adequate indicator sensitivity, a sufficient number of genetically encoded voltage indicator (GEVI) molecules need to be 51 52 excited for changes in fluorescence associated with action potential firing to be reliably detected above 53 photon shot noise statistics. To achieve adequate signal-to-noise, current approaches for one-photon (1P) 54 and two-photon (2P) voltage imaging have relied on "high photon flux" regimes in which excitation light 55 is concentrated on a small population of tens of neurons (Adam et al., 2019; Villette et al., 2019). One-56 photon cellular resolution voltage imaging is achievable using widefield illumination and high frame-rate 57 cameras but has limited depth penetration (Abdelfattah et al., 2019; Piatkevich et al., 2019). Due to light 58 scattering, only neuronal populations at shallow depths or those sparsely labeled can be imaged with 59 minimal signal degradation. Alternatively, 2P microscopy enables deeper imaging of densely labeled tissue at kilohertz acquisition rates but has been limited to a small field of views (FOVs) due to existing excitation 60 61 and scanning strategies (Villette et al., 2019; Wu et al., 2020; Zhang et al., 2019).

62 High photon flux is partially driven by the characteristics of currently used 2P-compatible GEVIs 63 that fluoresce at resting membrane potential and decrease their fluorescence during action potentials 64 (negative slope fluorescence-voltage relationship, i.e., "negative going" indicator) (Chamberland et al., 2017; Jin et al., 2012; Villette et al., 2019). This means that, in addition to potentially mislocalized proteins. 65 GEVI molecules at resting state also contribute to background fluorescence and light scatter (Abdelfattah 66 67 et al., 2020; Piatkevich et al., 2019), further reducing detectable spike-related changes in fluorescence. Under both 1P and 2P imaging conditions, high photon flux prevents sustained voltage imaging due to rapid 68 photobleaching which limits recording times to short durations (i.e., several minutes) (Abdelfattah et al., 69 70 2020; Piatkevich et al., 2019; Villette et al., 2019). Further, high photon flux excitation cannot be scaled for imaging larger neuronal populations. This is due to a second fundamental limit related to the total 71 72 amount of excitation power that can be delivered into the brain without introducing photodamage 73 (Podgorski and Ranganathan, 2016). Thus, when imaging at high speeds, the overall photon budget forces 74 a trade-off in which the amount of excitation light available to each neuron decreases as the number of 75 imaged neurons increases.

76 To achieve ultra-fast voltage imaging across large neuronal populations, these fundamental limits 77 need to be overcome. We adopted a multidisciplinary approach to address this, integrating protein 78 engineering, optical engineering, and deep learning. We developed a high-speed, positive-going 2P GEVI, 79 a kilohertz-scanning, large-FOV 2P microscope, and a deep convolutional neuronal network for image 80 denoising. Through this synergistic combination of technologies, we provide a new framework for population-level 2P voltage imaging in the awake behaving animal. 81

83 RESULTS

82

Development of positive-going two-photon compatible genetically encoded voltage indicator 84

85 We recently showed that we can manipulate the direction of the voltage-dependent fluorescence response

- of GEVIs by modifying amino acid residues determining the chromophore protonation state (Platisa et al., 86 87
- 2017) (Figure 1A). By mutating amino acid residues, D147A and H148A, within the green fluorescent

3

protein (GFP) of the "negative-going" indicator ArcLight (Jin et al., 2012), we produced GEVI Marina (Platisa et al., 2017). This "positive-going" indicator is more advantageous for *in vivo* application due to lower background and improved photostability. However, despite optimal voltage sensitivity and optical properties, spike detection with Marina is limited by its suboptimal kinetics (tau ~10 ms). Therefore, to develop a high-speed, positive-going voltage indicator, we devised a directed evolution strategy targeting the fastest available, negative-going, 2P compatible GEVIs, ASAP2f and ASAP3 (Chamberland et al., 2017; Villette et al., 2019).

95 We began by creating site-directed mutagenic libraries of amino acid residues within the ASAP2f 96 fluorescent protein (S149, and H150; from the starting Met of ASAP2f) that are homologous to those in 97 ArcLight/Marina (D147 and H148; numbering from starting Met of SuperEcliptic pHluroin GFP). Additional libraries targeted four residues at the linker region between the voltage-sensitive and FP domains 98 99 (L145, S146, F147, and N148). Functional screening showed that most variants produced signals that were 100 either smaller or similar to the parent constructs. However, as seen with Arclight and Marina, mutation of H150 residue produced several variants with reversed signal polarity, i.e., a modest increase in fluorescence 101 upon depolarization (data not shown). We then used primers with the degenerative codons NNKNNK to 102 create an insertional library targeted between residues H151 and N152 in ASAP3 (Figure 1B). To ensure 103 that all 400 potential variants were screened, we tested 1104 mutants produced in two separate PCR 104 105 reactions. Out of all mutants screened, a ~ 150 positive-going variants were detected (Figure 1C). In one mutant, a large $\Delta F/F_0$ of +30% was observed using field stimulation (Figure 1C-D). Subsequently, 106 107 simultaneous high-speed 1P imaging (500-1000Hz) and whole-cell patch-clamp electrophysiology of HEK cells transiently expressing this mutant showed an average $+18.7 \pm 1.1\% \Delta F/F_0$ response to 100 mV step 108 109 depolarization (n = 8 cells) (Figure 1E-F). Sequence analysis of this indicator revealed the insertion of 110 amino acids DS; this indicator was named SpikeyGi.

Along with our efforts to generate positive-going 2P GEVIs, we developed additional negative-111 going 2P GEVIs with improved signal characteristics. We created site-directed libraries targeted to the 26 112 amino acid residues between the circularly permuted sfGFP and S4 sequences of the voltage-sensitive 113 114 domain of ASAP3 (residues 370-395 in ASAP3 from starting Met, Figure 1A-B). Two subsequent mutagenesis and functional screening rounds (at positions 394 and 395) led us to the double mutant M394W 115 P395V that shows sensitivity optimized to detect depolarizations (Figure 1E and F). Compared to parent 116 117 GEVI ASAP3, this novel variant, named SpikeyG, showed increased sensitivity to transient 100mV step depolarizations ($\Delta F/F_0$: SpikeyG, -55.7 ± 1.9%, n = 6 cells; ASAP3, -38.1 ± 1.6%, n = 8 cells, Student's t-118 test; p < 0.00002; Figure 1D). At the same time, sensitivity to transient -50mV step hyperpolarization (-119 120 110mV voltage step from -70mV holding potential) decreased for SpikeyG ($\Delta F/F_0$: SpikeyG, 24.6 ± 2.1%, 121 n = 5 cells; ASAP3, 51.4 ± 4.2%, n = 8 cells. Student's *t*-test; p < 0.0002; Figure 1E). Overall, our directed 122 mutagenesis of fast two-photon-compatible GEVIs produced two improved candidates with the potential 123 for increased sensitivity for spike detection.

124

125 In vitro characterization of SpikeyG and SpikeyGi with high photon flux excitation

To determine the suitability of SpikeyG or SpikeyGi for action potential detection in neurons, we first 126 127 characterized responses of SpikeyG and SpikeyGi in vitro with simultaneous whole-cell patch clamp electrophysiology and two-photon imaging. We targeted layer 2/3 neurons in brain slices from animals 128 129 virally expressing either SpikeyG or SpikeyGi. Fluorescence responses across different membrane 130 potentials were measured under voltage-clamp mode. We imaged single neurons under high photon flux 131 conditions using a conventional two-photon microscope with an 80 MHz repetition rate laser source, similar 132 to previous studies (Bando et al., 2019; Chamberland et al., 2017). Using frame scanning (128x85 pixels, $0.3-0.6\mu$ m/pixel. 2.8µs dwell time, 35-45mW average power), the targeted cell received ~5x10⁵ pulses per 133 50ms time bin (Figure 2A). SpikeyG showed linear changes in response to voltage steps, with negative 134 135 steps producing an increase in fluorescence and positive steps producing a decrease in fluorescence. SpikeyGi responded in the opposite direction with positive steps increasing fluorescence (Figure 2B-C). 136 137 At +50mV (-120mV voltage step from -70mV holding potential), SpikeyGi showed larger magnitude fluorescence changes than SpikeyG ($\Delta F/F_0$: SpikeyGi, 29.4 ± 4.7%; SpikeyG, -14.6 ± 5.1%). We next 138

4

characterized single action potential responses of SpikeyG and SpikeyGi triggered at 100-ms intervals (average of 10 traces). Line scans at 3-5 kHz were performed along the cell membrane (24-54 pixels, 2.8 µs dwell time), equivalent to ~9000 excitation pulses per 1ms time bin. Both GEVIs showed clear responses to action potentials with similar kinetics. SpikeyGi exhibited greater magnitude peak responses compared to SpikeyG ($\Delta F/F_0$: SpikeyGi, 32.6 ± 0.9%; SpikeyG, -21.2 ± 0.4%; Student's *t*-test, *p* < .001) (**Figure 2D**). These results demonstrate that both SpikeyG and SpikeyGi are capable of reporting single APs under high photon flux conditions.

146

147 Ultra-fast two-photon microscope design and performance

To perform two-photon voltage imaging across a large population of neurons, we sought to design an ultra-148 fast two-photon microscope capable of imaging a 400x400µm² field-of-view (FOV) at a kilohertz frame 149 rate. While existing ultra-fast two-photon microscopes operate in a high-photon photon flux regime, we 150 151 set out to construct a system optimized for "low-photon flux" excitation (Figure 3A). Two-photon 152 excitation is achieved through pulsed lasers. Hence, the pulse repetition rate determines the total FOV that can be excited within a one-millisecond time bin. A minimum of one pulse is needed per imaging voxel to 153 154 cover the entire FOV. To increase the FOV size while maintaining full coverage, the effective repetition 155 rate of the imaging system needs to increase proportionally. This can be achieved through either temporal 156 or spatial multiplexing. Temporal multiplexing creates multiple excitation beamlets that are delayed in 157 time such that the resulting fluorescence detected by a single photomultiplier tube (PMT) can be disambiguated by their timing (Amir et al., 2007; Chen et al., 2016; Cheng et al., 2011; Clough et al., 2021). 158 However, the degree of temporal multiplexing is limited by the fluorescence lifetime of the excited 159 160 fluorophore which places an upper limit on the effective repetition rate using this approach. In contrast, there is no limit to the effective repetition rate formed by spatial multiplexed beamlets in which multiple 161 pulses are delivered into different regions of the tissue simultaneously (Kim et al., 2007; Zhang et al., 2019). 162 Resolution of spatially multiplexed beamlets does require spatial detection using cameras or multi-anode 163 164 PMTs (MAPMTs). Consequently, spatial multiplexing is depth limited as crosstalk between neighboring detectors increases with depth due to scattered fluorescence. This spatial crosstalk can be reduced by 165 increasing the spacing of the beamlets at the sample. 166

To maximize the effective repetition rate used in the UF2P microscope, spatial and temporal 167 168 multiplexing were combined in the same system (Figure 3B, S1). For the excitation source, we selected a 920nm 31.25MHz fiber laser which enables beamlets to be temporally multiplexed four times at 8-ns 169 pulse intervals, sufficient to resolve GFP-based indicators with minimal cross talk. Compared to 80 MHz 170 171 Ti:sapphire lasers traditionally used for two-photon imaging, the lower repetition rate also provides >2.5x greater pulse energy at the same average power, providing more efficient excitation per laser pulse (Charan 172 et al., 2018). Each temporally multiplexed beamlet was then split into a pair of spatially multiplexed beams 173 174 positioned 200µm apart at the sample to minimize scatter-related crosstalk. This generated a total of 8 175 beamlets (4 temporal X 2 spatial). The result is an illumination source with an effective 250 MHz repetition 176 rate, which is >3x higher than traditional systems scanning a single beam with 80MHz repetition rate. For 177 raster scanning, we used a resonant mirror with 24kHz line rates for x-scanning and a galvanometric mirror for y-scanning. Since fast scanning is achieved along the x-axis, the beamlets were linearly arranged along 178 179 the slower y-axis. The four temporally multiplexed beamlet pairs were spaced 50µm apart at the sample (Figure 3C). Thus, each beamlet scanned a 400x50 μ m sub-area, when tiled together, resulting in a total 180 FOV of $400x400\mu m^2$. Each sub-area was slightly offset in the x-axis as a result of the patterning of the 181 beamlets with respect to the position of the resonance scanner. By scanning 24 lines per subarea either, 182 unidirectionally or bidirectionally, we achieved 803 or 1000 Hz frame rates, respectively. A linearly 183 184 arranged MAPMT and matching detection optics were designed to project the imaging plane onto the detectors such that each anode collected fluorescence from a corresponding spatially multiplexed beamlet 185 186 pair. The fluorescence collected from the MAPMT was subsequently demultiplexed to resolve the 187 temporally multiplexed beamlets while minimizing crosstalk fluorescence in neighboring anodes.

We first evaluated the optical performance of the microscope. To check if each beamlet providessimilar excitation and resolution in each sub-area, the point spread function (PSF) of each beamlet was

190 individually measured using fluorescent beads (n=7-11 beads per beam, Figure 3D, S2). The optical performance was similar for each sub-area. Across all sub-areas, the microscope achieved an average PSF 191 192 of $0.9\pm0.1\mu$ m(X)/1.1±0.1 μ m(Y) lateral resolution and $4.1\pm0.4\mu$ m axial resolution, demonstrating sub-193 cellular resolution performance. To determine the degree of temporal and spatial cross talk across all 8 194 beamlets in scattering tissue, a cranial window was implanted in a mouse with virally expressed SpikevGi in the primary somatosensory cortex (S1). SpikeyGi fluorescence was measured as a function of cortical 195 196 depth for each beam across all detected sub-areas (Figure 3E). We observed that crosstalk caused by spatial 197 multiplexing increased as a function of imaging depth but remained under 10% as far as 300µm below the 198 pial surface. Crosstalk as a result of temporal multiplexing remained under 5%, independent of imaging 199 depth. Overall, this demonstrates that the UF2P microscope can achieve large FOV kilohertz frame scan 200 imaging into deep tissue.

201

202 Self-supervised denoising improves action potential detection below photon shot noise limits

While increasing the effective pulse rate with the UF2P microscope enables imaging FOVs to be increased 203 while maintaining high frame rate, photon flux is still magnitudes lower then high photon flux regimes. 204 Assuming a photon flux of ~0.1 μ s dwell time per μ m² voxel, each neuron only receives ~200 excitation 205 pulses per 1ms time bin. Under such imaging conditions, shot noise dominates pixel-wise measurements. 206 207 Recently, self-supervised deep learning denoising algorithms have been developed to remove independent 208 noise sources in calcium imaging data without any ground-truth "clean" (high SNR) measurements (Lecoq 209 et al., 2021; Li et al., 2021). We expanded upon this approach, developing a deep convolutional neural network (CNN) to denoise voltage imaging data (DeepVID) (Figure 4A). DeepVID combines self-210 supervised denoising frameworks that infers the underlying fluorescence signal based on a learned model 211 212 of the independent temporal and spatial statistics of the PMT measurements that is attributable to shot noise 213 (Krull et al., 2018; Lecoq et al., 2021). The CNN was trained to estimate a single center frame (N_{θ}) based 214 on information from prior (N_{pre}) and subsequent (N_{post}) neighboring frames within a time series. Simultaneously, the CNN was trained to estimate a few "blind" pixels (p_{blind} , in %) within the central frame 215 based on information from all remaining pixels within that frame. Based on the rise time of SpikeyG and 216 217 SpikeGi and imaging frame rate of the UF2P microscope, a model with $N_{pre}=3$ and $N_{post}=3$ was chosen to maximize inference accuracy while preserving the fast action potential kinetics of the indicators. We first 218 219 assessed the frame-to-frame variability in fluorescence signal in the raw data and confirmed that the 220 fluctuations in each pixel is proportional to the square root of the mean fluorescence (Figure 4B), as expected for shot noise limited signals. DeepVID drastically reduced the frame-to-frame variability, 221 222 resulting in a 15-fold improvement in SNR when comparing denoised and raw image data (SNR: $0.567 \pm$ 223 0.002, raw; 8.858 \pm 0.027, denoised, n = 8,000 pixels) (Figure 4C). By breaking this fundamental noise constraint, the underlying fluorescence signal can be more accurately inferred at individual time points 224 225 (Figure 4D).

226 To assess how DeepVID improves the reliability of spike detection in voltage imaging data, we measured neuronal responses using SpikeyGi in S1 during whisker stimulation. Whisker deflections using 227 air puffs produce well-timed single AP responses in L2/3 S1 neurons (Feldmeyer et al., 2012). Whisker 228 stimulation was delivered in the form of single or trains of 5 air puffs to the contralateral whisker pad. We 229 compared raw and denoised fluorescence traces. The reduction in shot noise fluctuations in denoised traces 230 231 readily allowed for the identification of potential sensory-evoked and non-evoked spiking events (Figure 4E). Fluorescence traces were converted into timeseries of SNR levels used for spike detection (Figure 232 233 4F). Denoising appeared to improve the SNR of putative spike events. Analysis of the fluorescence response (spiking and non-spiking) to single air puffs in denoised vs. raw traces show that peak responses 234 were increased in denoised traces, owing to better estimates of the baseline fluorescence levels ($\Delta F/F_0$ raw: 235 236 $9.3 \pm 1.0\%$; denoised, $11.1 \pm 0.2\%$; Student's t-test, p < 0.001) (Figure 4G-H). We compared the percent 237 of putative sensory-evoked spikes detected at varying SNR thresholds (Figure 4I). At low SNR levels (< 3), the percent of detected spikes was highly overestimated in raw traces whereas the likelihood of false 238 positives was greatly reduced in denoised traces. In contrast, denoising improved the detection of spikes at 239 high SNR thresholds (> 3) compared to raw traces. Overall, these results demonstrate that reduction in shot 240

6

noise provided by DeepVID significantly improves the reliability for spike detection in 2P voltage imaging
 data.

243

244 Positive-going GEVIs outperforms negative-going GEVIs *in vivo*

Using DeepVID, we next compared responses of SpikevG and SpikevGi in vivo under low photon flux 245 conditions. Imaging across the full 400 x 400 µm² FOV, cells were imaged at similar laser power compared 246 247 to *in vitro* conditions (~30 mW per beamlet) but at lower resolution (x: $1.0 \mu m/pixel$, y: $2.1 \mu m/pixel$). We 248 first compared the fluorescent responses to single air puffs (Figure 5A). SpikeyGi showed larger peak 249 responses compared to SpikeyG ($\Delta F/F_0$: SpikeyGi, 11.1 ± 0.2%; SpikeyG, 5.6 ± 0.1%; Student's t-test, p < 0.001). For SpikeyGi, the detection of APs across different SNR thresholds were consistently higher than 250 SpikeyG repeated measures ANOVA, group interaction: F = 407.41, p < 0.001) (Figure 5B). We further 251 252 evaluated the ability to perform population imaging with SpikeyGi using the UF2P microscope. We 253 simultaneously imaged 129 SpikeyGi neurons across the FOV and could identify sensory-evoked APs (SNR > 4) across neurons to both single and trains of air puffs (Figure 5C-D, S3). We evaluated the 254 temporal fidelity of SpikeyGi responses to 5-10 Hz trains of the air puff. Since spiking probability to 255 256 whisker stimulation can be variable from cell-to-cell in S1 neurons, we generated average traces to detected APs for each air puff in the stimulus train (Figure 5E). At both 5 and 10 Hz stimulation frequencies, the 257 258 averaged traces show well isolated spike responses to each air puff within the stimulus train. These results 259 demonstrate that SpikeyGi in combination with the UF2P microscope is suitable for population-level 260 voltage imaging.

261

262 Low photon flux enables sustained population-level voltage imaging.

263 We finally assessed the capacity to perform sustained *in vivo* voltage imaging at low photon flux using the UF2P microscope. We first tested photobleaching seen SpikeyGi and SpikeyG under in vitro high photon 264 flux conditions. This was performed in a 512 x 512-pixel field of view at 1 Hz with laser power of 35mW. 265 266 Intermittent imaging (10s on, 5s off) was performed across 12.5 minutes. Fluorescence was normalized to the first frame of the recording. SpikeyGi showed little reduction in fluorescent output over the course of 267 268 the recording and showed significantly less bleaching than SpikeyG (repeated measures ANOVA group x time interaction: $F_{749, 14231} = 2.221$, p < 0.0001) (Figure 6A). Next, we compared photobleaching rates in 269 vivo at low photon flux conditions using UF2P microscope under similar intermittent imaging conditions 270 271 (9s on, 4s off) across 60 min. For both SpikeyGi and SpikeyG, the fluorescence rapidly decreased by 11-272 13% within the first 5 minutes but then more slowly reduced to 22-25% after one hour. Unlike under high photon flux conditions, no difference in photobleaching was observed between SpikeyGi and SpikeyG 273 274 under low photon flux conditions. These results demonstrate that low photon flux excitation benefits both positive- and negative-going indicators. For SpikeyGi, we assessed the action potential (AP) detection rate 275 276 to air puff stimulation across the one hour of imaging (Figure 6B-C). Despite the changes in fluorescence, 277 no differences in action potential detection were observed across the period.

Given that the UF2P microscope delivers a total of 240mW across 8 beams (30mW per beamlet) 278 279 during *in vivo* imaging, we tested for signs of photodamage or toxicity after 1 hour of sustained imaging (Podgorski and Ranganathan, 2016). Animals were perfused 16 hours after imaging, and immunostaining 280 was performed for four markers of tissue damage and inflammation: astrocytic (anti-GFAP), microglial 281 282 (anti-Iba1), heat shock (anti HSP-70/72), and apoptotic pathway activation (anti-Caspase-3). We measured average fluorescence levels in the laser-exposed areas of the cortex and in control areas of the cortex which 283 284 were not exposed to laser scanning (Figure 6D). We found that laser exposure did not produce an increase in any of the four markers of photodamage. There was no significant difference between laser-treated and 285 control areas of the cortex for all four markers (paired Student's *t*-test, n=4: GFAP, t = 2.27, p = 0.11; Iba1, 286 287 t = 0.06, p = 0.95; HSP, t = 1.75, p = 0.18; Caspase-3, t = 0.39, p = 0.72). In summary, low photon flux imaging using UF2P microscope in combination with SpikeyGi provides safe and sustained population-288 289 level voltage imaging.

- 290
- 291

292 DISCUSSION

Here we developed a novel, multidiscipline approach to chronically record fast voltage transients from large sets of neurons in deep brain tissue in behaving animals. The performance gains of the system are possible by the combination of three independent novel advances; a high performing, positive-going, fast, voltage indicator (SpikeyGi), an ultra-fast, large FOV multiphoton microscope and a purpose-built AI denoising process (DeepVID). With these innovations we demonstrate sustained large-scale, ultra-fast, two-photon, voltage imaging for the first time.

299 Our approach uses a positive fluorescence-voltage slope relationship GEVI (SpikeyGi). During its 300 characterization in vitro under high photon flux illumination, Spikey Gi and its "negative going" counterpart (SpikeyG) showed similar SNR performance. However, under the low photon flux imaging conditions of 301 our UF2P microscope, SpikeyGi significantly outperforms its "negative-going" counterpart. While we have 302 303 not yet pursued the basis for this difference, we hypothesize reduced baseline noise and the higher photon 304 count during action potentials combined in SpikeGi result in significantly improved SNR. This indicates that *in vitro* characterization is not the best predictor of *in vivo* performance, but rather an approach which 305 combines probe and imaging method engineering is likely to be more successful. 306

307 In the study, we utilize temporal and spatial multiplexing to develop the UF2P microscope. By increasing the effective pulse rate of the excitation source while maintaining low detection cross talk, the 308 309 UF2P microscope can achieve kilohertz frame scanning in deep tissue at twice the FOV of current kilohertz-310 scanning two-photon systems (Table 1). The FOV size and imaging depth are comparable to standard 2P 311 microscopes performing calcium imaging at very low speed. This inherently results in lower photon flux per imaged neuron. Low photon flux excitation results in reduced photobleaching and photodamage, 312 enabling sustained and chronic voltage imaging. However, it is challenging to achieve reliable 313 314 fluorescence imaging under shot noise limiting conditions.

To improve imaging at low photon flux levels we developed a self-supervised, deep learning denoising method (DeepVID). DeepVID denoising was achieved without sacrificing spatial or temporal resolution and without access to "ground-truth" high-SNR measurements. It achieved a 15-fold increase in the single-pixel SNR, which is comparable to the improvements reported in 2P calcium imaging (Lecoq et al., 2021; Li et al., 2021). Applying DeepVID to sensory-evoked measurements revealed more reliable spike detections.

The combination of these tools represents a new direction of further technology development to 321 322 scale up two-photon voltage imaging. For the UF2P microscope, FOV can be further increased while maintaining overall photon budget levels by a combination of additional judiciously placed spatially 323 324 multiplexed beamlets to increase the effective repetition rate and beam shaping to improve excitation 325 efficiency (Demas et al., 2021; Weisenburger et al., 2019). Ongoing developments in denoising can further reduce the photon flux needed for reliable detection. The denoising performance of DeepVID can be 326 327 improved by incorporating more advanced network architectures, such as spatiotemporal convolution and 328 attention module (Vaswani et al., 2017). Improvements in SpikeyGi can further reduce photon flux 329 requirements by increasing fluorescence response amplitude to membrane potential changes, decreasing 330 resting fluorescence to reduce background, and improving subcellular localization to reduce inactive SpikeyGi molecules. Overall, low-photon flux imaging pushes the boundaries for monitoring neuronal 331 activity across spatial and temporal scales. Additionally, this framework can be adopted for other in vivo 332 333 imaging applications that track signal changes in molecules of low abundance across large areas at fast 334 temporal resolutions.

335

336 ACKNOWLEDGEMENTS

We thank K Khait for assistance in Scope software development; N Manjrekar for assistance in photodamage experiments; Pieribone Laboratory scientific staff J. Wojciekofsky, L. Delgado, and P. O'Brien for technical assistance; the Pierce Laboratory Instrument shop including J. Buckley, A. Wilkins, T. D'Alessandro, and A. DiRubba for help with instrumentation. This work was supported by grants from a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (J.L.C.), the Richard and Susan Smith Family Foundation (J.L.C.), Elizabeth and Stuart Pratt Career Development Award (J.L.C.), the Whitehall Foundation (J.L.C.), NSF Neuronex Neurotechnology Hub NEMONIC
#1707287 (J.L.C.), NIH New Innovator Award DP2NS111134 (J.L.C.), NIH BRAIN Initiative Awards
R01NS109965 (J.L.C.), UF1NS107705 (J.L.C. and V.A.P.), R21EY030016 (L.T.), U01NS103517 (V.A.P),
U01NS090565 (V.A.P), and DARPA N6600117C4012 (V.A.P.), and N660119C4020 (V.A.P.).

348 AUTHOR CONTRIBUTIONS

J.L.C. and V.A.P. initiated and supervised the study. J.P. engineered SpikeyG and SpikeyGi. J.P. carried
out cell culture experiments and analyzed data. X.Y. designed, built, and characterized the ultra-fast twophoton microscope. C.L. developed DeepVID algorithm and analyzed performance. A.M.A. performed
animal surgeries for slice and *in vivo* experiments. A.M.A. performed slice experiments and analyzed the
data. X.Y. and A.M.A. performed *in vivo* experiments. X.Y., A.M.A., and J.L.C. analyzed *in vivo* data.
I.A.C., I.G.D., L.T., V.A.P., and J.L.C. provided input and guidance. J.P., X. Y., A.M.A., C.L., L.T., V.A.P.,
and J.L.C. prepared the manuscript.

356

347

357 MATERIALS AND METHODS

358 Plasmid construction. The starting constructs used in this study, pcDNA3.1/Puro-CAG-ASAP3b and 359 pcDNA3.1/Puro-CAG-ASAP3b-Kv2.1, were a generous gift from Michael Z. Lin (Villette et al., 2019). 360 The identification of the expressing cells during automated functional screening (see below) was facilitated 361 with the addition of a self-cleaving T2A peptide sequence (GSGEGRGSLLTCGDVEENPGP) followed by nuclear-localized tag-FPs (mCherry) at the C-terminus of GEVI ASAP3. For neuronal expression, GEVI 362 variants SpikevG and SpikevGi were subcloned into the pAAV-hSvn-eGFP (Addgene #50465) by 363 364 replacing eGFP using KpnI and NheI restriction sites. Additionally, a fusion of the C-terminal cytoplasmatic segment of the Kv2.1 channel (the 65AA long proximal restriction and clustering signal) to 365 366 the C-terminus of the GEVI variants via the GSSGSSGSS linker facilitated restricted expression targeted to the neuronal soma and proximal dendrites (Villette et al., 2019). All constructs were manufactured using 367 368 InFusion Cloning System (Clontech, USA), with all the products confirmed by sequencing (Keck DNA Sequencing Facility, Yale). 369

370

Virus production. AAV2/PhP.eB-hSyn-SpikeyGi-Kv2.1 (9.7x10¹² gc/mL) was obtained from Boston
Children's Hospital Viral Core. AAV2/8-hSyn-SpikeyGi-Kv2.1 (1.0x10¹⁰ gc/mL) and AAV2/8-hSynSpikeyG-Kv2.1 (1.0x10¹⁰ gc/mL) were produced and purified in the house using an established protocol
(Challis et al., 2019) and commercial purification and titration kits (Takara Bio, USA).

375

376 **Library production**. The production of GEVI site-directed mutagenic libraries was described previously 377 (Platisa et al., 2017, 2020). Briefly, the mutagenic PCR reaction was run with a mix of the three forward 378 primers containing three degenerative codons (NDT, VHG, or TGG) and the single reverse primer. This 379 combination of degenerative primers resulted in site-directed mutagenic libraries encoding all 20 amino 380 acids with repeats for two amino acids, Lysine and Valine. The insertional library that resulted in the 381 development of the SpikeyGi was produced with a single set of PCR primers with the forward primer containing degenerative codon NNK targeted between ASAP3 amino acid residues D151 and N152 382 383 (Villette et al., 2019). The 15bp long overlapping extension with a sequence identical to a vector at the insertion site in forward primers facilitated vector circularization. For the PCR reaction, we used 384 CloneAmpTM DNA polymerase, and the parent template was removed with *DpnI* restriction enzyme 385 digestion. For the ligation of the amplified mutagenic vectors, we used In-Fusion HD enzyme premix and 386 for bacterial transformation Stellar Chemically competent cells (Clontech, USA). The mutagenic libraries 387 388 were produced in the 96- well plate with 46 bacterial colonies selected for each (two libraries per 96-well 389 plate; four wells were controls). The cDNA was purified using an automated liquid handling robot 390 (epMotion 5057; Eppendorf, USA), and the library complexity was confirmed by sequencing 10% of 391 selected colonies.

392

393 **Cell culture.** Functional testing on the semi-automated screening platform was done using spontaneously 394 spiking HEK cells (kind gift from Dr. Adam Cohen of Harvard University; Park et al., 2013; #CRL-3269, 395 ATCC, USA) expressing GEVI mutants. The stable expression of NaV 1.3 and KiR 2.1 ion channels creates 396 spike-like electrical activity in these cells. The cells were cultured in DMEM/F12, 10% FBS, 1% penicillin 397 (100 U/mL), streptomycin (100 µg/ml), geneticin (500 µg/mL) and puromycin (2 µg/mL) (Sigma-Aldrich). For simultaneous patch-clamp and imaging GEVI testing, we used HEK 293 cells (# CRL-1573, ATCC, 398 399 USA). The cells were kept in Dulbecco's Modified Eagle Medium (DMEM, High glucose; Invitrogen, USA) 400 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA). The cultures were maintained in 401 a humidified incubator at 37°C in a 5% CO2 environment, and cells were experimentally used for up to 25 passages. For functional testing, cells were plated either on glass-bottom 96-well black dishes (Cellvis, 402 USA) or on 12-mm coverslips (Carolina Biological, USA) coated with poly-D-lysine hydrobromide 403 404 (Sigma-Aldrich, USA). For transient expression of GEVI variants, we used Lipofectamine 200 (Invitrogen, 405 USA) at the half of the manufacturer's recommended reagent amount (for DNA 0.1 µg per 96-well or 0.4 μg per 12 mm coverslip in 24-well dish, for Lipofectamine 2000 0.25 μl per 96-well or 1 μl per 24-well). 406 407

408 Functional testing of mutagenic libraries under widefield excitation. The functional screening of GEVI mutants is done as previously described (Platisa et al., 2020; Platisa et al., 2017). In short, a semi-automated 409 410 screening platform was built around a Nikon Eclipse Ti-E inverted microscope equipped with a Perfect 411 Focus System and a motorized Prior Proscan II stage (Prior Scientific, Inc., USA). The custom-made 412 imaging chamber holds 96-well plates under constant temperature (37°C) and humidity during experiments. For imaging, we used a Nikon Plan Apo 20x 0.75 NA objective (Nikon, Japan), a pE-300 (CoolLED Ltd, 413 U.K.) light source, and an ORCA Flash 4.0 sCMOS camera (Hamamatsu, Japan). For ASAP-based 414 415 constructs (GFP), we used a 470/40 nm excitation filter, 495 nm dichroic mirror, and 525/25 nm emission filter (# 49002, Chroma Technologies Corp., USA). The nuclear-localized tag protein, mCherry, was 416 417 visualized with a 560/40 nm excitation filter, 585 nm dichroic mirror, and 630/75 nm emission filter 418 (#49008, Chroma Technologies Corp., USA). For field stimulation (Grass S48 Stimulator, USA), we used 419 a custom-made field electrode and actuator system (Thorlabs, USA) attached to the roof of the imaging 420 chamber. The image collection, electrical stimulation, and signal detection were done using a custom 421 application written in LabView (National Instruments, Inc., USA). The fluorescence intensity of nuclearlocalized mCherry was used to identify expressing cells and select for the field of views within each well. 422 423 For functional screening, images were collected at 100 fps in 2500 ms long sweeps with a single pulse of 70V 0.5 ms applied at 400 ms from the beginning. The fluorescence signal in each cell in response to field 424 425 stimulation was quantified as $\Delta F/F$. Each GEVI variant was screened in four separate plates, and the 426 selection of the best mutants was based on the maximum response amplitude across cells and wells. 427

428 Electrophysiology and widefield imaging in HEK293 cells. For whole-cell patch-clamp experiments, 429 HEK239 cells were kept in a perfused chamber at 33-35°C (Warner Instruments, USA) with the constant running bath solution (129 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, and 10 430 mM HEPES, pH 7.4 and was adjusted to 310 mOsm with sucrose). The 3-5 M Ω glass patch pipettes 431 (capillary tubing with 1.5/0.75 mm O.D./ID, WPIP, USA) were pulled on a P-97 Flaming/ Brown type 432 micropipette puller (Sutter Instrument Company, USA). The pipette solution contained 125 mM K-433 434 gluconate, 8 mM NaCl, 0.6 mM MgCl2, 0.1mM CaCl2, 1 mM EGTA, 4 mM Mg-ATP, 0.4Na-GTP and 10 mM HEPES, pH 7.2 and adjusted to ~290 mOsm. Voltage-clamp recordings in the whole-cell configuration 435 436 were performed using MultiClamp 700B amplifier, digitizer Digidata Series 1400A and pClamp software (Molecular Devices, USA). Depending on the experiment, the voltage was changed from a holding potential 437 of -70mV to i) +30 mV (a single-step depolarization experiments), ii) -110, -90, -50, -30, -10, 10, and 30mV) 438 439 (in a series of 20mV incremental subsequent steps), or iii) -120, -20, +30 and +80 mV (a series of steps). Imaging was performed on an Olympus BX61WI upright microscope using a LUMPlan FL 40x N.A. 0.80 440 water immersion objective (Olympus, USA) and a 488 nm 50 mW laser light excitation (DL488-050, USA). 441 442 We used a GFP filter set, a 495 nm dichroic mirror, and a 520/35 nm emission filter (Semrock, USA), and the laser power measured at the preparation was 13-18 mW/mm2. Additionally, the light intensity was 443

10

adjusted for each recording session using a continuous circular neutral density filter (ThorLabs, Inc., USA)
to the minimum required to record optical signals. The images were collected with a fast-speed NeuroCCD
camera controlled by NeuroPlex software (RedShirt Imaging, USA) at a frame rate of 500 or 1000Hz. For
the image demagnification, we used either an Optem zoom system A45731 0.13 or Optem C-to-C mount
25-70-54 0.383 (Qioptiq LINOS, USA).

449

450 Widefield imaging analysis. The data were analyzed using NeuroPlex, Excel, and custom scripts written 451 in Igor and Matlab. All the results are presented as a mean value and the standard error of the mean (s.e.m.). 452 The values for the resting fluorescence and the bleaching rate were derived from recordings on the screening platform. The resting fluorescence was calculated as the mean value recorded across all the cells within the 453 454 field of view at the first five frames at the beginning of the recording and prior stimulation. The bleaching 455 rate is calculated as a percent change between resting fluorescence at the beginning and end of the trial. The optical traces are spatial averages of the intensity of the pixels within the region of interest (ROI) that 456 covers the cell body. The ROIs were visually identified using the Neuroplex feature Frame Subtraction. 457 The amplitude of fluorescence change was measured as the difference between the averaged values for 50 458 459 frames before stimulation and 5 (for SpikeyGi) and 100 (ASAP3 and SpikeyG) frames around the peak of the response. Data are presented as the voltage-dependent change in fluorescence divided by the resting 460 461 fluorescence, $\Delta F/F$. For bleach correction, the portion of the trace outside the stimulus was fitted with a 462 double exponential curve.

463

Animal preparation. All experimental procedures were approved by the Institutional Animal Care and 464 Use Committee for the Charles River Campus at Boston University. For in vitro slice experiments, viral 465 466 injections were performed in neonatal (P7-P9) C57Bl/6 mice. Mice were bred in house in the Boston University animal care facility, with standard housing, a 12-hour light/dark cycle, and *ad lib* access to food 467 and water. Pups were removed from the mother, anesthetized with 1-3% isoflurane, and placed in a custom 468 469 stereotaxic holder for neonatal mice. A small incision was made in the scalp and a two small holes were 470 made with a 30-gauge needle in the skull of the left hemisphere. Mice were injected with AAV2/8-hSyn-471 SpikeyGi-Kv2.1 or AAV2/8-hSyn-SpikeyG-Kv2.1 (300 nL) at 250 µm below the pial surface. Incisions were closed with Vetbond Tissue Adhesive (3M), and animals were treated post-operatively with 472 buprenorphine (0.05-1 mg/kg, s.c.). Animals were returned to their mother immediately after surgery and 473 474 weaned at 3 weeks of age.

475 For in vivo experiments, adult (6-8 week old) C57Bl/6 mice, stereotaxic viral injections of GEVIs 476 were performed in L2/3 and L5 of primary somators ensory cortex, 300 and 500 μ m below the pial surface 477 (600 nL total volume). Either AAV2/PhP.eB-hSyn-SpikeyG-Kv2.1 (1:8 or 1:12 in saline) or AAV2/8hSyn-SpikeyG-Kv2.1 were injected. There were two injection sites per mouse, both targeting the left 478 479 hemisphere sensorimotor (S1) cortex (AP -1.1 mm, ML 2.9 mm and AP -1.1 mm, ML 3.3 mm). To enable 480 optical access, a 4 mm cranial window was implanted over S1 (Margolis et al., 2012). A metal headpost was implanted over the right hemisphere to enable head fixation. Animals were treated post-operatively 481 with buprenorphine (0.05-1 mg/kg, s.c.) and given 2-3 weeks for viral expression to take place. Animal 482 were handled and habituated to head fixation for 3-5 days before imaging experiments began. 483

484

485 **Slice electrophysiology.** For *in vitro* characterization of GEVIs, whole-cell slice electrophysiology was performed on layer 2/3 cortical neurons. To obtain brain tissue slices, mice were deeply anesthetized with 486 487 ketamine/xylazine and trancardially perfused with modified ACSF (in mM, 124 NaCl, 75 sucrose, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO3, 1.3 ascorbic acid, 0.5 CaCl₂ and 7 MgCl₂). Brain tissue was 488 sliced into 300 µm coronal sections and maintained with oxygenated (95/5% O2/CO2) ACSF (in mM, 124 489 490 NaCl, 26 NaHCO3, 20 sucrose, 3 KCl, 1.25 NaH2PO4, 2 CaCl2 and 1.5 MgCl₂). Pipettes were pulled from 491 thin-walled borosilicate capillaries without filament (3-6 M Ω) (Sutter Instruments), and filled with internal solution containing (in mM, 135 K-gluconate, 10 HEPES, 2 MgCl₂, 2 MgATP, 0.4 EGTA, 0.5 Na₃GTP, 10 492 493 phosphocreatine disodium). The process of approaching the cell, sealing onto the membrane, and breaking in to establish a whole-cell configuration was controlled by Autopatcher (Neuromatic Devices). Electrical 494

11

signals were recorded with a Multiclamp 700B amplifier (Axon Instruments), filtered at 10 kHz and
digitized at 20 kHz (National Instruments PCI-6321), and recorded with custom MATLAB software
(Mathworks). Simultaneous 2P imaging was performed at with a conventional 2P microscope (Ultima,
Prairie Technologies, Middleton WI) using a 40x/0.8NA water immersion objective (Olympus) and Dodt
contrast imaging. For laser source, a 80Mhz Ti:sapphire laser (Mai Tai HP, Spectra Physics) was tuned to
920nm with 35-45mW delivered at the sample.

501 For characterization of fluorescence responses to varying membrane potentials, fluorescence 502 intensity was measured while neurons were held in voltage clamp mode. One-second trials consisted of a 503 200ms baseline period at resting membrane potential (-70 mV), followed by a 400 ms voltage step, then a return to resting membrane potential for 400ms. Trials were repeated 10 times across 10 voltage steps 504 ranging from -130 mV to +50 mV (20 mV increments). For image acquisition, frame scans of the targeted 505 506 cell were acquired at 20Hz across 128 x 85 pixels at ~0.3µm/pixel resolution and 2.8µs pixel dwell time. 507 For analysis, the cell membrane was segmented by isolating pixels 1.3-2.0x above background. For 508 characterization of action potential responses, neurons were recorded in current clamp mode with single action potentials evoked at 100ms intervals (triggered by 10ms pulses of 800-1200 pA), with 10 500-ms 509 sweeps of 5 action potentials each. Fluorescent responses were recorded by line scans along the cell 510 511 membrane at 24-54 pixels per line, scanned at 3-5 kHz, 2.8µs pixel dwell time. $\Delta F/F_0$ was calculated where 512 F₀ corresponded to fluorescence at resting membrane potential.

513

514 Microscope design. The optical design was performed using Zemax OpticStudio software (Zemax LLC), and the opto-mechanical design was performed using AutoDesk Inventor (Autodesk Inc.). A 920nm, 2W 515 fiber laser (ALCOR-920, Sparks Lasers, 100fs pulse duration, 31.25MHz repetition rate) was used as the 516 517 light source. The total laser power was controlled by a Pockel's Cell (350-80, ConOptics). The laser beam 518 was split into 4 beam paths using polarizing beamsplitter (PBS) cubes (PBS123/ CCM1-PBS253, Thorlabs). 519 A half-wave plate (AHWP05M-980, Thorlabs) was placed before each beamsplitter cube (three in total) to 520 allow power control of individual paths. Two half-wave plates were mounted on manual rotation mounts (CRM1PT/M, PRM05/M, Thorlabs) The third half-wave plates mounted on a motorized precision rotation 521 522 stage (PRM1/MZ8, Thorlabs) for remote control when selecting between 8 beam and 1 beam imaging. Temporally multiplexed beams were delayed by 8ns relative to each other using delay lines. After the delay 523 line, all beams were reduced to obtain a 1.5mm beam diameter (BE052-B, AC127-075-B, ACN127-025-B, 524 525 or BE02-05-B, Thorlabs).

526 Temporally multiplexed beams were routed into customized beamsplitter plates. Beamsplitter 527 plates serve two functions. First, they split each temporally multiplexed beam into pairs of spatially 528 multiplexed beams. Second, they arrange the beamlets linearly to definite the position of each subarea. A beamsplitter plate consisted of an assembly of half-wave plates (AHWP05M-980, Thorlabs) on rotation 529 530 mounts (PRM05/M, Thorlabs), PBS cubes (PBS123, PBS053, Thorlabs) and half-inch gold-coated mirrors 531 (PF05-03-M01, Thorlabs) on miniature mirror holders (LMMH-12.7R-N, OptoSigma). One beamsplitter routed 4 beams through independent f=100mm relay lenses (KPX034AR.16, Newport) which were held in 532 533 a customized lens holder and separated by 8.5mm, corresponding to 100um spacing at the sample. Two beamsplitter plates were used to combine a total of 8 beams using a 2-inch PBS cube (PBS513, Thorlabs) 534 and positioned such that beams from each beamsplitter plate were interleaved at 50µm spacing at the sample. 535 536 A beam blocker mounted onto a linear actuator (L12-P, Actuonix) was placed after each lens holder and allowed selection between all 8 beams or any arbitrary individual beam. 537

All 8 beamlets were sent to a customized f=330mm scan lens assembly (SLB-50-300N, SLB-50-450P, SLB-50-250P, OptoSigma), and conjugated to a 2kHz resonant scanner (CRS 12kHz, 5.0mm x 7.2mm aperture, Cambridge Technology) and galvo scanner (6215H, Cambridge Technology). The scan lens (S4LFT0089/094, Sill Optics GmbH & Co.) and tube lens (AC508-500-B, AC508-750-B, Thorlabs) expanded the beam to fill the back aperture of the objective (N16XLWD-PF, 16x, Nikon). The laser light was reflected to the objective by a short-pass filter (FF01-720/SP-25, AVR Optics).

The emitted light passed through the short-pass filter and then was separated by a secondary dichroic (FF556-SDi01-40x45, Semrock) into green and red fluorescent channels. Red fluorescence excited

12

from a single beam was detected using an achromatic lens (AC508-080-A, Thorlabs) and an eyepiece
(19mm, Televue) focused onto a hybrid PMT (R11322U-40, Hamamatsu). For green fluorescence, a
customized lens assembly was used to magnify and reshape the square FOV to match the geometry of a
16x1 multi-anode photomultiplier (H13123, GaAsP 16-channel MAPMT, Hamamatsu). The assembly
included two achromatic lenses (AC508-100-A, Thorlabs) and two cylindrical lenses (LJ1567RM-A,
Thorlabs, and CKX019, Newport) positioned orthogonal to each other.

552 Using a customized 16-to-4 adder (Marina Photonics Inc.), signals from the 16 MAPMT anodes 553 were amplified, digitized, and then combined into four detector subgroups each consisting of signals from 554 4 adjacent anodes. Using custom electronics, the subsequent LVPECL signal was converted and fanned out to two LVDS signals (NB6N11S, ON Semiconductor). The signals were collected by an FPGA (PXIe-555 7965R, National Instruments Corp.) with a 20-channel digital I/O board (NI-6587, National Instruments 556 557 Corp.) operating at 1Gbit/s sampling rate. For a given detector subgroup, the two fanned out signals were inputted into two I/O channel wherein one of the two fanned out signals was delayed by 0.5ns. This 558 provided a 2Gbit/s sampling rate for each detector subgroup when combined on the FPGA. 559

Each detector subgroup contained fluorescence from two beamlets which were then temporally 560 demultiplexed on the FPGA using a synchronization signal provided by the laser and electronic delay box 561 (DB64, Stanford Research Systems) providing de-multiplexing with 0.5ns precision. Additional time-562 563 dependent gating was implemented on the FPGA to minimize spatial multiplexed crosstalk from neighboring detector subgroups (LabVIEW, National Instruments). For sample positioning, a lifting stage 564 565 (HT160-16-DC, Steinmeyer Mechatronik GmbH) mounted on an XY stage (KT310-200-DC, Steinmeyer Mechatronik GmbH) was used. The microscope system was controlled by a customized C++-based 566 software, Scope. The software controlled the resonant and galvo scanner (imaging FOV), the pockel's cell 567 568 (laser power) and the shutter through a DAQ module (PXI-6259, National Instruments Corp.).

569

570 **Point spread function characterization.** For each beamlet in the UF2P microscope, the point spread 571 function (PSF) was measured using 0.5µm diameter fluorescent beads with (T7281, Invitrogen). Images were taken one beamlet at a time. The beads were embedded in 1.5% agarose and an image stack of the 572 573 bead was taken at 0.1x0.1x0.5um³ voxel resolution with 50 frames at each plane. Motion correction was 574 performed at each plane before averaging. A summed Z-intensity projection of the image stack was taken and the maximum intensity pixel was identified as the center of the bead along the X/Y-axis. The center 575 576 along the Z-axis was defined as the plane with the maximum total signal. The profile was plotted through 577 the center along each axis. The PSF value reported was the full-width-at-half-maximum (FWHM) of each 578 profile.

579

580 **Crosstalk characterization.** To determine the degree of temporal and spatial crosstalk observed in scattering tissue, a mouse virally expressing SpikeyGi implanted with a cranial window was used. Tissue 581 was scanned using one beam at a time (\sim 30mW) and fluorescence was collected across all 8 subareas. 582 583 Images consisting of 50-frame averages acquired at 389Hz were taken at 30µm steps from 0- 300µm below 584 the pial surface. For the excited subarea in each image, pixels corresponding to SpikeyGi fluorescence were identified as those whose intensities were >95th percentile compared to other pixels in the same subarea. 585 586 Percent crosstalk was calculated as the mean signal measured in each of the non-excited subareas divided by the mean signal in the excited subarea contain SpikeyGi fluorescence. 587

588

Intrinsic signal optical imaging. To identify the location of viral expression relative to S1, intrinsic signal 589 imaging was performed under light anesthesia (1-1.5% isoflurane). The cortical surface was illuminated 590 591 with a 625 nm LED (Thor Labs), and two individual whiskers (B2 or C2) were stimulated at 10 Hz with a 592 piezo-electric stimulator. Reflectance images were recorded using a f = 25 mm lens (Navitar) and a CMOS 593 camera with a 30 Hz frame rate, 6.5 µm pixel size, 4 x 4 binning, and 512 x 512 binned pixels (Hamamatsu). 594 Cortical activation in the barrel column was determined by comparing changes in reflectance during whisker stimulation versus periods of non-stimulation, expressed as $\Delta R/R_0$ (150 frame average). Barrel 595 columns were identified as signal minima after averaging intrinsic reflectance signals over 10 trials. 596

13

597

Self-supervised deep learning denoising of voltage imaging data. DeepVID combines self-supervised 598 frameworks implemented in DeepInterpolation and Noise2Void (Lecoq et al., 2021; Li et al., 2021). The 599 600 network architecture of DeepVID was based on the DnCNN (Zhang et al., 2017), a fully convolutional network with residual blocks. This architecture was chosen to better accommodate the 8:1 aspect ratio in 601 602 the sub-image scanned by each beamlet. The network was constructed with 2D convolution layers (Conv), 603 batch normalization (BN) layers and Parametric Rectified Linear Unit (PReLU) activation layers, with 16 604 repeated residual blocks in the middle. Each residual block contained two 3x3 Conv layers with BN layers 605 followed, and an PReLU activation layer was appended after the first BN layer. The skip connection was 606 added to link low dimensional and high dimensional features by adding the feature map of the input and 607 the output for each residual block (Figure 4A).

608 DeepVID was designed to denoise a single frame from each sub-area at a time. It was trained to 609 predict the central frame N_0 using an input image time series, consisting of $N_{\rm pre}$ frames before and $N_{\rm post}$ frames after the central frame, in addition to a degraded central frame with several "blind" pixels. A random 610 set of pixels (*p*_{blind}) in the central frame were set as blind pixels using a binary mask, whose intensities were 611 replaced by a random value sampled from randomly selected pixels in the frame. The hyperparameters ($N_{\rm pre}$ 612 = 3, $N_{\text{post}} = 3$, $p_{\text{blind}} = 10\%$) were optimized to maintain the temporal dynamic of voltage signal spikes while 613 614 recovering a high single-frame spatial resolution. The loss function was the mean squared error (i.e. L2 loss) 615 between the original and denoised central frame and was calculated only on the blind pixels. The training 616 was performed using the Adam optimizer with 360 steps per epoch and a batch size of 4. The training stopped after going over all samples in the data set one time to avoid overfitting. The learning rate was 617 initialized at 5×10^{-6} and reduced to 1×10^{-6} when the loss on the validation set did not decrease in the past 618 619 288,000 samples.

The training data set consisted of 1181 videos, each of which contained 1000 frames acquired at 620 621 803 Hz. Each image time series was preprocessed by detrending and normalization before sending into the 622 DeepVID network. For detrending, the trend was approximated by a second order estimator by fitting to the time trace of the intensity mean of each frame. The scalar in the trend at each frame was subtracted from 623 624 all the pixels in the corresponded frame. The detrended video was then normalized by subtracting the mean and dividing by the standard deviation of all pixels in the image time series. The network was trained on a 625 graphics processing unit (Nvidia P100, 12 GB VRAM) using TensorFlow 2.2.0, Python 3.8 and CUDA 626 627 11.2. Once DeepVID was trained, inference denoising of subsequent image data using the trained model was performed frame-by-frame by feeding each corresponding 7-frame image time series. It can be 628 629 performed at approximately 200 frames per second on a single Nvidia P100 GPU. To quantify the performance of DeepVID, the single-pixel SNR was defined as the ratio of the mean divided by the standard 630 deviation of the pixel intensity along the temporal axis. 631

632

633 In vivo voltage imaging. To measure sensory-evoked voltage responses, whisker stimulation was performed on awake, head-fixed mice. Air puffs (25ms) were delivered to the whisker pad contralateral to 634 the imaged hemisphere. Stimulus patterns consisted of alternating trains of 1 puff or 5 puffs separated at 4 635 second intervals. Trains of 5 air puffs were delivered at either 5 or 10 Hz stimulus frequency. Using the 636 UF2P microscope, voltage imaging in S1 was performed 803Hz at frame rate across 400x192 total pixels 637 (400x24 per subarea) at x: 1.0 µm/pixel, y: 2.1 µm/pixel resolution, ~30mW per beamlet, ~0.1µs dwell 638 time. For image analysis, each subarea was analyzed independently in MATLAB (Mathworks). For 639 640 correction of brain motion, brain motion was estimated using a 2D rigid fast-Fourier transform based on images from one subarea and then a rigid transform correction was applied to all 8 subareas. Images for 641 each subareas were then denoised using DeepVID. Neurons were then manually segmented and 642 643 fluorescence trace extracted for each ROI.

644

Action potential analysis. For analysis and detection of spiking-related voltage signals, slow fluctuations
 in fluorescence signals for each cell were first removed by baseline subtraction along a moving average
 across 2.5 seconds of recordings. Given the ~5ms rise and ~12.5ms decay time (peak-to-trough) of tested

14

648 GEVIs, a putative spike trace was generated by calculating the difference in fluorescence intensity across 649 every 10th imaging frame (12.5ms) across the time series. The putative spike trace was normalized by the 650 cell's noise level, defined as the mean of the absolute difference between each time point, to produce an 651 SNR trace. Spikes were identified as transient events exceeding a given SNR threshold. Sensory-evoked 652 action potentials were identified as detected spikes occurring within a 20 frame window (25ms) following 653 air puff delivery. The sensitivity of GEVIs for spike detection was assessed by quantifying the percent of 654 detected action potentials evoked for each air puff across a range of SNR thresholds.

655

Photobleaching. For *in vitro* photobleaching measurements, cortical tissue was prepared in the same manner and imaged using the same setup for slice electrophysiology. Areas of SpikeyGi or SpikeyG expression were positioned in the field of view using an epifluorescent microscope. Imaging data was recorded at 1 Hz in an intermittent pattern with 10s on (shutter open) and 5s off (shutter closed) for 12.5 min. Laser power of 35 mW was used, with 1.13 μ m per pixel and dwell time of 2.8 μ s. ROIs corresponding to a single cell were extracted manually, and each data point corresponded to the mean fluorescence intensity in a single frame.

In vivo photobleaching measurements were performed using the UF2P microscope on awake, head-663 fixed mice with implanted cranial windows. Imaging was performed using a total laser power of 240mW 664 665 at the sample (30mW per beam). The tissue was scanned at a framerate of 803Hz in an intermittent pattern consisting of 9s on (shutter open) and 4s off (shutter closed) for 60 min. To assess action potential responses 666 667 during photobleaching, voltage imaging with whisker stimulation was performed at 15 minute intervals. ROIs corresponding to a single cell were extracted manually. Each data point corresponded to the mean 668 fluorescence intensity of every 20th frame across the 9 seconds of imaging. Photobleaching rates were 669 670 determined by normalizing all data points to the first data point.

671

672 Photodamage. Animals previously injected with virus and implanted with cranial windows were used for 673 experiments. A location for laser exposure away from the viral injection sites was selected based on a widefield blood vessel map under the cranial window. Each photo-damage session consisted of one-hour of 674 675 continuous laser scanning with 240mW total power (30mW per beamlet). Sixteen hours after laser exposure, animals were anesthetized with 1.5-3.0% isoflurane and cranial windows were removed to expose the 676 cortex. The blood vessel map was used to locate the laser exposure site, and a lipophilic dye (SP-DiIC₁₈(3); 677 678 ThermoFisher Scientific; D7777) was injected to mark the four corners of the laser exposure field of view. 679 Animals were then transcardially perfused with 0.1 M PBS and 4% paraformaldehyde. Brains were 680 postfixed in 4% paraformaldehyde for 24 hours, transferred to 0.1 M PBS, then were sliced with a vibratome into 50-um coronal sections. Slices were first incubated in a blocking solution (10% normal goat serum and 681 1% Triton X-100) and washed three times in 0.1M PBS. Alternating slices were labeled with sets of primary 682 683 antibodies in 5% normal goat serum and 0.1% Triton X-100. One set of slices were stained with primary 684 antibodies for mouse monoclonal anti-GFAP (G3893; Sigma-Aldrich; 1:1,000 dilution) and rabbit anti-Iba1 (019-19741; Wako Chemicals; 1:500 dilution). The other set of slices were stained with mouse anti-685 HSP70/HSP72 (ADI-SPA-810-D; Enzo Life Sciences; 1:400 dilution), and rabbit anti-cleaved caspase-3 686 (Asp175) (9661; Cell Signaling; 1:250 dilution). Slices were then washed three times in 0.1 M PBS and 687 incubated in secondary antibodies. Iba1 and caspase-3 were labelled with goat anti-rabbit Alexa Fluor 555 688 689 (Invitrogen, A21429; 1:500 dilution), and GFAP and HSP were labelled with goat anti-mouse Alexa Fluor 647 (Invitrogen, A21235; 1:500 dilution). Slices were washed in 0.1M PBS and mounted with 690 Fluoromount-G mounting medium (0100-01, SouthernBiotech). The lipophilic dye was used to identify 691 which sections contained the laser exposure site, and where it was located on the medial-lateral axis. Slices 692 were imaged with a Nikon ECLIPSE Ni-E microscope and NIS-Elements software (Nikon Instruments). 693

Photodamage was determined increased antibody labeling in the laser exposed region relative to the corresponding area on the contralateral hemisphere. The relative fluorescence was determined by dividing the mean fluorescent intensity (a.u.) on the treated hemisphere by mean intensity on the contralateral hemisphere. Fluorescence was measured in two areas of the cortex, the laser exposure site and a control area that was at least 1 mm away from the site of laser exposure. The control area was intended

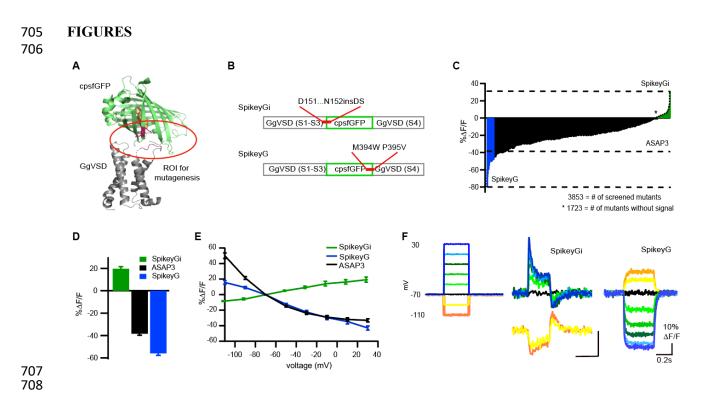
699 to capture protein expression that may have been caused by chronic window implantation and/or virus 700 injection, but was not caused by laser exposure.

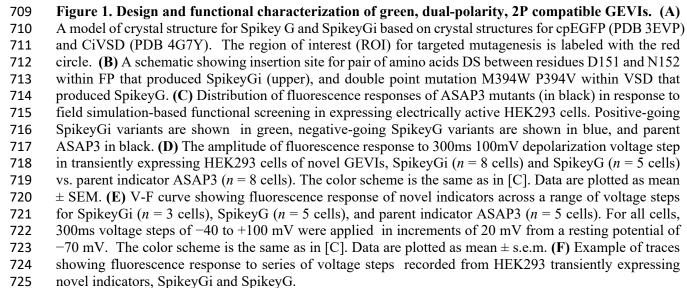
701

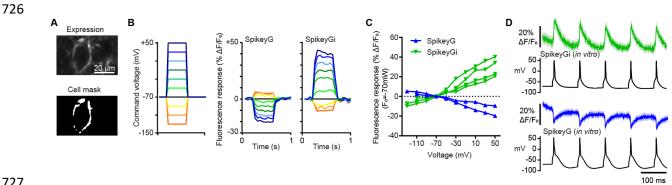
Material and data availability. All sequence information will be available at NCBI, all plasmids and 702 rAAVs will be available through Addgene and UNC Vector Core. All codes will be available at 703

https://github.com/common-chenlab/ and https://github.com/bu-cisl/DeepVID 704







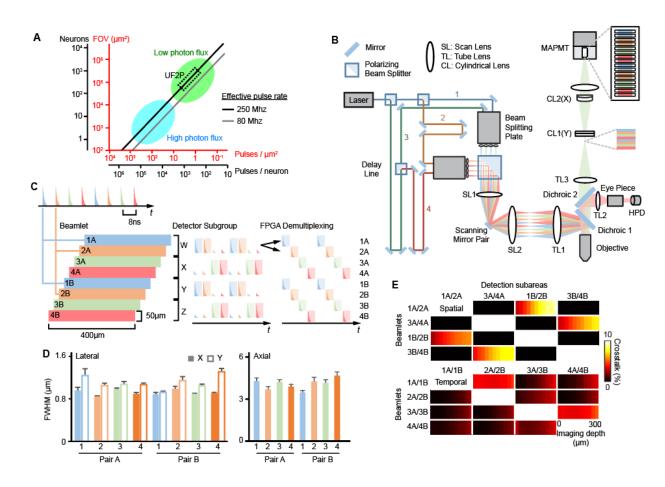


727

Figure 2. In vitro performance of SpikeyGi and SpikeyG. (A) Example of field of view of neuron imaged 728 729 for slice experiments (top). ROI mask used for image analysis (bottom). (B) Example command voltages 730 applied during voltage clamp mode (left). Corresponding fluorescence responses measured in example (C) Fluorescent responses to steady-state voltage steps in slice 731 SpikeyG and SpikeyGi cells). 732 electrophysiology for individual cells (Normalized to -70 mV; SpikeyG, n=2 cells; SpikeyGi, n=4 cells; 10 733 trials per step). (D) Fluorescent responses to 10Hz action potential trains evoked by current injection in

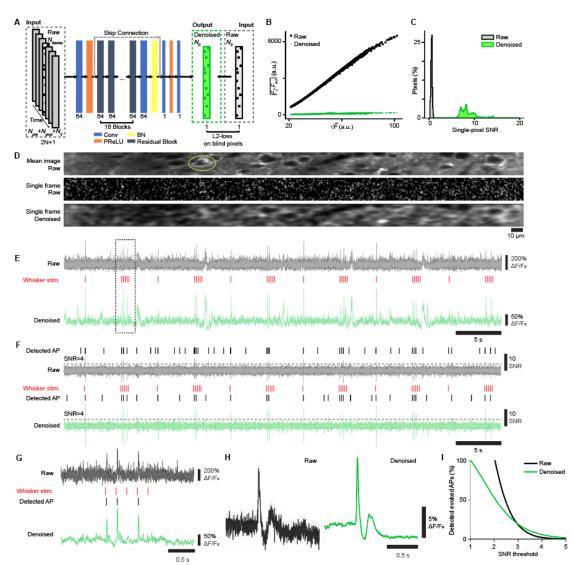
vitro (n = 10 trials). Shaded region; s.e.m. 734

18



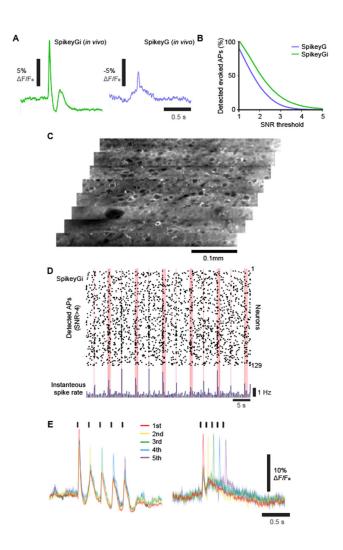
735

Figure 3. Design and performance of ultra-fast two-photon microscope. (A) Relationship between 736 excitation pulses per neuron and total neurons imaged at 1 kHz sampling rate assuming 1 μ m² voxel across 737 738 different effective pulse rates. (B) Schematic of the ultra-fast two-photon microscope. Laser beam was first split into 4 beamlets (blue - 1, orange - 2, green - 3, red - 4) using polarizing beam splitters (PBS). Beamlets 739 were temporally multiplexed using delay lines and then split into spatially multiplexed beamlet pairs. 740 Using two beam splitting plates, beamlets were spatially arranged, combined with a 2-inch PBS into a linear 741 arrangement, and projected onto the objective back pupil. Scan and tube lenses were matched to the beam 742 diameter between resonant/galvo scanner pair and the size of the objective back aperture. The detection 743 path enabled single color imaging with all 8 beamlets or dual color imaging with a single beam. A dichroic 744 separated green from red fluorescence. Red fluorescence excited from a single beam was detected with a 745 746 single hybrid PMT. For green fluorescence, cylindrical lenses in the detection path reshaped the collected fluorescence to match a linearly arranged 16 x 1 MAPMT detector. Signals from each anode were 747 independently collected when imaging using 8 beamlets or summed when excited with a single beam. (C) 748 749 Schematic of detection and demultiplexing algorithm. The 16 anodes on the MAPMT were summed into 4 detector subgroups (W, X, Y and Z). Each detector subgroup received photons from 2 temporally 750 751 multiplexed subareas that were subsequently demultiplexed using FPGA programmed digital gates (shaded area). Additional time-dependent gating was implemented on the FPGA to minimize spatial multiplexed 752 crosstalk from neighboring detector subgroups. (D) Lateral and axial PSF measurements for each beamlet. 753 754 (n=7-11 beads per beamlet). Error bar indicates the s.e.m. (E) Average detected crosstalk as a function of imaging depth due to spatial multiplexing (top panel) or temporal multiplexing (bottom panel). (n = 5755 756 imaging stacks). See also Figure S1-S2.



757 758 Figure 4. DeepVID reduces photon shot noise to improve action potential detection. (A) Training strategy and network structure of DeepVID. The voltage signals from blind-spot pixels in the central frame 759 (N_0) are inferred from the rest of unmasked pixels in the central frame and the neighboring frames (N_{pre} and 760 N_{nost}) in the input image data. DeepVID is a deep convolutional neural network with residual blocks, where 761 Conv is 2D convolution, BN is batch normalization, and PReLU is the parametric rectified linear unit. 762 763 (B) Frame-to-frame noise (mean of the absolute intensity difference) as a function of fluorescence signal (square-root of the mean intensity) for raw vs. denoised pixels for representative image data. 764 (C) Distribution of pixel-level SNR (temporal mean over S.D. for each pixel time series) from a 765 representative raw and denoised data. (D) Example of single frame image denoising with DeepVID. Images 766 from one subarea acquired with the UF2P microscope. Raw in vivo image averaged across 1000 frames 767 (top panel) showing SpikeyGi-expressing neurons. Single frame is shown before (middle panel) and after 768 denoising with DeepVID (bottom panel). (E) Raw and denoised fluorescence traces from neuron [circled 769 770 in D]. Air puff whisker stimulus are shown. (F) Putative spike events based on SNR levels in raw and denoised traces. Detected events at SNR>4 are shown. (G) High temporal resolution view of example raw 771 and denoised traces [box in E] showing spike-related fluorescence changes. (H) Average raw and denoised 772 773 fluorescence traces in response to single air puff stimulus. (I) In vivo detection of sensory-evoked APs with SpikeyGi across SNR thresholds for raw and denoised traces (n = 214 cells, 3 animals). Shaded region in 774 775 E, F,G and H equals S.E.M.

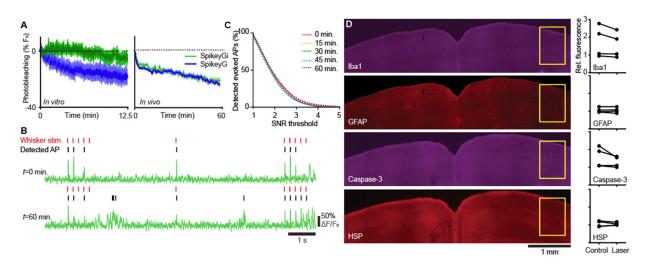
20



776

777 Figure 5. SpikevGi outperforms SpikevG for *in vivo* two-photon population imaging. (A) Average raw and denoised fluorescence traces in response to single air puff stimulus for SpikeyGi and SpikeyG. (n =778 779 214 cells, 3 animals; SpikeyGi, 135 cells, 2 animals; SpikeyG). (B) In vivo detection of sensory-evoked 780 APs across SNR thresholds for SpikeyGi and SpikeyG. (C) Example FOV from UF2P microscope of L2/3 neurons expressing SpikeyGi. (D) Detected in vivo spike trains at SNR>4 for 129 simultaneously imaged 781 782 neurons expressing SpikeyGi. Red lines indicate air puffs. Instantaneous spike rate across the population 783 are shown at the bottom. (E) Average fluorescence responses to detected APs (SNR >4) for individual air 784 puffs in 5Hz (left) or 10Hz (right) stimulus trains (n = 214 cells, 3 animals, 5Hz stimuli, and n = 206 cells, 785 3 animals, 10Hz stimuli). Shaded region; s.e.m. See also Figure S3.

bioRxiv preprint doi: https://doi.org/10.1101/2021.12.07.471668; this version posted December 9, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.



786

787 Figure 6. Low photon flux excitation facilitates sustained two-photon voltage imaging. (A) Left panel shows *in vitro* photobleaching curves for SpikeyGi and SpikeyG under high photon flux conditions (n = 8788 789 cells, 1 FOV, SpikeyGi; 13 cells, 3 FOV, SpikeyG). Right panel In vivo photobleaching curves for SpikeyGi and SpikeyG under low photon flux conditions (n = 33 cells, 3 FOV, SpikeyGi; 33 cells, 3 FOV, 790 SpikeyG). (B) Example SpikeyGi fluorescence traces and detected action potentials (SNR>4) of a neuron 791 792 across 1 hour of intermittent in vivo imaging (9s on, 4s off). (C) In vivo detection of sensory-evoked APs 793 across SNR thresholds for SpikeyGi across one hour of imaging. (D) Left panels show example coronal 794 sections of immunostained tissue assaying photodamage after one hour of sustained imaging. Yellow box 795 denotes imaged region. Right panels show relative fluorescence in imaged region compared corresponding 796 to contralateral region areas across immunostained tissue (n = 4 animals). Scale bar: 1 mm. Shaded region; 797 s.e.m.

22

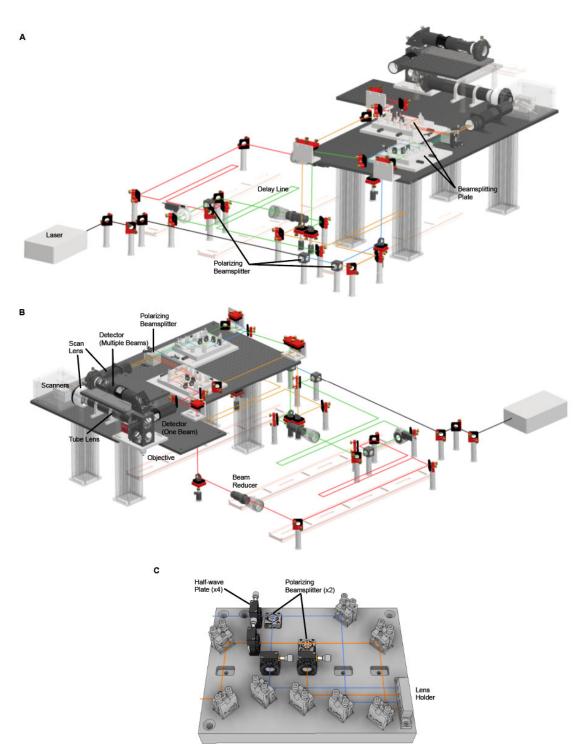
798 SUPPEMENTARY INFORMATION

Microscope	FACED	ULOVE	KHz2P	SLAP	UF2P
Reference	(Wu et	(Villette et	(Zhang et	(Kazemi	This manuscript
	al., 2020)	al., 2019)	al., 2019)	pour et	
				al.,	
				2019)	
FOV (μm x μm)	50x200	150x150*	700x135	250x250	400x400
# Foci	80	1	400 (spa.)	4 lines	8
(Temporal / Spatial)	(temp.)			(temp.)	(4 x 2)
Foci Spacing (µm)	0.625	N/A	15	0.2	50x200
					(temp. x spa.)
Total avg. power	75	20	280	96	270
(mW)					
Peak energy (nJ/foci)	0.94	0.25	3.5	19.2	0.96
Laser pulse per voxel	1.1	8000	0.4	4.2	2.5
Effective pulse rate	80	80	40	5	250
(Mhz)					
Spatial Crosstalk	None	None	High	High	Low
In Vivo GEVI	Yes	Yes	No	No	Yes
Imaging?					

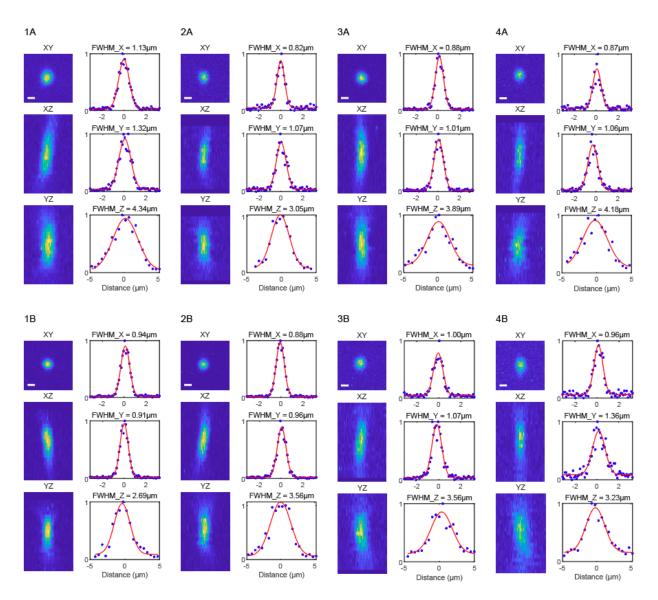
799

800 Table 1. Comparison of kilohertz-scanning two-photon microscopes. *indicates Random access

scanning.



802 803 Figure S1 related to Figure 3. Opto-mechanical design of UF2P microscope. (A-B) CAD design Rendering of the Uf2P microscope. (C) CAD design rendering of the beam splitter plate. 804



805

806 Figure S2 related to Figure 3. Example point spread function measurements for each beamlet in the

807 UF2P microscope.

		manne			And and the spectrum day and	Varansiana		un hanna		(Participant Company and Co	
		,									
and works	and the second	hall have the adviser the	in and a start of a st	man a manager adapt	manistrainmenterset	Mary Markenski alterede		and the low for the state of the		And the second	and the second second second
and the state of t		[.] بر ماند رویس شمانی کرد. در ماند رویس شمانی رویس کرد.	nangsandal pala nan tanàng manganana amin'ny saraharana Ny saraharana paositra mananasana amin'ny saraharana	and a set of the second s	an and free and a state of a state of a	And the second s	***************************************	And a state of the	net in piter and a family and		۲۰۰۰٬۰۰۰ ۱۹۹۰٬۰۰۰ ۱۹۹۰٬۰۰۰ ۱۹۹۰٬۰۰۰ ۱۹۹۰٬۰۰۰
-		And the second s	Renning and the second and	and the second second	Anna and a shared and	and the second s					man manufacture and and
21.000,100,000,000 21.000,000,000	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ر ای بی می این ای بی ای ای ای ای ای ای ای ای این این این ای		nersity of the first state in the	and the second sec	New Contract of the Second sectors	า และสาวมารถอนุรัฐมีกรี่ เป็นเราที่สาวกันประสารสาวมุรัน กระการสาวมารถสาวมารถสาวมารถสาวมารถสาวมากระ	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	، ۵۰ مارین میکور به این کرد بر این ماری ماری میکور این میکور این میکور این میکور این میکور این میکور این میکور ۱۹۹۵ میکور با میکور این میکور ای	National Contraction of Contractiono	
naigner alleis affine	constraints provide the second	and the second second second	Mainghading Search and an annumber of the providence of the second second second second second second second se	Recorded to the second second	and a second state of the		en e	utore hereimenty mergelmeiteten		alter and get at any the state	and the state of the product providence of
ani, mane la	and the second design as a second desig	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	۲۰۰۰٬۰۰۰ ۲۰۰۰٬۰۰۰ ۱۰۰۰٬۰۰۰٬۰۰۰٬۰۰۰٬۰۰۰٬۰۰۰٬۰۰۰٬۰۰۰		······································	and a second		and the second sec	A REPAIR OF THE	man marine marine	مري المريحية المريحية المريحية المريحية المريحية المريحية المريحية المريحية المريحية المريحة المريحة المريحة ال المريحية المريحية الم
war, wary and		hand a service the second and		- Marine Marine	Martin Martin Contract	person the second product		and a service an		and the last march	unawormeest weter whether
and the second parts		and a second	Paralleland and a second	and the second s	And a second	have been the strategies and the			and an and the sugar services	1 martin martine	,
in the second		narrow interest and and	man hard and a second s	hard an and a strange of the	to the second	And a second second second		www.weiner.	and the second	make Merman, which we	and the second second second
alana Taganakana Artigian ang kang	Sulpriside Protocol State	مر من المراجع والمراجع المراجع ا مراجع المراجع ال	۵، میلاد است. ۱۹۹۵ میلی در میلود است. ۱۹۹۵ میلی در میلود است.	any maker and sized how		And the second s	a fan de sense en de sense de la serie de la serie La fan de la serie de la se	and a set a second and a second and a second as a s	,	in multaine state of a string	and the second
arts frequest frequest	and real and the second strange and the		man have attended attended	and the second second second	Antonio and and a second			and an experiment and a state of the second s		and a start and	****
and reaction and the second	and any department of the property of	na haquiquetta a superiora	All and the state of the state	an a	A reason for a serie of the series of the se		, ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	and the state of t	and the second of the second s	And and March and March Sheet	
معقر محمير محمد بالمان محمود المعينات		ور بر المرجع		Para National Anthrophy	و میکاند. برهانید بیکرا و په افغانیونې	and the second state of the second		all a second and a second s	ingen, stannista, et fingen, en traiseantais An saisiste seita, fit finiste error an MA		an a
		**************************************	No. 10 and a construction of the second s	and a construction of the second	A strike set and a set of the set of the set	Contractor and a state of the s	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	and a second state of a second se	and the property of the state o		ang ng mang ng mga n Ng mga ng mga
		ىدەلىدەر بىر بەرەم بىرىت كۈچۈ بار بەرىيە بىرى بەرەر بەرىيە بىرى بەرەر ب	Provide and a second	- Antonio and	a second and a second second second	and the second second second		- marine and a second		have the construction	······································
,		and the second s	-	- Manunantanan	Now water and the second	and a second and a second second	-	- marine marine marine	a and a second		
وريد المراجع ا المراجع المراجع		an a	angentroph related and which any other	makerin unknowned to a series	when in relation to the second second	and a complete the second	Waahuuringen-erineyayuunah	national angle (in the company of the first	and a merical state of provide with a survey of	and the manufacture of the second	۵. ۲۹۳۹ - ۲۹۳۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰۰۹ - ۲۰
			manine the second standard and		a man with more index days to get	and the second states and the second	una wanter and a manual sea			a mar shall a shell a shell a shell a shell a	an and a second and a second
oprily contractions	and the second	And the state of the second	Participation and a second	an a second second second second	and a standard and a	Million as a share a political form	teriastary/suttanentereteris	and the second		When the state of the state of the	****
and a state of the	and a state of a state of a state of			the same and the second	and a rate of the second state of the second s			and any of Augustic and Martin August Harry		APP of minury of passes of dory of any	
Maria Production			Anyong Magdan and Manakan share and an	*******	**********	and the second	مرينيم مرينية منطق ماريسي المرينية المرينية المرينية المرينية المرينية المرينية المرينية المرينية الم	and the second second second		፟ኯ፟ኯ፟፼፟ኯኯኯ፼ጞቘ፝ኯጞኯኯኯኯኯ፟ቚቜዀቜቘኯኯ፟ቜቜኯ ዀቚዸዀኯኯጞጞዀዀዀ፟ዄዄዀኯዀዀዀ	weigestilligeterstellen verstellige von
		And an and a second sec	Channel and States and the states of the sta	and Paraget data and share and	alter and a state of the state		······································			and the second sec	
when the the second design in		Manusuling Mining Instigutions	Aurophics, construction physics and	A STATILITY IN CONTRACT OF A STATE	Participation and an and a second second					and the second se	
was the same from	an and the second second	to a second a second	-	www.comerconares	Wingson Manager and Manager and	1 mar mail man and the to	and the second state of th	when the stranger of		Annow May to make the second	
and a second second second	where we are a set of the set of	۲۰۰۱، میتروند با به میتروند. هر موندر کاما از مانا بر روانده میتروند که	A TANG A TANK A TANK A TANK A TANK	and the state of the second		and the second second			The Contract of the Contract of the Contract of the Contract of Co		neresta anti a constructione de la construction de la construcción de la construcción de la construcción de la Neres autores a construcción de la c
amphistore	مەربەيلەرتىلەرمۇمۇرمۇمۇرمۇرىدۇمۇرىيەتەر بەردۇر	Patra straw been by the party strategy and	And property of the same of the party of the same	a water and a state of the stat	were a service and the service	a national states and a second states of	alantanager an the second second		www.co.co.co.co.co.co.co.co.co.co.co.co.co.	and a second second second	
sand first surgers				A second second second		and the second s		The second second		and a state of the second s	and the second
			- and a second and a second second		Comparative Manager and Providence		,		and of the second s		
and a second		and the second s	10000000000000000000000000000000000000	Maria Mari		and a second	an a		and a second	and the second sec	
	and the second	and the second state of the second	and the spin of a surface of the	مر مرد مرد مرد مرد المرد ال	And interest of the second sec			- Andrew The Street of Street	and the second	and the state of t	
	and the state of the second data and and the state of the second data and the second d		The set of the second s		and a state of the second s						
and the second second	and the second	1.480.0000000000000000000000000000000000	Princet - section in a system of a state	- Augusta	Annual second		and a state of the	-	and a standard and a standard and a standard	And Anna and	
and a state of the state	New propriet and the second	A market and a second	an a	marl and and married a	anthing damager and here and	and the state of t		And a Manager of the state of the	Non- Andrew Street and	، در بیان میران بر با این از این از در بیان این از این	
information		Pharman Charles Construction		www.menson			and a second and a second and a second	and the second second	ويترا والإردوان ومعاليا أسهرار والاحاص و	*****	
And Party of the		Landar and an interesting		· · · · · · · · · · · · · · · · · · ·	**************************************	Mana Sources and with source	and the mount of the	and a subscription of the second s		ter have been been all and a start and	
	wine and a second second	him and the second	Anna and the second second second	har get with a second	Anterna the second second second	Normanian	a hard a constraint and a constraint and	-	********		
	-	renewalk the second	معين المعالية المراجل المالي المحالي المحالية المحالية المحالية المحالية المحالية المحالية المحالية المحالية ال	maplifusicities and when	and and the second and a second and a second	1	Mrianna Mappine Montana Ada	and the second state of th	an anti-angle and a second	and the state of t	ne jage division a provint of the state of the state
A Strange Strange		and the second second second		and an all and a second s	and a state of the second	A star and the second star				Warner Marine Marine	
- Agentalia	and a state of the		·	and a second second second	and and a state of the state of	Contraction of the second second	an a	and the second	1	And a second second	
and the second second	underente ^{ren} terenterenterenteren Generalderste der Stadier Stadierter	and a state and a state of the set the	Party and a state of the second state of the s	A Contractor and the second	Construction and the second se	and the second s	ي من راهندر از اللي الريمة معادية مراجع اليوسي ا من من المراجع التي تعريف التي تعريف الريمة	and the state of t	and the state of t		and the second
-	and an and the second second		Parrow and the second second	- Antonio -	frank ward	have a second and the				the contraction	and the second s
					Accession of the second second	And an advertising the second second				and a second	
minine		and an analysis and an and	and the second	and the state of t			*****		······································		
يوانل ميروابندي ومرام (معرو د الاجرو ر زايا م	ĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸ ĸĸĸĸĸĸĸ		ĨŢġĸĸġŊŢĦĊŢĔĸĸĸĬŢĬĬĬĔĹĸĔĬĊĸĬĬĊĸĔŎĬĬŎŎĬĬŎ ġſĸĹĬĬĿĹĬĬĹŢĿŎĿĸġĬſĸŇĿŎĬſĸĿĸŎſŎĿŎĬĬĬŎŎŎ	م بر	**************************************	۱۹ پارد ۸ در ۱۹ م ۲۰ رهنده ۲ بلیزید (بعد ۱۹ مرکز ۲۰ م ۲۰ م (۱۹ مرکز ۲۰ مرکز ۲۰ مرکز ۲۰ مرکز ۲۰ میکند ۲۰	an a	المرجعين الموركي المرجع من مرجع مع المرجع المرجع مرجع المرجع من المرجع المرج	المراجعة المراجعة المراجع المريحة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المر مراجع المراجعة المراجع	ؾڂڎڔ؞ ^ڛ ڡ؇ڹؾ؋ڔڣڔ؞؞ڹڣؿٵڡٷ ڡٷ ٩٩٩ ٩ ٩٩ من اردي اور ۹۵ مردي - ۲۰ ۱۹۹۹ م	۲۰۰ مارد است. ۱۹۰۰ میروند بازیکر میروند بازیکر میروند و از مارد میروند. ۱۹۰۰ میروند انتخاب است. است. است. استان میروند و از م
shipman	and the second sec	م م د د رو ا ما ^ر رو ا ک ^{ر س} ار د د و ساله ا	- care and the second and the second se	- marine and a start of the sta	have been and the second	Management	handle and many sectors and the	and the second and	monte and the monte of the	to be a start way of a start	
	nersen sin her site her site and site of the second second second second second second second second second se	an a	ania/2010/2010/00/00/00/00/00/00/00/00/00/00/00/00/	And the second sec	a philipped in the set of a strategy of the set of a strategy of the set of t	and a second s	e / pinyter diget on general de la la service en la la generaliste en tenter en general de la service de la	۵٬۰۰۱ می در ۱۳۵۰ میلی است. ۲۰۰ میلی می است. ۲۰۰۰ - ۲۰۰۰ میلی در این است. ۲۰۰ میلی ۲۰۰ میلی می مواند.	and a start of the second of the		مودر المرجع عوامه (الرجع و الان المحمول من المحمول من المرجع و المحمول المحمول من المحمول المحمول المحمول الم المحمول المراجع المرجع المحمول ا
and the second second		Property relations	and the and water and the	and the second second second		and a superior and the second s	المرجود المرجود المرجود المرجود المرجود		and-frank-prest-scatter, 17/100	and the second second second	*****
			, 1996, 1996, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997, 1997	1000 - 10000 - 1000 - 1000 - 1000 - 1000 - 1000 - 1000 - 1000 - 1000 - 1	and a property in the part of	Protection and a second s	and a second	***************************************	an an international and a state of the state	, 1997 - 2017 - 2017 - 2019	an a
			ويهدد المندسين المهارسي مناكلتها مناسبة	and an			- apropries and a second s	and the second second second			
and the second	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	and the state of the particulation of the state of the st	Standard and a strategic and a	- Weinerstein hunderen	and the second	Mark Australia Transferdance Milling		A second s	and the standard and the s	a the second the second the second	**************************************
	angup sa baran sa	and a second and a second s	MUNING CHANGE CONTRACT	the set of	halestate the second se		*****	1000 - 1000 - 1000 - 1000 - 1000 - 1000	1	*****	an a
1. Martin Contractor	uellan han manangrup di panjan ja	and the party set to be the set	a fa sea a state a sea a s	an far an	Anna yan daga su dana fan ana ana an	Landon al marine and an and an and an	and a second state of the second s	and the second se		and a state of the	an the subscription of the second
With Mailing Ma			An any specification in a second state of the	Manager and the second	An and an and an and a second s	And an and a state of a state of a	tariny ay projected from a main takan manakaya. Badil di Kabupatén d	and the second second second	and a second	and an alter of the second attended on the	and the second
www.coderwee.com		A start of the start and a start of the star	- Antonio antonio antonio antoni				AN STATISTIC AND	**************************************			and find and play that the second second second
،مەتل _ى تەرىپىدەردەر بەرىچىرىيە رەجەر تەرىپەدۇرار بارىتەر يەر	Vergeneze , Ma lfregenisten megafisten Vergenisten Mensken visiten Benderbaren	Construction of the second	Provide State State Street Street and Street	And the second	And a second	Particular South Contraction	ngalan Judo ya Nanangi Ata yana Masha y Najina kuma, malaku wa kuta ya kuta ya	and a state of the	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	and the second	**************************************
angraphia		bere and the second of the	-	and interaction	Palware stationer op all dramatica at	manner and	and the second second second second	Participant and the second second	and the second se		
alahan ang ala print Kanang ang ang ang ang ang ang ang ang an	agendersch fürste Visigt vor son verbieten.	the for her in the second s	de ingenijetski jednijetski filozova – filozovat si odvoda Namenijetski poslateni na odvane na poslateni da odva	1000 1000 1000 1000 1000	Construction of the second states of the second sta	Non-water and the second	nanazithdayatintiyithyon,wakimtey Aatta anadataa				and a second
		and a state of the second second	and the second state of th	and the second s	and the state of t	have a new second second	and a second second second second second	and a survey of the survey of	and the second sec	and the second state of the second	1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1
میکرد. میکرد و اطلاب است.	ſŊĊŦĹŊĊĸĊŦġŦŦŔŊĬĬĊĸĿĸĊŊĬĊĬŦŊĊĸŢŎĸŎĸ ĿŊĹŎĸŖĔĸſĊŢŎŦĸŊĿĸĔĸĊĬŔĬĬĊĿĸĿŊŦĬĸ	and a second second second second	Parapateter May Print Astronomy Para	Martin Contraction	And the product of th	Particular programmer and			and a second	and the second	
and and an and a start of	والمادية المربي والمحاصر والمحاصر والمحاصر	Richmon Print Printer	and the second states and the second se	- Marthanim ration	and the property and the	and a second sec				Canada and a state of the state	*****
and a stand of the st	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	والمراجع والم	an de la composition de la composition En composition de la c	and the second	Autorian Calendary and Calendary and Calendary	and the second s	and the second sec	and a second	and all the providence of the	a de la secono de la construcción d La construcción de la construcción d	n fan sen fan sen fan sen fan ferster sen ferster sen ferster sen ferster sen ferster sen ferster sen ferster s
white posterior	when a win weather the fight the fight the	en an haaf ganer hat the strategy is served	and the second second second second		anone was builded a survey of		www.www.www.www.www.www.	a line repairing research and	a three works where the		
and a Constant of the			Participation (PP) and a solution of the solut	and the second sec	Annanista fanta (alter a fanta)	Contraction to a traction for the		The second s			
-	- Marine the second second second		and the second	and a state of the second s	and a set of the set o	and the second second second	ter show when a factor of the starting	appinter man provide and a series of the	ter manual a state of the state of the		
Warning the					And a state of the	The second s	an the second	and a second of the second			appear the share of the second
And the second party		Parament Prophers and	A service and a se		Annual Contraction of the second s	And the second of the second s		and the second		ALALITA HOUSE AND ADDRESS OF ADDR	
	alalasiyilig ^a rminiyi waladaasiinida Ayaraaladaafiinina alaasiini dag		Manuter and all the manufactures all the	and the second state of th	₽,576	President and the President of the Presi	ang bergina yang bergina di kanan di k Kanan di kanan di kan	and the second	19. June 1	and a second	
and the second design of the	, , , , , , , , , , , , , , , , , , ,	ny a many analysis, have been stop	and a state of the second state		aller and the strength of the strength	والارار والمالية والمسيقة والمسيقة والمحالية	ورهي مورفون بودر برواف برواد ردوم و		-	the state of the s	
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	tentetintelleten sindette algemetten an ukterintette Physical State Constantion	Manufacture and an address of the second s	manglithanglapittin quadri atta ngi pyr Mana i ngi pagihina minipali angi pananana ti			and a second	andri (12) Minter, April Brukering, Supering and Statements and Statement and	//////////////////////////////////////	<u></u>		and a standard the state of the
				- mar monoring		and a state of the	-			المرجود الجراية الايرمين المراواتين	
and the second second		المرجع المرجع والمرجع والمرجع والمراجع والمراجع والمرجع	Paparate and a second and a second se	and an and the second	**************************************		*****	م المراجع الم المراجع المراجع	۲. د د او	الدخاني المحصور والمحالي والمحالية	
		portaneous and the second		and the statement of the second second second		and the second second second					and the second sec
and Married Married	*(154)************************************	And the second s	Freenonderservices and a standard and	مان می الاستان بازیکاری الارتباط مان می استان مان بیش الارتباط	an and the second second second	And the second s	lan han padamaterin yén di sen di sen di sen se Na Mala sen selada sen sen da sen sen di sen s			and a survey and a survey of	
-	and a second	and any of respected and and		netro in attraction and parts	and the second	An an an a second providence of the		and a man was a first of the			
			And a second		and the second sec	Conference and the second s				and the second	
mathinkin	without a state bags to a to a street	Marrie Marrie Constraint	and a second sec	and the providence of the second	anone winder	مردونه والدوال والمرادر أوالي المدر المرادي	······································				and the second second second
wei/window		tone and the second		and the second s	* JADuran Constant and Maria Maria Cal	A subscription of the second second	had a free way and the second states and the second states and the second states and the second states and the	angel all the second second second	and a provide a second s	haran and and the state	
	*****	New York Water Strate Station	man provident and the second	new trainwitten of the process	and the second		and the second shared and the second	and the second state of th	and and a state of the second of the	ماريد موري معري المحمد الم	-
and a second	na na ang ang ang ang ang ang ang ang an	10000000000000000000000000000000000000		and the second	and a second	and the second sec	and the state of the state of the solution of				
No. March								***	ومقير محتفر واسترويه محمول منع		
A Carlo Carlo	مى مەرىمىرىكى بىرىمىيە يەلىلىرى بىر تەرىر ئەرىھا بىر ئاتامىيە يەلىلىرىر بېر تەرىز	have a support of the second second		and a state way and a state of the	Provinsion and the second second	And an and a second from the second s		and the second s	······································	Carlo and a second and a second	and the second s
the second second		the hard a strange of the second			annual and the second strategicture of			and the second			
Alt, 100,000,000		Party and the second second second second								Parter for the second second second	
			·	n - 1	i 10	00			1		
	mulation										5 s
sker stin			1111	1		1			1111	1	
1			a constant to be	المعد الكلابات	والدور وأبال وبالرواهما	LALA Ashia	ببابات أحادتها	سيقلعت مقاديت	hand a land	بعابير والعديرية	an maharata a s
1	anteski i Domini	فيشرقه فيتحد المتحد والأهار	HANKARANI KAMPINA	AVMY THUM THE	auto/selitebrail. April 1986	****	radio di Padama	all and a second	a yaqaa ahaa yaqaa yaqaa	alaa hada hada hadaa hada hada hada hada	wany many sites in a second
1	air-at-			14							
1	est-statestime	an the second	,								
1	an the second				1 N	الماليمان بتدييه الخسطناني	mathematican .	ومعيدية بالعبة سيبط	A		أسيبية ليتبعاد الأشيط
1	******	and have a second	www.	Mary Adverte	ANT PROPERTY AND A DESCRIPTION OF A DESC			Inder The Property of the second seco	With the second second	ستطرحه الزواب الجمريحة المجزم	A REAL FRANCE
1	**************************************	Marrador and and	www.	*******	ant Madamata Abarah	and the second second		water water		all a subsection of the second se	hasethered
1	aning day of history generation and a second	Manager and the second se		*******	IIIII WW			and the Made		alandan da ang ang ang ang ang ang ang ang ang an	1111
1	84444 ⁴ 4444 ⁴ 444444444 ₁	Maria Malan White and the		*********		. Mada	1		. ME-16-04-4-64/14		1111
1	estydyddiaethaeth gangelan yw	han ha		******	Munhum	who War Manusia				arren ar A a a a a a a a a a a a a a a a a a a a	how
isker stir I WWWMM	entertennen ministernen ministernen	all and a state of the state of		**************************************	n hundern	martin War administration			. nashering was a shere	arten ander ander ander Reference ander ander	1111
hitele Martine maragericans autoreactor	genegiskingernegister mynage/ ^{se} ndersende	and the second sec		**************************************	Munhum	number Man Manusking/	*~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		nirtan satur site and and and and and a second s	hunn
hitele Martine maragericans autoreactor	genegiskingernegister mynage/ ^{se} ndersende	Maria and a second		┺┺╪╌╗┍┍┝┶┶╌┍╄┓ ╘╾╼╲╿╽╱╼╼┍╕╌╿╼╕ ┲┺╼═┠┎╼╼┠╸═┺╧╱┶╴	n famburn	nutur ^{Man} dunising/		harrow and an	ana an	بىنىلىرىيەتلەرىمەتلىرىمەتلىر سەتورلىدىمىرەتسىرى الىرىيەتلىرىمىرىلەتلىرى	hunn
hitele Martine maragericans autoreactor	genegiskingernegister mynage/ ^{se} ndersende	hterrow hterrow		┺┺┙╼┲╍┶┍┸┶╍┍┸┶╛ ┺╾╾╲╵└╻╴┺╼┍╡╌┑┺╕ ┲┺┲╼┟╵┅╍┝╘╽┍╄╅╱╘╕		nun Martanan		harrow warnes	nan ya na	arran warmen arrender Anners yn rei yn yn yn Hyddydd fawraid yn yd	hunny
h h h h h h h h h h h h h h h h h h h	genegeleiten och som	hterrow hterrow				n.n./Www.unu.		hannen kunnen hannen kunnen hannen kunnen	nanienienienienienienienienienienienienien	arrana arrana Arrena arrana Arrena arrana	hunny
h h h h h h h h h h h h h h h h h h h	genegeleiten och som	hterrow hterrow				nun Martania Nadarda Balladayi		hannen annen hannen annen	nannannurrainn nannannurrainn Nurgipherplechtigener e. dalainn an taint	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	hunny
h h h h h h h h h h h h h h h h h h h	genegiskingernegister mynage/ ^{se} ndersende	hterrow hterrow				nun Martanungu Ngalertaripatisat Agripatistagi kerang		hannan hannan hannan	anteriorani anteriorani anteriorani anteriorani	arran arran arran ar Arran ar an an ar an ar Arran feystyr reid ar	hunner hunner
h h h h h h h h h h h h h h h h h h h	genegeleiten och som	hterrow hterrow				nun Markanainus Nadardan Paddadaga Afartastariya Namara	an a	hanner anner hanner anner hanner anner Vanner anner		annen annen annen annen Lannen annen annen Lannen annen annen annen Lannen annen ann	hunner hunner
hinder hander hander hander hander hander hander	anantanana man Manana pertember Manana Pertember Manana	ul hannon Mananan Mangadananan		white	4./w/194644-4-4-4-4	nun Martanusa Nadar santarikadan Narias kirafi karana		hannan hannan hannan		net in the second	hyhmannon hyhianyahyanyahya hyhianyahyanyaanya
hinder hander hander hander hander hander hander	genegeleiten och som	ul hannon Mananan Mangadananan		white	4./w/194644-4-4-4-4	nun Maranay Nyaipataipatai Nyaipataipataipatai Nyaipataipataipa	annen er fan	hannann hannann hannann hannann		net in the second	hunner hunner

Figure S3 related to Figure 5. *In vivo* population imaging of SpikeyGi from UF2P microscope. (A)
 Fluorescence traces from simultaneous recordings across 129 S1 neurons from the UF2P microscope
 acquired at 803 Hz. Raw images were denoised with DeepVID. For visualization purposes, traces are

812 detrended with 2.5 sec moving average and low pass filtered at 200 Hz. Red lines indicate air puff whisker

813 stimulus. Detected action potentials are plotted in Figure 5B. (B) Magnified view of example traces across

814 different cells. Squares denote traces corresponding to shaded regions of the same color indicated in [A].

815 Red lines indicate air puff whisker stimulus.

27

### 816 **REFERENCES**

- 817
- Abdelfattah, A.S., Kawashima, T., Singh, A., Novak, O., Liu, H., Shuai, Y., Huang, Y.-C., Campagnola, L.,
- 819 Seeman, S.C., Yu, J., *et al.* (2019). Bright and photostable chemigenetic indicators for extended in vivo
- voltage imaging. Science *365*, 699-704.
- Abdelfattah, A.S., Valenti, R., Zheng, J., Wong, A., Team, G.P., Podgorski, K., Koyama, M., Kim, D.S., and
- 822 Schreiter, E.R. (2020). A general approach to engineer positive-going eFRET voltage indicators. Nat
- 823 Commun 11, 3444.
- Adam, Y., Kim, J.J., Lou, S., Zhao, Y., Xie, M.E., Brinks, D., Wu, H., Mostajo-Radji, M.A., Kheifets, S., Parot,
- 825 V., et al. (2019). Voltage imaging and optogenetics reveal behaviour-dependent changes in hippocampal
- 826 dynamics. Nature *569*, 413-417.
- 827 Amir, W., Carriles, R., Hoover, E.E., Planchon, T.A., Durfee, C.G., and Squier, J.A. (2007). Simultaneous
- imaging of multiple focal planes using a two-photon scanning microscope. Opt Lett *32*, 1731-1733.
- 829 Bando, Y., Sakamoto, M., Kim, S., Ayzenshtat, I., and Yuste, R. (2019). Comparative Evaluation of
- Genetically Encoded Voltage Indicators. Cell Rep 26, 802-813 e804.
- 831 Chamberland, S., Yang, H.H., Pan, M.M., Evans, S.W., Guan, S., Chavarha, M., Yang, Y., Salesse, C., Wu,
- H., Wu, J.C., et al. (2017). Fast two-photon imaging of subcellular voltage dynamics in neuronal tissue
- 833 with genetically encoded indicators. Elife 6.
- 834 Charan, K., Li, B., Wang, M., Lin, C.P., and Xu, C. (2018). Fiber-based tunable repetition rate source for
- deep tissue two-photon fluorescence microscopy. Biomed Opt Express *9*, 2304-2311.
- 836 Chen, J.L., Voigt, F.F., Javadzadeh, M., Krueppel, R., and Helmchen, F. (2016). Long-Range population
- dynamics of anatomically defined neocortical networks. Elife 5, e14679.
- 838 Cheng, A., Goncalves, J.T., Golshani, P., Arisaka, K., and Portera-Cailliau, C. (2011). Simultaneous two-
- photon calcium imaging at different depths with spatiotemporal multiplexing. Nature methods *8*, 139-142.
- 841 Clough, M., Chen, I.A., Park, S.W., Ahrens, A.M., Stirman, J.N., Smith, S.L., and Chen, J.L. (2021). Flexible
- simultaneous mesoscale two-photon imaging of neural activity at high speeds. Nat Commun *12*, 6638.
- 843 Demas, J., Manley, J., Tejera, F., Barber, K., Kim, H., Traub, F.M., Chen, B., and Vaziri, A. (2021). High-
- 844 speed, cortex-wide volumetric recording of neuroactivity at cellular resolution using light beads
- 845 microscopy. Nat Methods *18*, 1103-1111.
- 846 Feldmeyer, D., Brecht, M., Helmchen, F., Petersen, C.C., Poulet, J.F., Staiger, J.F., Luhmann, H.J., and
- 847 Schwarz, C. (2012). Barrel cortex function. Progress in neurobiology.

- 28
- 848 Huang, L., Ledochowitsch, P., Knoblich, U., Lecoq, J., Murphy, G.J., Reid, R.C., de Vries, S.E., Koch, C.,
- Zeng, H., Buice, M.A., et al. (2021). Relationship between simultaneously recorded spiking activity and
- 850 fluorescence signal in GCaMP6 transgenic mice. Elife 10.
- Jin, L., Han, Z., Platisa, J., Wooltorton, J.R., Cohen, L.B., and Pieribone, V.A. (2012). Single action
- potentials and subthreshold electrical events imaged in neurons with a fluorescent protein voltage
- 853 probe. Neuron *75,* 779-785.
- 854 Kazemipour, A., Novak, O., Flickinger, D., Marvin, J.S., Abdelfattah, A.S., King, J., Borden, P.M., Kim, J.J.,
- Al-Abdullatif, S.H., Deal, P.E., *et al.* (2019). Kilohertz frame-rate two-photon tomography. Nat Methods *16*, 778-786.
- Kim, K.H., Buehler, C., Bahlmann, K., Ragan, T., Lee, W.-C.A., Nedivi, E., Heffer, E.L., Fantini, S., and So,
- P.T.C. (2007). Multifocal multiphoton microscopy based on multianode photomultiplier tubes. Opt
- 859 Express 15, 11658-11678.
- Krull, A., Buchholz, T.-O., and Jug, F. (2018). Noise2Void Learning Denoising from Single Noisy Images.
  arXiv:181110980 [cs].
- 862 Lecoq, J., Oliver, M., Siegle, J.H., Orlova, N., Ledochowitsch, P., and Koch, C. (2021). Removing
- independent noise in systems neuroscience data using DeepInterpolation. Nat Methods 18, 1401-1408.
- Li, X., Zhang, G., Wu, J., Zhang, Y., Zhao, Z., Lin, X., Qiao, H., Xie, H., Wang, H., Fang, L., et al. (2021).
- 865 Reinforcing neuron extraction and spike inference in calcium imaging using deep self-supervised
- 866 denoising. Nat Methods 18, 1395-1400.
- 867 Piatkevich, K.D., Bensussen, S., Tseng, H.A., Shroff, S.N., Lopez-Huerta, V.G., Park, D., Jung, E.E.,
- 868 Shemesh, O.A., Straub, C., Gritton, H.J., et al. (2019). Population imaging of neural activity in awake
- 869 behaving mice. Nature *574*, 413-417.
- 870 Platisa, J., Han, Z., and Pieribone, V.A. (2020). Different categories of fluorescent proteins result in GEVIs
- with similar characteristics. bioRxiv, 2020.2005.2006.081018.
- 872 Platisa, J., Vasan, G., Yang, A., and Pieribone, V.A. (2017). Directed Evolution of Key Residues in
- 873 Fluorescent Protein Inverses the Polarity of Voltage Sensitivity in the Genetically Encoded Indicator
- 874 ArcLight. ACS Chem Neurosci 8, 513-523.
- 875 Podgorski, K., and Ranganathan, G. (2016). Brain heating induced by near-infrared lasers during
- 876 multiphoton microscopy. J Neurophysiol *116*, 1012-1023.
- Sjulson, L., and Miesenbock, G. (2007). Optical recording of action potentials and other discrete
- physiological events: a perspective from signal detection theory. Physiology (Bethesda) 22, 47-55.

- 29
- Vaswani, A., Shazeer, N., Parmar, N., Uszkoreit, J., Jones, L., Gomez, A.N., Kaiser, Ł., and Polosukhin, I.
- 880 (2017). Attention is all you need. Paper presented at: Advances in neural information processing
- 881 systems.
- 882 Villette, V., Chavarha, M., Dimov, I.K., Bradley, J., Pradhan, L., Mathieu, B., Evans, S.W., Chamberland, S.,
- 883 Shi, D., Yang, R., *et al.* (2019). Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake
- 884 Behaving Mice. Cell *179*, 1590-1608.e1523.
- Weisenburger, S., Tejera, F., Demas, J., Chen, B., Manley, J., Sparks, F.T., Martinez Traub, F., Daigle, T.,
- Zeng, H., Losonczy, A., et al. (2019). Volumetric Ca(2+) Imaging in the Mouse Brain Using Hybrid
- 887 Multiplexed Sculpted Light Microscopy. Cell *177*, 1050-1066 e1014.
- 888 Wilt, B.A., Fitzgerald, J.E., and Schnitzer, M.J. (2013). Photon shot noise limits on optical detection of
- neuronal spikes and estimation of spike timing. Biophys J *104*, 51-62.
- 890 Wu, J., Liang, Y., Chen, S., Hsu, C.L., Chavarha, M., Evans, S.W., Shi, D., Lin, M.Z., Tsia, K.K., and Ji, N.
- 891 (2020). Kilohertz two-photon fluorescence microscopy imaging of neural activity in vivo. Nat Methods
- 892 17, 287-290.
- Zhang, K., Zuo, W., Chen, Y., Meng, D., and Zhang, L. (2017). Beyond a Gaussian Denoiser: Residual
- Learning of Deep CNN for Image Denoising. IEEE Trans Image Process 26, 3142-3155.
- Zhang, T., Hernandez, O., Chrapkiewicz, R., Shai, A., Wagner, M.J., Zhang, Y., Wu, C.H., Li, J.Z., Inoue, M.,
- 896 Gong, Y., et al. (2019). Kilohertz two-photon brain imaging in awake mice. Nat Methods 16, 1119-1122.

897