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# Scanless two-photon voltage imaging

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# 1 Scanless two-photon voltage imaging

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15  
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17 generated holography

## 18 19 **Abstract**

20  
21 Parallel light-sculpting methods have been used to perform scanless two-photon photostimulation of  
22 multiple neurons simultaneously during all-optical neurophysiology experiments. We demonstrate that  
23 scanless two-photon excitation also enables high-resolution, high-contrast, voltage imaging by efficiently  
24 exciting fluorescence in a large fraction of the cellular soma. We present a thorough characterisation of  
25 scanless two-photon voltage imaging using existing parallel approaches and lasers with different repetition  
26 rates. We demonstrate voltage recordings of high frequency spike trains and sub-threshold depolarizations  
27 in intact brain tissue from neurons expressing the soma-targeted genetically encoded voltage indicator  
28 JEDI-2P-kv. Using a low repetition-rate laser, we perform recordings from up to ten neurons simultaneously.  
29 Finally, by co-expressing JEDI-2P-kv and the channelrhodopsin ChroME-ST in neurons of hippocampal  
30 organotypic slices, we perform single-beam, simultaneous, two-photon voltage imaging and  
31 photostimulation. This enables in-situ validation of the precise number and timing of light evoked action  
32 potentials and will pave the way for rapid and scalable identification of functional brain connections in intact  
33 neural circuits.

## 34 Introduction

35 Deciphering the logic and syntax of neural computation is a central goal in neuroscience and requires  
36 methods to record (read-out) and manipulate (write-in) the activity of individual neurons.  
37 Electrophysiological methods have proven instrumental towards achieving this goal since they can read  
38 and write neural activity with high fidelity. However, while extracellular probes can record from large  
39 populations, they have limited spatial resolution and cannot excite or inhibit specific neurons. In contrast,  
40 whole-cell patch-clamp methods can manipulate and record the electrical activity of targeted neurons but  
41 are hard to achieve *in vivo* even for a handful of neurons simultaneously and are unsuitable for longitudinal  
42 (chronic) studies. Furthermore, all electrophysiological methods have limited access to smaller cellular  
43 compartments of neurons (such as axons, distal dendrites, spines and boutons). These limitations have  
44 stimulated the development of a plethora of minimally invasive photonic approaches, combining advanced  
45 optical methods with light-sensitive proteins, such as genetically encoded fluorescent indicators and  
46 optogenetic actuators, for recording and manipulating neural activity, respectively<sup>1-3</sup>.

47  
48 In the nervous system, calcium ions regulate a broad range of processes and generate versatile intracellular  
49 signals<sup>4</sup>. Since action potentials lead to a large elevation of intracellular calcium, which can last an order of  
50 magnitude longer than the action potentials themselves<sup>5</sup>, the developments of synthetic<sup>6</sup> and genetically  
51 encoded<sup>7</sup> fluorescent calcium indicators (GECIs) capable of reporting changes in intracellular calcium were  
52 extremely important scientific breakthroughs. GECIs can be targeted to sub-cellular compartments and  
53 specific cell types<sup>8,9</sup>. Their long-term expression in intact tissues and organisms<sup>2</sup> enables the repeated  
54 observation of individual cells. Calcium transients last significantly longer than the underlying voltage  
55 fluctuations, facilitating the detection of neural activity, but also limiting the quantification of spike firing rate  
56 and timing. Furthermore, GECIs are not well-suited for detecting sub-threshold voltage changes and  
57 hyperpolarizations resulting from synaptic and neuromodulatory inputs<sup>10</sup>.

58  
59 Voltage indicators, which generate optical signals whose magnitude varies as a function of membrane  
60 potential, promise to address many of the aforementioned limitations of GECIs<sup>11</sup>. Following the first optical  
61 recordings of membrane potential with a synthetic dye<sup>12</sup>, voltage-sensitive indicators have undergone  
62 continual advancements, including improved synthetic dyes<sup>13</sup>, genetically encoded voltage indicators  
63 (GEVIs) and hybrid GEVIs<sup>14</sup>. However, detecting voltage spikes with GEVIs requires millisecond-timescale  
64 imaging, two orders of magnitude faster than generally required for GECIs. This technical challenge has  
65 limited the broad adoption of GEVIs for population imaging with cellular resolution.

66  
67 The majority of voltage imaging experiments have relied on widefield, one-photon (1P) illumination and  
68 detection. The resulting mesoscopic observations of population activity have enabled investigation of the  
69 functional organisation and dynamics of cell-type specific excitatory and inhibitory cortical circuits<sup>15,16</sup>. The  
70 lack of optical sectioning of 1P widefield microscopy has been overcome using sparse labelling strategies<sup>17</sup>

71 or sculpted illumination<sup>18–21</sup>. However, these strategies are not suitable for multitarget voltage imaging in  
72 densely labelled scattering samples with cellular resolution, such as mammalian in-vivo preparations.

73

74 In principle, the optical sectioning inherent to two-photon excitation can be used to overcome these  
75 problems<sup>22</sup>, and two-photon laser scanning microscopy (2P-LSM) is commonly used to perform calcium  
76 imaging in scattering tissue<sup>23</sup>. However, the acquisition rate of conventional 2P-LSM is limited and  
77 millisecond transients such as action potentials can only be detected by drastic reduction of the field of  
78 view<sup>24–27</sup>. As a result, several specialised scanning-based techniques have been developed to image neural  
79 activity across larger areas at kilohertz rates<sup>28–35</sup> and have yielded spectacular results, such as recording  
80 the voltage dynamics of cortical neurons in layer 5 in awake behaving mice. However, these methods are  
81 extremely technically demanding, have thus far been limited to imaging a few cells simultaneously, and  
82 have not yet been demonstrated to be compatible with two-photon optogenetics, as required for two-photon,  
83 all-optical neurophysiology experiments.

84

85 Here, we propose an alternative approach for high-contrast, high-resolution, voltage imaging in densely  
86 labelled samples that is compatible with simultaneous two-photon optogenetic stimulation. Our method  
87 leverages existing scanless two-photon excitation approaches<sup>36–39</sup> and the recently developed soma-  
88 targeted GEVI JEDI-2P-kv<sup>40</sup>. We demonstrate that, in combination with temporal focusing (TF)<sup>41–43</sup>, the  
89 three light-sculpting approaches commonly used for scanless two-photon photoactivation —Generalised  
90 Phase Contrast (GPC)<sup>36,38</sup>, low numerical aperture (NA) Gaussian beams (such as 3D-SHOT)<sup>39,44</sup> and  
91 Computer-Generated Holography (CGH)<sup>45,46</sup>— enable voltage imaging in mammalian cells. By performing  
92 simultaneous imaging and electrophysiology, we provide a thorough quantitative comparison of these  
93 illumination modalities. Next, by viral expression of JEDI-2P-kv in mouse hippocampal organotypic slices,  
94 we show that 2P-TF-GPC enables high spatiotemporal resolution voltage imaging of neural activity in  
95 extremely densely labelled preparations. We further demonstrate the detection of high-frequency spike  
96 trains and subthreshold membrane depolarizations with amplitudes on the order of excitatory postsynaptic  
97 potentials (PSPs). Capitalising on the overlapping spectra of JEDI-2P-kv and the channelrhodopsin  
98 ChroME-ST<sup>44</sup>, we demonstrate simultaneous two-photon voltage imaging and photostimulation in multiple  
99 cells. This approach enables *in-situ* characterisation of the light-induced spiking properties of a population  
100 of neurons. Collectively, these results pave the way for studying neural function with two-photon all-optical  
101 neurophysiology in highly-scattering, densely labelled preparations.

## 102 **Results**

### 103 **Scanless two-photon voltage imaging with sculpted, temporally focused excitation**

104 The optical setup (Figure 1a, Supplementary Figure 1 and Table S1) was comprised of two independent  
105 excitation paths, one designed to generate temporally focused (TF) Generalized Phase Contrast (GPC)  
106 patterns<sup>36,38</sup> or low NA Gaussian spots (similar to 3D-SHOT<sup>39</sup>), and the second for TF-Computer Generated  
107 Holography (CGH)<sup>37</sup>. These paths were combined prior to the microscope objective with a polarising beam  
108 splitter. Each excitation path was designed to generate temporally focused spots with dimensions matching  
109 the typical size of a neuronal soma (12  $\mu\text{m}$  lateral full width at half maximum (FWHM) and  $\sim 9$   $\mu\text{m}$  axial  
110 FWHM for all modalities (Figure 1b, c, Supplementary Note 2, Supplementary Figures 2-5)). The  
111 fluorescence from 1  $\mu\text{m}$  microspheres excited with a 12  $\mu\text{m}$  GPC spot was recorded and found to have an  
112 axial FWHM of 3.7  $\mu\text{m}$  demonstrating sub-cellular axial resolution (Figure 1c, right panel). In all cases,  
113 emitted fluorescence was detected by an sCMOS camera, effective pixel size 0.1625  $\mu\text{m}$ . The nominal field  
114 of excitation of each of the light sculpting approaches was 250 x 250  $\mu\text{m}^2$ <sup>6,34,35</sup>. However, the effective  
115 imaging field of view was limited in one dimension by the number of sCMOS rows readout simultaneously  
116 at a given acquisition rate (see Methods). The system was equipped with three different laser sources, two  
117 high repetition rate oscillators (as commonly used for two-photon laser scanning microscopy; 80 MHz, 920  
118 or 940 nm, 100 fs, 12.5 nJ and 50 nJ pulse energies) and a third low repetition rate, high pulse energy laser  
119 (250 kHz, 940 nm, 100 fs, up to 2.5  $\mu\text{J}$  energy per pulse).

120 We compared the performance of three excitation modalities (2P-TF-GPC, 2P-TF-Gaussian and 2P-TF-  
121 CGH) for scanless two-photon voltage imaging using a high repetition rate laser source, as typically used  
122 for conventional 2P-LSM. We transiently expressed a recently developed, negative-going, voltage indicator  
123 optimised for two-photon excitation (JEDI-2P-kv<sup>40</sup>), in mammalian (CHO) cells (Figure 2a). We controlled  
124 the membrane potential of individual cells using whole-cell patch-clamp electrophysiology and  
125 simultaneously performed two-photon voltage imaging. We used three different protocols, hereafter named  
126 1, 2 and 3 (Figure 2b), to test the feasibility of scanless two-photon voltage imaging and to assess the  
127 advantages and disadvantages of each parallel approach.

128 Protocol 1 was used to quantify the voltage sensitivity of fluorescence of cells expressing JEDI-2P-kv. The  
129 responses of patched cells to three 100 ms, 100 mV voltage steps were recorded at 100 Hz under  
130 continuous illumination (power density: 0.88  $\text{mW } \mu\text{m}^{-2}$ , 100  $\text{mW}$  per cell) for 3 seconds. Voltage responses  
131 were clearly observed as a decrease in fluorescence with 2P-TF-GPC, 2P-TF-Gaussian and 2P-TF-CGH  
132 (Figure 2b, left panel). For most cells, we observed variability between the response amplitude measured  
133 at different membrane locations. Since differences in voltage responsivity of the fluorescence originating  
134 from different portions of the membrane were random, this is plausibly due to differences in plasma  
135 membrane trafficking and protein folding.

136 All data acquired using protocol 1 (n = 41 cells) were pooled and used to establish and validate an analysis  
137 pipeline capable of automatically identifying and segmenting neurons and of detrending the optical traces  
138 (Supplementary Figure 6). Due to the similarities between the data obtained with scanless two-photon  
139 voltage imaging and single photon voltage imaging with widefield detection, it was possible to develop an  
140 analysis pipeline based on existing open-source packages. Compared with results obtained by calculating  
141 the unweighted mean of all pixels within segmented cells, the regression-based pixel weighting  
142 algorithm<sup>14,49-51</sup> (Methods, Supplementary Note 3), which improved segmentation, was found to increase -  
143  $\% \Delta F / F_0$  for all modalities ( $33.8 \pm 9.5$  vs.  $43 \pm 11.7$ , mean  $\pm$  s.d.,  $p < 0.00001$ , n=41, Supplementary Figure  
144 7b), resulting in values in accordance with those previously reported<sup>40</sup>. No significant difference in SNR  
145 (signal amplitude divided by the standard deviation of the baseline signal) was found between the two  
146 approaches ( $59.4 \pm 30.2$  vs.  $59.6 \pm 31.3$ ,  $p = 0.9158$ , n=41, Supplementary Figure 7c), a result of the fact  
147 that the improved segmentation contained approximately half of the pixels of the initial segmentation ( $16263$   
148  $\pm 2180$  vs.  $8962 \pm 2676$ ,  $p < 0.00001$ , n=41, Supplementary Figure 7d). However, the location of these  
149 pixels coincided with the exterior cell membrane (Supplementary Figure 7d, inset), the most voltage-  
150 sensitive, which compensated for the effective reduction in photon count. We found that the final traces  
151 generated using the weighted pixel mask exhibited slightly more photobleaching than the traces generated  
152 with the original segmentation ( $0.82 \pm 0.02$  vs.  $0.80 \pm 0.03$ ,  $p < 0.00001$ , n=41, Supplementary Figure 7e).  
153 This is likely the result of two factors. Firstly, responsive pixels imaged with high contrast are more likely to  
154 be retained in the second segmentation step. These pixels are those where the cellular equator coincided  
155 with the focal plane, where the excitation power density (and presumably photobleaching) is highest.  
156 Secondly, the voltage responsive fluorophores are more likely to be tethered to the membrane, less mobile  
157 and hence more susceptible to photobleaching.

158 Having established the analysis pipeline, we then compared the three excitation modalities (Supplementary  
159 Figure 8). 2P-TF-GPC, 2P-TF-Gaussian and 2P-TF-CGH were all found to be suitable for scanless two-  
160 photon voltage imaging. Data obtained with 2P-TF-CGH exhibited the highest signal-to-noise ratio ( $81.6 \pm$   
161  $35.3$ , n = 15), almost double that of 2P-TF-GPC ( $48.5 \pm 19.2$ ,  $p = 0.00222$ , n = 17) and 2P-TF-Gaussian  
162 ( $43.9 \pm 20.3$ ,  $p = 0.00608$ , n = 9). We hypothesised this was because the high spatial density of photons in  
163 speckle grains results in more efficient two-photon excitation. This hypothesis was confirmed by simulations  
164 (Supplementary Note 2, Supplementary Figure 5).

165  
166 Since the high density of photons in speckles also increase the likelihood of non-linear photophysics (for  
167 instance photobleaching<sup>52</sup>), we designed and used a different protocol (Protocol 2) to investigate the extent  
168 of these non-linear effects as a function of the excitation power density ( $0.66 - 1.55 \text{ mW } \mu\text{m}^{-2}$  corresponding  
169 to  $75 - 175 \text{ mW}$  per cell). Protocol 2 consisted of three 100 ms, 100 mV voltage steps, 200 ms illumination  
170 pulses centred on each voltage step, and 2.5 s inter-pulse intervals (Figure 2b, middle panel). For all  
171 modalities, the baseline fluorescence ( $F_0$ ) increased quadratically as a function of power density (Figure  
172 2c, first panel), as expected with two-photon illumination and indicating that fluorescence excitation was not

173 saturated at any of the powers used. Furthermore, the SNR increased linearly as a function of power density  
174 (Figure 2c, second panel,  $R^2 = 0.999$  (2P-TF-GPC),  $R^2 = 0.995$  (2P-TF-Gaussian),  $R^2 = 0.998$  (2P-TF-  
175 CGH)), confirming that experiments were performed in the shot-noise limited regime rather than being  
176 limited by the read noise of the detector. On this basis, all SNR estimates stated hereafter were calculated  
177 as  $SNR = (-\Delta F/F_0) \sqrt{F_0}^{53}$ .

178

179 The SNR of the responses to 100 mV steps (Protocol 2) was higher at all excitation power densities with  
180 2P-TF-CGH than with 2P-TF-GPC or 2P-TF-Gaussian, (Figure 2c, second panel, Supplementary Figure  
181 9a), though the 2P-TF-CGH fluorescent transients exhibited a systematically lower average  $-\% \Delta F/F_0$  than  
182 when using 2P-TF-GPC. This difference increased as a function of power density (Figure 2c, third panel,  
183 Supplementary Figure 9b). Photostability, defined as the ratio between the integral of the baseline  
184 fluorescent trace to  $F_0 \cdot n_t$  where  $F_0$  represents the fluorescence in the first frame and  $n_t$  the number of  
185 baseline fluorescence timepoints (schematic diagram, Figure 2c, fourth panel, inset), decreased as a  
186 function of excitation power in all cases (Figure 2c, fourth panel). No significant difference was observed  
187 between the different modalities (Supplementary Figure 9c). Photorecovery, quantified as the ratio of  
188 fluorescence after dark intervals to the original fluorescence (schematic diagram, Figure 2c, fourth panel,  
189 inset), was over 97% following 2.5 s dark inter-pulse intervals when excited with 2P-TF-Gaussian and 2P-  
190 TF-GPC, which is consistent with previous observations<sup>28</sup>. In the case of 2P-TF-CGH, the photorecovery  
191 decreased as a function of excitation power density and was lower than the other modalities (<95% for  
192 power densities greater than  $0.88 \text{ mW } \mu\text{m}^{-2}$  (100 mW per cell),  $p=0.01$ , Figure 2c, fifth panel, Supplementary  
193 Figure 9d).

194

195 Finally, we used protocol 3 (Figure 2b, right panel) to assess the detection of short, action-potential like  
196 transients with scanless two-photon voltage imaging. Cells were illuminated continuously for 500 ms (power  
197 density:  $1.33 \text{ mW } \mu\text{m}^{-2}$ , corresponding to 150 mW per cell) and the fluorescence response to a 20 Hz train  
198 of 10 rectangular pulses (100 mV amplitude, 3 ms duration) was recorded with a 1 kHz acquisition frequency  
199 (see Methods). The transients recorded using 2P-TF-GPC, 2P-TF-Gaussian and 2P-TF-CGH, had  $-\% \Delta F/F_0$   
200 values of  $45 \pm 14 \%$ ,  $42 \pm 16 \%$  and  $26 \pm 6 \%$  ( $n = 8-11$ ) respectively (Supplementary Figure 10b). For all  
201 modalities, the average SNR was greater than 11, demonstrating that action-potential-like signals can be  
202 reliably detected in single trials with scanless two-photon voltage imaging. As per data presented in  
203 supplementary figures 10c and d, the highest SNR data was acquired using 2P-TF-CGH ( $20.5 \pm 6.2$ )  
204 compared with  $13.9 \pm 3.6$  (2P-TF-GPC) and  $11.5 \pm 4.1$  (2P-TF-Gaussian),  $n = 8-11$ ), at the cost of lower  
205 photostability ( $0.86 \pm 0.07$  (2P-TF-CGH) versus  $0.92 \pm 0.08$  (2P-TF-GPC) and  $0.89 \pm 0.12$  (2P-TF-  
206 Gaussian),  $n = 8-11$ ).

207

208 Overall, these results confirm that 2P-TF-GPC, 2P-TF-Gaussian and 2P-TF-CGH can successfully be  
209 applied to scanless two-photon voltage imaging, albeit with different advantages and limitations. Since the



210 SNR of data acquired using 2P-TF-CGH was significantly higher than for 2P-TF-Gaussian or 2P-TF-GPC,  
211 we consider it the optimal modality for imaging large numbers of cells simultaneously, for short periods,  
212 with a given incident power. For prolonged recordings (continuous illumination for hundreds of milliseconds  
213 or more) of neurons labelled with JEDI-2P-kv, we would recommend 2P-TF-GPC or 2P-TF-Gaussian, since  
214 we observed lower photobleaching and higher photorecovery with these methods than with 2P-TF-CGH.  
215 Although no significant performance differences were found between 2P-TF-Gaussian and 2P-TF-GPC  
216 (Figure 2c, Supplementary Figures 8 and 9), 2P-TF-Gaussian requires higher power at the laser output for  
217 a given SNR. Specifically, uniform illumination of the somal membrane was achieved with 2P-TF-Gaussian  
218 by expanding and subsequently cropping the beam (Supplementary Note 2, Supplementary Figure 4) and  
219 thus was ~3 times less power-efficient than 2P-TF-GPC<sup>54</sup>. However, it is perhaps the simplest approach to  
220 implement, and hence a good solution given a sufficiently powerful laser source.

221

### 222 **Scanless voltage imaging of neural activity in hippocampal organotypic slices with two-photon,** 223 **temporally focused Generalised Phase Contrast**

224 We set out to identify the imaging conditions (specifically the power densities and acquisition rates) required  
225 to observe neural activity ranging from high-frequency spike trains to sub-threshold depolarizations in  
226 densely labelled samples. We also aimed to determine whether the necessary imaging conditions perturb  
227 neural activity or otherwise impact cellular physiology. We performed simultaneous 2P-TF-GPC imaging  
228 and whole cell-patch clamp recordings of granule cells located in the dentate gyrus (DG) of organotypic  
229 slices bulk-transduced with JEDI-2P-kv (see Methods). Expression of JEDI-2P-kv in the granule cells of the  
230 DG was well localised to the plasma membrane, with no evidence of intracellular aggregation (Figure 3a).  
231 Even though granule cells are extremely closely packed in DG, due to the optical sectioning conferred by  
232 temporally focused, targeted illumination, we were able to image individual neurons with high-contrast and  
233 high-resolution in this challenging preparation (Figure 3b).

234 Using protocol 1, we confirmed we could detect 100 mV depolarizations in densely labelled, scattering  
235 organotypic slices with comparable  $-\% \Delta F/F_0$  ( $43 \pm 8$ ) to that obtained in CHO cells ( $51 \pm 11$ ) ( $n > 15$  cells,  
236 accounting for differences in the resting potential between neurons and CHO cells, Supplementary Figures  
237 11 and 12). No significant difference was observed in SNR ( $69 \pm 25$  (CHO),  $50 \pm 30$  (organotypic slices),  $n$   
238  $> 15$  cells, Supplementary Figure 12e) or photostability between results obtained in hippocampal  
239 organotypic slices and CHO cells. The effective lateral and axial resolution of the scanless two-photon  
240 imaging system, quantified as the relative  $\Delta F/F_0$  of an electrically evoked spike as a function of the distance  
241 between the excitation spot and the soma, was found to be approximately isotropic and of similar  
242 dimensions to the neuronal soma ( $14 \mu\text{m}$  lateral and  $13 \mu\text{m}$  axial FWHM, Supplementary Figure 12h),  
243 confirming the cellular resolution of scanless two-photon voltage imaging.

244 Next, we recorded the fluorescence from patched cells while 50 action potentials (APs) were evoked  
245 electrically by injection of current (700 - 900 pA, 2 ms) into the soma at a rate of 1 Hz. Electrically evoked  
246 APs were imaged with 3 different acquisition rates: 500 Hz, 750 Hz, and 1 kHz (corresponding to per-frame  
247 exposure times of 2, 1.33 and 1 ms respectively) as previously used for 1P widefield voltage imaging<sup>21</sup>. In  
248 all conditions, individual APs could clearly be identified from single trials in the raw fluorescence traces  
249 (representative traces for single cells plotted in Figure 3c, power density:  $1.11 \text{ mW } \mu\text{m}^{-2}$ , corresponding to  
250  $125 \text{ mW}$  per cell). Putative APs were identified by template matching, based on the most prominent peaks  
251 originally identified in each fluorescence trace<sup>50</sup>. The 1 kHz recordings exhibited a higher  $-\% \Delta F/F_0$  than 500  
252 Hz recordings across all powers ( $30.5 \pm 2.2$  vs  $25.7 \pm 1.2$ ,  $n > 5$  cells, Figure 3d). However, consistent with  
253 previous reports<sup>17</sup>, higher SNR was achieved with 500 Hz recordings than for 1 kHz (for example for  $0.66$   
254  $\text{mW } \mu\text{m}^{-2}$  ( $75 \text{ mW}$  per cell):  $14.2 \pm 0.3$  vs  $11.9 \pm 0.3$ , Figure 3d), because the increase in the number of  
255 photons collected per action potential more than compensated for the reduced  $-\% \Delta F/F_0$ .

256 Having established that it was possible to record APs with high SNR in single trials at different acquisition  
257 rates, we next tested whether we could also monitor individual spikes within high-frequency trains of action  
258 potentials (such as bursts) under these conditions. We observed that an acquisition rate of 500 Hz was  
259 sufficient to track individual APs in trains with frequencies up to 100 Hz (Figure 3e, Supplementary Figure  
260 13) and using power densities as low as  $0.66 \text{ mW } \mu\text{m}^{-2}$  ( $75 \text{ mW}$  per cell). As a result of increased SNR  
261 recordings with lower acquisition rates, at low power densities, the detection probability (fraction of correctly  
262 identified APs) was higher (Figure 3f). Note that the difference in SNR between results presented in Figure  
263 3d and Figure 3f is the result of using different cameras and different excitation wavelengths, as specified  
264 in the Methods and Supplementary Tables 1 and 2. However, an acquisition rate of 500 Hz was insufficient  
265 for robustly tracking spikes in 125 Hz trains due to a reduction in  $-\% \Delta F/F_0$  (Supplementary Figure 13b),  
266 which led to a deterioration in detection probability and fluorescence response compared with the data  
267 acquired at 1 kHz at power densities  $\geq 0.66 \text{ mW } \mu\text{m}^{-2}$  (corresponding to  $75 \text{ mW}$  per cell, Figure 3f,  
268 Supplementary Figure 13). For all power densities  $> 0.66 \text{ mW } \mu\text{m}^{-2}$  ( $75 \text{ mW}$  per cell) sub-millisecond  
269 precision of AP timing estimation was obtained, as measured with respect to the electrophysiology trace  
270 (Figure 3f).

271 We next examined whether these conditions (power density:  $1.11 \text{ mW } \mu\text{m}^{-2}$ , corresponding to  $125 \text{ mW}$  per  
272 cell, 1 kHz acquisition rate) were also suitable for imaging sub-threshold changes in membrane potential.  
273 To emulate excitatory PSPs, patched cells were clamped to  $-75 \text{ mV}$ , while the membrane potential was  
274 varied in  $0.5 \text{ mV}$  steps from  $0$  to  $2.5 \text{ mV}$  for  $20 \text{ ms}$ . This protocol was repeated 50 times. Since it was not  
275 possible to detect these transients from individual recordings (Figure 4a, b,  $n=6$ ), we averaged data from  
276 different trials to improve SNR. Averaging data from 25 repeats was sufficient to stabilise the magnitude of  
277 the fluorescence transient ( $-\% \Delta F/F_0$ ) for a given depolarization (Figure 4a, c) and to increase the SNR  
278 above 1 for all depolarizations larger than  $0.5 \text{ mV}$  (Figure 4d).

279 Next, we tested the capability of scanless two-photon voltage imaging to record spontaneous network  
280 activity, a fundamental feature of developing neural circuits<sup>54</sup>. We performed simultaneous  
281 electrophysiological (whole cell patch clamp, (current clamp)) and fluorescence recordings (2P-TF-GPC,  
282 power density:  $1.33 \text{ mW } \mu\text{m}^{-2}$  (150 mW per cell), 1 kHz acquisition rate) of spontaneous activity from  
283 neurons in hippocampal organotypic slices which exhibited a range of different resting potentials ( $n > 10$   
284 cells; 5 slices). We were able to observe several hallmarks of spontaneously generated activity, large slow  
285 depolarizations, bursts of action potentials, rhythmic sub-threshold depolarizations and hyperpolarizations  
286 (Figure 5 and Supplementary Figure 14). These results confirmed the capability of scanless two-photon  
287 voltage imaging for high temporal precision, single trial recordings of action potentials and sub-threshold  
288 events, even though the sensitivity curve of JEDI-2P-kv is not optimized for sub-threshold recordings.  
289 Collectively, the results presented in Figures 3-5 parameterize the necessary imaging conditions required  
290 to detect neural activity with sufficient SNR using scanless two-photon voltage imaging.

291 To test the robustness of our approach for long-term recordings, we repeated the same protocol (2P-TF-  
292 GPC, 30 s continuous illumination,  $1.33 \text{ mW } \mu\text{m}^{-2}$  (150 mW per cell), 1 kHz acquisition rate), for a maximum  
293 recording time of 20 minutes. Due to limitations of the prototype experimental configuration (primarily data  
294 transfer rates), there was a dark period (<10 seconds) between consecutive acquisitions. To increase the  
295 duty cycle of the recordings, we reduced the acquisition speed to 500 Hz which enabled us to perform  
296 longer continuous recordings with a shorter dark period of <5 seconds (2P-TF-GPC, 1 min continuous  
297 illumination,  $1.33 \text{ mW } \mu\text{m}^{-2}$  (150 mW per cell), 500 Hz acquisition rate). As indicated by the data presented  
298 in Supplementary Figure 15, we were able to record spontaneous activity from single neurons for a  
299 maximum recording time of 20 minutes without a significant decrease in SNR (Supplementary Figure 15b).  
300 The imaging period was primarily limited by axial sample drift which decreased the SNR (data not shown),  
301 but could be overcome in future experiments with a tandem construct containing the voltage indicator and  
302 a spectrally shifted, fluorescent reporter used to track the sample drift and to dynamically update the co-  
303 ordinates of the multiplexed spots.

304 Having established the conditions necessary to observe neural activity ranging from high-frequency trains  
305 of action potentials to sub-threshold membrane potential depolarizations, we next investigated whether  
306 such imaging conditions induced physiological perturbations. There are two main sources of light-induced  
307 perturbations. The first is heating, due to linear absorption of the infrared light (mostly by water), which has  
308 been reported to affect ion channel conductances<sup>45</sup> and action potential waveforms<sup>46</sup>. The second is non-  
309 linear photodamage, due to higher order light-matter interactions which occur because of high  
310 instantaneous photon density in the focal volume and can ultimately induce apoptosis and cell ablation<sup>47-  
311 49</sup>. We performed experiments (10 ms strobed illumination, 50 cycles, 1 Hz, total illumination time 500 ms,  
312 >15 cells per region, targeted sequentially: identical to the protocol used to detect APs (Figure 3b-c)) at  
313 power densities we found necessary to observe neural activity with sufficient SNR and above ( $0.66 - 1.55$   
314  $\text{mW } \mu\text{m}^{-2}$ , corresponding to 75 – 175 mW per cell). Following these experiments, we used

315 immunohistochemistry to detect heat-shock proteins (anti-HSP70/72 immunostaining) and activation of  
316 apoptotic pathways (anti-activated-Caspase-3 immunostaining). Fixed slices were imaged using confocal  
317 microscopy. No difference in fluorescence intensity was observed between any of the illumination powers  
318 used and the control slices (not illuminated) in the case of Caspase-3 (Supplementary Figure 16a). In  
319 contrast, we observed that levels of anti-HSP increased as a function of excitation power above 1.1 mW  
320  $\mu\text{m}^{-2}$  (125 mW per cell), which indicates that the physiological damage induced by the high repetition rate  
321 laser sources, is predominantly heating. Since the damage threshold of 1.1 mW  $\mu\text{m}^{-2}$  (125 mW per cell)  
322 identified using immunohistochemistry is an upper bound, we also investigated whether there was any light  
323 induced changes in the electrical properties of neurons using electrophysiology. We did not observe any  
324 light-induced changes in action potential amplitude or width at any of the tested powers (Supplementary  
325 Figure 16b), however we found that the latency of action potential firing slightly increased at all powers  
326 tested (0.1 ms, Supplementary Figure 16b). This effect was observed 15 seconds before a similar increase  
327 in latency was seen in control experiments (Supplementary figure 16b). Whilst a 10 percent change is not  
328 huge, the immunohistochemistry and electrophysiology results imply a laser-induced perturbation of  
329 physiology for powers > 125 mW per cell.

### 330 **Scanless two-photon voltage imaging of multiple targets with low repetition rate lasers**

331 To test the capability of multiplexed 2P-TF-GPC to image multiple neurons simultaneously we used a  
332 custom low-repetition rate source (940 nm, pulse duration 100 fs, repetition rate 250 kHz, 600 mW average  
333 output power). Low-repetition rate sources, used for two-photon optogenetics, can provide higher peak  
334 energies and lower average power, hence potentially minimize photoinduced thermal effects and scale up  
335 the number of neurons that can be imaged simultaneously. Low-repetition rate lasers are particularly well  
336 suited for techniques with long times, such as scanless two-photon imaging. We found that using the low  
337 repetition rate laser, action potentials could be detected in single trials with power densities as small as  
338 0.01 mW  $\mu\text{m}^{-2}$  (1.5 mW per cell, Supplementary Figure 17). In contrast to the results obtained using the  
339 high repetition rate source (80 MHz, previous section), no changes in action potential properties were  
340 detected at any of the tested powers (Supplementary Figure 17). Immunohistochemistry targeted against  
341 HSP70/72 and activated-Caspase-3 (Supplementary Figure 17) did not reveal thermal or non-linear  
342 damage at power densities below 0.09 mW  $\mu\text{m}^{-2}$  (10 mW per cell) using 2P-TF-CGH, two-fold higher than  
343 the maximum powers we found typically necessary to image neural activity. Extrapolating from the results  
344 related to photobleaching and photostability obtained in CHO cells, we anticipate higher non-linear damage  
345 thresholds for 2P-TF-GPC and 2P-TF-Gaussian.

346 As a result of the increased energy per pulse of the low-repetition rate source, we were able to increase  
347 the number of neurons imaged simultaneously without exceeding the power damage threshold. For  
348 example, we recorded spontaneous activity in up to 8 neurons in the dentate gyrus of hippocampal  
349 organotypic slices simultaneously (2P-TF-GPC, power densities: 0.04 – 0.08 mW  $\mu\text{m}^{-2}$  (5 – 9 mW per cell,  
350 total power < 75 mW), Figure 6, Supplementary Figure 18). The sub-threshold activity of most neurons was

351 found to be highly synchronized, a characteristic feature of the immature hippocampus<sup>54</sup>. Control traces  
352 recorded adjacent to targeted neurons confirmed that this was not an artefact due to crosstalk. We were  
353 able to combine data from separate acquisitions to perform voltage imaging throughout a large region (200  
354 x 150  $\mu\text{m}^2$ ) as demonstrated in Figure 6. Since this data was acquired on a prototype system, the period  
355 between sequential acquisitions was on the order of seconds. However, by optimizing the acquisition  
356 pipeline, the period between sequential acquisitions could feasibly be reduced to milliseconds to enable  
357 scanless two-photon voltage imaging of populations of neurons.

358 The number of achievable targets per acquisition and/or the imaging depth could be further increased, as  
359 demonstrated for two-photon photostimulation of multiple cells<sup>55</sup> using high-power, low repetition rate,  
360 industrial light sources such as Ytterbium-doped fibre lasers which can provide much higher output powers  
361 (tens of Watts), and comparable pulse energies to the 940 nm source used in this work. These lasers are  
362 commonly fixed wavelength sources (1030 – 1040 nm), which means that they are not typically compatible  
363 with GFP-based fluorescent indicators. However, since the excitation spectrum of JEDI-2P-kv is slightly  
364 red-shifted as compared with previous GFP-based voltage indicators<sup>40</sup>, we tested whether it was possible  
365 to record neural activity using scanless two-photon voltage imaging with 1030 nm excitation. We repeated  
366 protocol 2 (see above) in CHO cells and imaged electrically evoked action potentials and spontaneous  
367 activity in sparsely labelled hippocampal organotypic slices (Supplementary Figure 19).

#### 368 **Scanless two-photon voltage imaging and photostimulation of multiple targets with a single beam**

369 Next, we performed simultaneous two-photon voltage imaging and photostimulation of neurons co-  
370 expressing JEDI-2P-kv and a soma-targeted channelrhodopsin. ChroME-ST and JEDI-2P-kv were co-  
371 expressed in the dentate gyrus of hippocampal organotypic slices by bulk transduction of two Adeno-  
372 Associated Virus (AAV) vectors (Figure 7a). We characterised the photophysical properties of ChroME-ST  
373 excited using the low-repetition rate laser using whole cell patch clamp electrophysiology (voltage-clamp).  
374 ChroME-ST mediated photocurrents in CHO cells saturated at 0.02  $\text{mW } \mu\text{m}^{-2}$  (2.5 mW per cell)  
375 (Supplementary Figure 20, n=4). In hippocampal organotypic slices, power densities between 0.02 and  
376 0.04  $\text{mW } \mu\text{m}^{-2}$  (2.5 - 5 mW per cell) generated sufficiently large photocurrents to reliably evoke APs with  
377 short (< 5 ms) latency and sub-millisecond jitter (Supplementary Figure 20, n=7).

378 We performed simultaneous photostimulation, imaging and whole-cell patch clamp recordings on neurons  
379 co-expressing ChroME-ST and JEDI-2P-kv and confirmed that optically evoked action potentials could be  
380 detected in voltage imaging recordings (Figure 7b). We next performed an all-optical characterisation of  
381 ChroME-ST. By modifying the power, duration and frequency of the illumination, we explored the joint-  
382 parameter space of imaging and stimulation conditions to optimize the probability of optically evoking and  
383 recording action potentials (Figure 7c). Unlike in neurons exclusively expressing JEDI-2P-kv, at power  
384 densities below 0.02  $\text{mW } \mu\text{m}^{-2}$  (2.5 mW per cell), the SNR of action potentials from single trials did not  
385 exceed the SNR threshold and hence could not be detected optically (Figure 7c), although  
386 electrophysiological recordings performed simultaneously indicated that the probability of optically evoking

387 an AP was greater than 75% (Supplementary Figure 20b). We attribute the reduction in SNR to a reduction  
388 in the expression efficiency of JEDI-2P-kv as a result of co-expressing a voltage indicator and  
389 channelrhodopsin, which results in a difference between the optical and electrophysiological results.  
390 However, for power densities above  $0.02 \text{ mW } \mu\text{m}^{-2}$  (2.5 mW per cell), we were able to detect action  
391 potentials in single trials and measured similar latencies to those obtained using whole-cell patch clamp  
392 recordings ( $4.3 \pm 0.2 \text{ ms}$ , mean  $\pm$  s.e.m.) and jitter on the order of a millisecond (Supplementary Figure  
393 20c-d).

394 The action potential probability decreased as a function of stimulation frequency, feasibly a result of  
395 channelrhodopsin desensitization at the saturating powers used, although in some cases it was possible to  
396 stimulate and image action potentials at 50 Hz (Figure 7c, right panel). Based on this characterisation, we  
397 determined that the optimal photostimulation and imaging parameters to robustly optically evoke and detect  
398 action potentials were a frequency of 5 Hz and 15 ms photoactivation pulses. The extended illumination  
399 time relative to typical photostimulation protocols was necessary for having sufficient baseline to calculate  
400  $-\% \Delta F / F_0$  and to robustly detect optically evoked action potentials. Results obtained for these parameters  
401 are summarized for 27 cells in the raster plot in Figure 7d with representative fluorescence traces of optically  
402 evoked APs shown in Figure 7e. For each cell, the power density was increased until a spike was detected  
403 optically in at least one of five repeats. The final set of power densities used was between 0.02 and 0.08  
404  $\text{mW } \mu\text{m}^{-2}$  (2.5 and 9 mW per cell), below the power threshold found to induce physiological perturbations  
405 (see above). The average  $-\% \Delta F / F_0$  of optically evoked action potentials was found to be  $20 \pm 8 \%$  ( $n=33$ ;  
406 Figure 7f), consistent with results obtained for electrically evoked action potentials (Figures 3d and f).

407 Next, we extended the unique capability of our approach of scanless two-photon voltage imaging to perform  
408 simultaneous two-photon photostimulation and imaging of multiple cells (Figure 8). In each experimental  
409 session, we first sequentially targeted cells that were expressing one or both constructs to quantify the  
410 probability of false positives (Figure 8a-c). We detected an increase in fluorescence when targeting cells  
411 only expressing ChroME-ST, due to excitation and detection of the nuclear-targeted fluorophore. No action  
412 potentials were identified in cells that were not co-expressing the two constructs but were detected optically  
413 in approximately fifty percent of co-expressing cells. We then simultaneously stimulated and imaged the  
414 same group of cells (Figure 8d) with no deterioration of SNR or  $-\% \Delta F / F_0$  of evoked and imaged action  
415 potentials (Figure 8d) and were able to determine the number and timing of action potentials evoked, and  
416 identify failures, during the stimulation period in multiple cells simultaneously (Figure 8e).

417 These results demonstrate that simultaneous, scanless two-photon voltage imaging and photostimulation  
418 can be performed in multiple cells simultaneously using high-energy, low-repetition rate lasers, using  
419 powers well-below the damage threshold.

## 420 Discussion

421 In this work, we introduced scanless two-photon voltage imaging and performed high-contrast, high-  
422 resolution voltage imaging of single and multiple neurons expressing the newly developed GEVI JEDI-2P-  
423 kv. Due to the axial confinement conferred by temporal focusing we were able to perform high-contrast two-  
424 photon voltage imaging in densely labelled intact brain slices.

425 We performed a thorough characterisation of three, temporally focused, parallel excitation modalities (2P-  
426 TF-Gaussian, 2P-TF-GPC and 2P-TF-CGH) for scanless two-photon voltage imaging. A strong advantage  
427 of 2P-TF-Gaussian illumination (similarly to 3D-SHOT) is that it is the easiest and most cost-effective  
428 approach to implement. 2P-TF-Gaussian beams have been used for volumetric Calcium imaging<sup>56,57</sup>.  
429 However, it is the least photon-efficient approach. Conversely, the photon-dense speckle grains in 2P-TF-  
430 CGH spots result in efficient two-photon excitation and hence the highest SNR. In the case of limited power  
431 budget, 2P-TF-CGH is thus the optimal modality for scanless multitarget voltage imaging. However, since  
432 we observed higher photorecovery with 2P-TF-GPC and 2P-TF-Gaussian, these modalities are preferred  
433 for prolonged (continuous illumination for hundreds of milliseconds or more) recordings such as imaging  
434 spontaneous activity. Looking ahead, one of the primary advantages of 2P-TF-GPC is the capability of  
435 sculpting well-defined lateral shapes<sup>36</sup> in order to target the most responsive regions of the cell membrane.  
436 In contrast with existing two-photon voltage imaging approaches, the high lateral resolution (0.1625  $\mu\text{m}$   
437 pixel size) of the experimental system presented in this manuscript would also be capable of sculpted,  
438 scanless two-photon voltage imaging of thin subcellular processes.

439 We used 2P-TF-GPC to demonstrate many of the theorized advantages of imaging neural activity with  
440 GEVIs by imaging action potentials (single-trial), subthreshold depolarizations and resolving single action  
441 potentials in high-frequency spike trains up to 125 Hz. We performed simultaneous imaging and  
442 electrophysiology to comprehensively characterize the performance of, and optimize, scanless two-photon  
443 voltage imaging with the genetically encoded indicator JEDI-2P-kv. Consistent with previous reports<sup>30</sup>, we  
444 found that it was generally possible to reduce the imaging speed down to 500 Hz (and consequently the  
445 required power) without a critical loss in the ability to determine the number and timing of action potentials.  
446 However, these imaging speeds reduce the accuracy of action potential detection for spike trains with  
447 frequencies  $> 100$  Hz. The optimal imaging conditions will also depend on the characteristics of the specific  
448 GEVI used. In principle, a major advantage of voltage versus calcium imaging in neuroscience is the ability  
449 to detect sub-threshold changes in somatic membrane potential. In our current configuration, we found that  
450 imaging small sub-threshold signals required averaging data from up to 25 individual trials to reach a SNR  
451 above 1 for depolarizations larger than 0.5 mV. Whilst these findings highlight the challenges of detecting  
452 subthreshold unitary PSPs ( $< 2.5$  mV) *in vivo* with current state-of-the-art GEVIs under two-photon  
453 excitation, they also indicate that such experiments ought to be possible using GEVIs optimized for sub-  
454 threshold voltage detection.

455 Since the current implementation of scanless two-photon voltage imaging requires continuous illumination,  
456 we carefully investigated whether the illumination conditions required to observe different aspects of neural  
457 activity induced any observable physiological perturbations. We found that single cells could be imaged  
458 using 100 fs, 80 MHz sources, at lower average powers than those commonly used for existing kilohertz  
459 scanning microscopes applied to two-photon voltage imaging<sup>28,58,59</sup> and did not observe any changes in AP  
460 properties at these powers. However, we found that the latency of electrically induced APs was increased  
461 slightly at all powers tested, and irreversible thermal damage was revealed with 2P-TF-CGH at the highest  
462 powers tested using immunohistochemistry. In contrast, Caspase-3 staining did not reveal any non-linear  
463 damage at the investigated powers. In fact, we found that one of the biggest impediments to long-term  
464 voltage imaging was sample drift, a problem we imagine will be significantly more severe for scanless two-  
465 photon imaging during future *in-vivo* experiments. To overcome this problem, we plan to use a tandem  
466 construct containing the voltage indicator and a nuclear targeted, spectrally shifted, fluorescent reporter in  
467 order to track the sample drift and to dynamically update the co-ordinates of the multiplexed spots. Use of  
468 such a construct would also allow segmentation of the field of view and automated estimation of neuron  
469 location, which also ought to increase the accuracy of spot position relative to the somal membrane.

470 We also demonstrated that scanless two-photon voltage imaging could be performed with a much lower  
471 effective repetition rate (250 kHz) than used with existing kilohertz scanning microscopes. Even with this  
472 low-repetition rate laser, fluorescence was excited with more than 50x the number of pulses per voxel than  
473 for the scanning approaches. As a result, scanless two-photon voltage imaging is much more robust to  
474 fluctuations in output laser power than the scanning approaches. We demonstrated AP detection in single  
475 trials using 15-30 times lower average power than required using the high-repetition rate laser. Under these  
476 conditions, we did not find any histological evidence of thermal stress or physiological perturbations with  
477 whole-cell patch clamp recordings. As demonstrated in the case of two-photon optogenetics<sup>55</sup>, the major  
478 advantage of low-repetition rate lasers is that multiple targets can be illuminated simultaneously, whilst the  
479 average power delivered to the sample is kept below the thermal damage threshold, and much lower than  
480 the average powers used for existing kilohertz scanning microscopes. Specifically, in this work we were  
481 able to perform simultaneous two-photon voltage imaging of spontaneous activity in multiple neurons (up  
482 to 8) using a 100 fs, 250 kHz, 940 nm laser source (with between 200 - 600 mW exit power, corresponding  
483 to a maximum average power of 60 mW at the sample). In order to increase the number of target cells, it  
484 would be necessary to replace the laser source used in our experiments with a higher-power, low-repetition  
485 rate industrial light source such as existing Ytterbium-doped fibre lasers (1030 nm, > 10 W output power).  
486 In this work, we demonstrated that electrically evoked action potentials and spontaneous activity in  
487 hippocampal organotypic slices could be recorded by exciting JEDI-2P-kv at 1030 nm. Based on the results  
488 presented in this manuscript, with a 1030 nm Ytterbium-doped fibre source it would be feasible to record  
489 the voltage of 40 cells simultaneously (5 mW per cell) using the existing configuration for scanless two-  
490 photon voltage imaging, whilst remaining below the damage threshold (200 mW total average power).



491 The use of an SLM for holographic light multiplexing of the temporally focused, sculpted light resulted in a  
492 nominal FOV of  $250 \times 250 \mu\text{m}^2$ . However, the effective FOV used for individual high-speed voltage imaging  
493 recordings was reduced in one dimension due to the maximum number of rows of pixels that could be read  
494 out at a given acquisition rate. We demonstrated that it was possible to perform voltage imaging throughout  
495 the  $250 \times 250 \mu\text{m}^2$  area and to combine data in post-processing, although this was not optimized on our  
496 prototype system, and increasing the lateral field of view is one of the most urgent future avenues of  
497 development. The field of view of scanless two-photon voltage imaging could be trivially increased by  
498 reducing the magnification of the detection axis, although this would not be suitable for all experiments.  
499 Furthermore, the sCMOS could be replaced with a detector capable of higher (full frame) readout speeds.  
500 A primary motivation behind the development of holographic light multiplexing was to enable multitarget  
501 photostimulation at axially distinct planes<sup>43,60,61</sup>. Although our system is also capable of targeting neurons  
502 in three-dimensions, we can currently only perform high-contrast imaging of a single plane. Performing  
503 scanless activity recordings from multiple planes simultaneously would require increasing the depth of field  
504 of the detection axis. For a very small number of discrete planes, this would be feasible using existing  
505 approaches, such as remote focusing<sup>62,63</sup>. More generally, camera-based volumetric voltage imaging will  
506 require the implementation of computational imaging approaches, such as variants of light-field microscopy,  
507 which have already been applied to imaging in scattering tissue<sup>64</sup>.

508 All experiments in this study were performed at relatively superficial depths ( $<50 \mu\text{m}$ ) in scattering tissue  
509 where the expression pattern of JEDI-2P-kv was confined due to the approach used for viral delivery (bulk  
510 transduction). The next step will be to monitor the membrane potential of multiple neurons simultaneously  
511 *in-vivo*. It has already been demonstrated that temporal focusing preserves the profile and axial  
512 confinement of sculpted light up to  $500 \mu\text{m}$  in scattering tissue for the three excitation modalities used in  
513 this work<sup>38,45</sup>, and camera detection has also been used to perform functional imaging with multi-spot  
514 excitation at depths up to  $300 \mu\text{m}$  *in-vivo*<sup>65,66</sup>. Hence it ought to be possible to perform scanless two-photon  
515 voltage imaging in the upper cortical layer. Reaching deeper brain structures could be achieved by  
516 combining camera detection with excitation through graded index lenses (GRIN) lenses<sup>67,68</sup> or via emerging  
517 computation approaches capable of overcoming scattering-induced ambiguity and of de-mixing the  
518 fluorescent transients emanating from different sources<sup>69</sup>.

519 We combined two-photon voltage imaging and optogenetics for the first time in proof-of-principle all-optical  
520 neurophysiology experiments. We capitalized on the overlapping excitation spectra of JEDI-2P-kv and the  
521 channelrhodopsin Chrome-ST to simultaneously evoke and record APs using a single beam. This approach  
522 could be incorporated into all-optical experiments to dynamically tune the incident power necessary for  
523 photostimulation and obtain the desired actuation on each of the targeted neurons *in situ*. In contrast to  
524 approaches relying on inferring neural activity from GCaMP fluorescence<sup>70</sup>, we demonstrated that voltage  
525 imaging provides a direct readout of the precise number and timing of optically-evoked action potentials  
526 with single spike precision at high spiking rates. The single beam approach to optically induce and read out

527 neuronal activity will also be a major addition in connectivity mapping to non-invasively confirm the  
528 successful optical induction of an action potential in the potential pre-synaptic cell population. We  
529 demonstrated that scanless two-photon imaging can be performed with any of the existing modalities used  
530 for parallel two-photon photostimulation. As seen in our all-optical recordings (Figure 7d), the excitability of  
531 the targeted cells can vary between cells and over time, advising a confirmation of the pre-synaptic spike  
532 in each instance. In contrast to current connectivity mapping approaches, which do not confirm pre-synaptic  
533 spiking<sup>71</sup> or use GECIs<sup>70</sup>, an approach using a voltage indicator would additionally reveal the precise timing  
534 of the pre-synaptic spike, which would facilitate correlation of the post-synaptic response and discrimination  
535 from noise. We anticipate that improved stoichiometric co-expression of the GEVI and the  
536 channelrhodopsin, possibly using a fusion-construct for tandem expression where they are covalently  
537 coupled, would facilitate the adoption of this approach.

538 The development of spectrally orthogonal voltage indicators and excitatory channelrhodopsins would  
539 facilitate the next-generation all-optical neurophysiology experiments. For example, it would be possible to  
540 record sensory-evoked activity patterns using voltage imaging, replay this activity using optogenetic  
541 stimulation and, also tune the excitation parameters in order to explore the logic and syntax of neural  
542 computation. Furthermore, this configuration would enable all-optical connectivity experiments whereby  
543 putative presynaptic neurons are stimulated optogenetically and sub-threshold post-synaptic responses are  
544 recorded optically. However, performing crosstalk-free all-optical neurophysiology experiments based on  
545 two-photon excitation is not trivial since the majority of two-photon compatible voltage indicators are  
546 optimally excited between 920 and 980 nm, a region of the electromagnetic spectrum where all commonly  
547 used channelrhodopsin variants are persistently activated<sup>72</sup>. While all-optical experiments with calcium  
548 indicators have been reported, similar results with GEVIs are more challenging due to the higher average  
549 powers required for high-SNR millisecond-timescale voltage imaging. The development of performant red-  
550 shifted genetically encoded voltage indicators, which could be combined with spectrally orthogonal blue-  
551 shifted channelrhodopsins, will remove these remaining challenges and fill an important gap in the  
552 optogenetic toolbox.

553 We anticipate that the description and thorough characterisation of scanless two-photon voltage imaging  
554 presented in this manuscript will motivate its application to deciphering the logic and syntax of neural  
555 circuits.

556

## 557 **Methods**

### 558 **Experimental setup for performing two-photon voltage imaging with temporally focused, sculpted** 559 **light**

560 All two-photon voltage imaging presented in this manuscript was performed using the experimental setup  
561 presented in Supplementary Figure 1. In the schematic diagram, all reflective spatial light modulators  
562 (SLMs) are shown as transmissive for illustrative purposes. Path 1 was used to generate temporally  
563 focused, multiplexed, GPC (12  $\mu\text{m}$  FWHM, 2PE) or low NA, apertured, Gaussian beams (12  $\mu\text{m}$  FWHM,  
564 2PE) respectively (upper path, Supplementary Figure 1). Path 2 (lower path, Supplementary Figure 1) was  
565 used to generate temporally focused holographic disks (12  $\mu\text{m}$  FWHM, 2PE). Three different laser sources  
566 were used to acquire all data presented, referred to as Lasers A, B and C respectively throughout the  
567 manuscript. The specific source used to acquire each dataset is specified in each case and all experimental  
568 configurations used to acquire the data presented in each figure are summarised in Supplementary Tables  
569 1 and 2. Laser A refers to a tuneable femtosecond source (Coherent Discovery, 80 MHz, 100 fs) tuned to  
570 920, 940 or 1030 nm (as specified). Laser B refers to a femtosecond source with a fixed wavelength output  
571 (Spark Alcor, 4 W, 80 MHz, 100 fs, 920 nm). Laser C refers to a custom OPA pumped by an amplified laser,  
572 also with fixed wavelength output (Amplitude Satsuma Niji, 0.2 - 0.6 W, 250 kHz, 100 fs, 940 nm). By virtue  
573 of the removable mirrors indicated in Supplementary Figure 1, light from each of the lasers could be directed  
574 through path 1 or path 2 during a given experiment as indicated by the dashed lines in Supplementary  
575 Figure 1. In all cases, the average laser power at the sample plane was controlled using a half-wave plate  
576 (Thorlabs, WPHSM05-980) mounted on a motorised rotation mount (Thorlabs, PRM1Z8) in combination  
577 with a polarising beam splitter (PBS), (Thorlabs, CCM1-PBS253/M). Prior to each experiment, the efficiency  
578 of each path was measured. The power at the sample plane was recorded using a handheld power meter  
579 (Thorlabs, S121C) and the power of the s-polarised light exiting the PBS at each laser output was measured  
580 with a second power meter (Ophir, 30(150) A-BB-18, Nova II). The power of the s-polarised beam was  
581 monitored continuously during experiments and used to update the rotation of the half-wave plate to deliver  
582 the desired power at the sample plane during a given acquisition (calculated using the experimentally  
583 measured efficiency of each path). All half-wave plates were externally triggered prior to each acquisition.  
584 The output of lasers A and B was modulated by a mechanical shutter (Thorlabs, SH05R/M) or using a high-  
585 speed modulator (Thorlabs, OM6NH/M) whereas the output of laser C was gated directly. In all cases the  
586 external trigger was a TTL signal generated by pCLAMP (Molecular Devices, Sunnyvale, CA) controlling  
587 an acquisition system (Molecular Devices, Axon Digidata 1550B).

588 In path 1, a telescope formed of two lenses (L1,  $f = 80$  mm, (Thorlabs, AC508-80-B) and L2,  $f = 300$  mm,  
589 (Thorlabs, AC508-300-B), for GPC and L1,  $f = 80$  mm, (Thorlabs, AC508-80-B) and L2,  $f = 200$  mm,  
590 (Thorlabs, AC508-200-B) for low-NA Gaussian illumination) was used to expand and project the beam onto  
591 a spatial light modulator (SLM1), (Hamamatsu, LCOS 10468-07,  $600 \times 800$  pixels, 20  $\mu\text{m}$  pitch). In the case  
592 of GPC, SLM1 was used to apply a  $\pi$  phase shift to the portion of the beam overlapping with the circular

593 spot and a phase shift of zero elsewhere. The modulated beam was Fourier transformed by L3,  $f = 400$   
594 mm, (Thorlabs, AC508-400-B), resulting in a spatial displacement between the low and high spatial  
595 frequency components of the field in the Fourier plane. The low spatial frequency components were  
596 selectively phase shifted by  $\pi$  using a phase contrast filter (PCF) with  $60 \mu\text{m}$  radius (Double Helix Optics,  
597 custom design) positioned in the Fourier plane of L3. The spatial frequencies were recombined in the image  
598 plane by L4,  $f = 300$  mm, (Thorlabs, AC508-300-B). For more details, refer to<sup>36</sup>. For parallel two-photon  
599 excitation using a low NA Gaussian beam, the corrective phase mask provided by the manufacturer was  
600 displayed on SLM1 and the PCF was displaced from the optical path as indicated in Figure 1. A blazed  
601 diffraction grating (Richardson Gratings, 600 lines/mm) located at the focal plane of L4, a conjugate image  
602 plane, disperses the different spectral frequency components of the ultrafast beam as required for temporal  
603 focusing. The grating was oriented at the blaze angle to maximize light throughput. The dispersed beam  
604 was collimated in one direction and Fourier transformed in the orthogonal direction by L5,  $f = 500$  mm,  
605 (Thorlabs, AC508-500-B), resulting in an asymmetric “line” illumination of SLM2 (Hamamatsu, LCOS  
606 10468-07,  $600 \times 800$  pixels,  $20 \mu\text{m}$  pitch), as described previously<sup>61</sup>. For this work, SLM2 was used for 2-  
607 dimensional multiplexing of the beam, although 3-dimensional multiplexing would be possible. Phase  
608 masks were generated using a weighted Gerchberg-Saxton algorithm as described previously<sup>48,73</sup>. For all  
609 data presented in Figures 1, 2 and 3, SLM2 was used to displace the sculpted light from the optical axis  
610 and the zeroth-diffraction order, which was removed using a physical beam block positioned in a conjugate  
611 image plane. Lenses 6 ( $f = 500$  mm, (Thorlabs, AC508-500-B)) and 7 ( $f = 300$  mm, (Thorlabs, AC508-300-  
612 B)) were used to de-magnify the beam to the back focal plane of the objective lens (Nikon, CFI APO NIR,  
613  $\times 40$ , 0.8 NA,  $f = 5$  mm) which projected the light (and re-combined the different spectral frequency  
614 components) onto the focal plane. A half-wave plate was included downstream of SLM2 in path 1 to convert  
615 s-polarised light to p-polarised.

616 In path 2, a Galilean beam expander formed of two lenses (L8,  $f = -75$  mm, (Thorlabs, LC1258-B) and L9,  
617  $f = 500$  mm, (Thorlabs, AC508-500-B)), expanded the beam onto SLM3, (Hamamatsu, LCOS X13138-07,  
618  $1272 \times 1024$  pixels,  $12.5 \mu\text{m}$  pitch). For the single cell experiments, 5 holograms designed to generate a  
619 single  $12 \mu\text{m}$  holographic spot (located in the same position) were computed prior to each session. For  
620 each recording, one of these phase masks was randomly selected and displayed on the SLM in order to  
621 minimize any effects of the variable speckle distribution on the resulting dataset. The holograms displayed  
622 on SLM3 were designed to generate multiple  $12 \mu\text{m}$  holographic spots targeted to chosen neurons  
623 throughout the field of excitation following the calibration procedure outlined in the Supplementary  
624 Information. All holograms were calculated using an iterative Gerchberg-Saxton algorithm<sup>74</sup>. The zeroth  
625 diffraction order was removed using a physical beam block positioned in a conjugate image plane. The  
626 modulated beam was Fourier transformed by L10 ( $f = 750$  mm, (Thorlabs, AC508-750-B)), to form the  
627 holographic disks in a conjugate image plane where a blazed grating (Thorlabs, GR50-0610, 600 lines/mm)  
628 was located. A 2” diffraction grating was used to maximise the field of excitation. The diffraction grating was  
629 oriented perpendicular to the optical axis and the illumination angle was chosen such that the first diffraction

630 order of the central wavelength propagated along the optical axis. This orientation did not coincide with the  
631 blaze angle, and hence was not the most efficient<sup>23</sup> but was allowed the temporal focusing plane of each  
632 of the holographic disks to coincide with the focal plane of the objective lens. A pair of telescopes comprised  
633 of L11 (f = 500 mm, (Thorlabs, AC508-500-B)), L12 (f = 300 mm, (Thorlabs, AC508-300-B)), L7 (f = 300  
634 mm, (Thorlabs, AC508-300-B)) and the objective lens (Nikon, CFI APO NIR, x40, 0.8 NA, f = 5 mm, water)  
635 was used to de-magnify and relay the holographic spots on to the sample plane. In experiments where non-  
636 temporally focused spots were used (1030 nm excitation), the diffraction grating was replaced by a mirror.

637 Excitation paths 1 and 2 were combined prior to the tube lens (L7) using a polarising beam splitter (Thorlabs,  
638 PBS253). The linear polarisation of the light exiting the objective was changed using a half-wave plate  
639 following the PBS. For each modality, the rotation of the half-wave plate was set to that which was found  
640 to maximise the two-photon excited JEDI-2P-kv fluorescence.

641 SLM1 was also used to optimise the alignment of the GPC path by translating the position of the  $\pi$  phase  
642 disk relative to the centre of the incident Gaussian beam and adding tip/tilt/defocus phases to translate the  
643 position of the focus with respect to the PCF filter. SLMs 1, 2 and 3 were also used to correct for system  
644 aberrations by adjusting the coefficients of Zernike modes (evaluated at the centre of each SLM pixel) in  
645 order to maximise the efficiency, uniformity and contrast of two-photon excited fluorescence excited in a  
646 thin rhodamine layer.

647 In all experiments, fluorescence was captured using a simple widefield detection axis comprised of a  
648 microscope objective (Nikon, CFI APO NIR, x40, 0.8 NA, f = 5 mm, water), a tube lens (TL), (Thorlabs,  
649 TTL200-A) and a scientific complementary metal-oxide semiconductor (sCMOS) camera (Hamamatsu  
650 ORCAFlash 4.0 or Photometrics Kinetix, as summarised in Supplementary Table 1). For both sCMOS  
651 detectors, the pixel size at the sample plane was 0.1625  $\mu\text{m}$  and 1x1 binning was used for all experiments.  
652 The fluorescence was separated from the excitation light using a dichroic mirror (Semrock #FF705-Di01,  
653 70 x 50 mm). Widefield, single photon, epi-fluorescence excitation was accomplished by means of two LED  
654 sources (Thorlabs M490L4, 490 nm to excite JEDI-2P-kv fluorescence and Thorlabs M430L5, 430 nm to  
655 excite BFP fluorescence), filtered by bandpass excitation filters (Semrock FF02-482/18, FF01-414/46) and  
656 focused onto the back focal plane of the objective lens by means of an achromatic lens (Thorlabs f = 50  
657 mm). Long exposure times (1s) and low excitation power densities were used to acquire all images based  
658 on single photon fluorescence. For single photon or dual-colour imaging (for instance during all-optical  
659 experiments), fluorescence was filtered using either a quad-band filter (Chroma ZET405/488/561/640) or  
660 individual bandpass filters (Chroma ET525/50, ET605/7). Infrared light used for two-photon excitation of  
661 fluorescence was blocked from the camera using a shortpass filter (Semrock #FF01-750sp).

662 Data were acquired using a control scheme based on custom scripts written to control *micro-manager 2.0*  
663 *Gamma*<sup>75</sup> from Python via Pycro-manager<sup>76</sup>. All experiments were controlled using two desktop computers  
664 running Windows 10. During voltage imaging experiments, the micro-manager acquisition engine was  
665 bypassed. Data from the camera was streamed directly to disk on one of the acquisition computers. The

666 first step of any experiment was to acquire 2 (JEDI-2P-kv and transmitted light) or 3 (JEDI-2P-kv, ChroME-  
667 ST (H2B-BFP2) and transmitted light) widefield images of the sample. These images were used to select  
668 targeted cells during a given experiment. The centroids of all targeted cells were written to file for all  
669 experiments. All widefield images presented in this text are background subtracted for visualization  
670 purposes (rolling ball background subtraction, ImageJ, rolling ball radius 50 pixels). All the voltage imaging  
671 data presented in this manuscript were acquired in “dynamic range”, 16-bit mode, which meant that a  
672 maximum of 266 rows of pixels could be acquired at 1 kHz ( $43 \times 250 \mu\text{m}^2$  FOV). In practice we used an  
673 exposure time of 1 ms resulting in an effective acquisition rate of 980 Hz following 0.02 ms readout (similarly  
674 for the recordings referred to as 500 and 750 Hz in the manuscript). For single cell experiments  
675 (corresponding to data presented in Figures 2-5), data were only acquired for a square region with diameter  
676 less than 266 pixels centred on a given cell. For the multi-cell experiments, neurons were grouped to find  
677 the maximal number which could be imaged within 266 pixels. The relative centroids of all targeted cells  
678 within this cropped region, and the upper left-hand coordinate of each cropped region were written to file  
679 for all experiments. These coordinates were used to “stitch” data from sequential acquisitions into a single  
680 dataset. In “dynamic range” mode, the field of view is inversely proportional to the exposure time, such that  
681 we could acquire data from 532 rows ( $86 \times 250 \mu\text{m}^2$  FOV) at 500 Hz. The field of view could be increased  
682 by a factor of 6 using “speed” mode, where data is read out at 8-bit. The camera was triggered using a 5  
683 V, TTL signal generated by pCLAMP (Molecular Devices, Sunnyvale, CA) controlling an acquisition system  
684 (Molecular Devices, Axon Digidata 1550B). During experiments, widefield images were visualised using  
685 the open-source image viewer Napari and voltage imaging traces were visualised using pyqtgraph.

686 All fluorescence traces were analysed using the same analysis pipeline written in Python, as outlined in the  
687 main text and Supplementary Information, derived from<sup>50</sup>. When multiple cells were imaged simultaneously,  
688 the (known) centroid of the excitation spot in camera co-ordinates was used to crop a rectangular region of  
689 interest (ROI) surrounding each cell (generally  $100 \times 100$  pixels). In rare cases where the ROIs of  
690 independent cells overlapped, a region of each independent cell was identified manually. Individual cells  
691 were then defined according by regression of each pixel in the ROI against the average fluorescence trace  
692 of the manually segmented pixels.

### 693 **Preparation of CHO cells**

694 CHO cells were acquired from Sigma (Sigma, 85050302) and cultured in T25 flasks (Falcon, 353107) in a  
695 medium consisting of DMEM-F12 + Glutamax (Fisher, Gibco™ 10565018), supplemented with 10% SBF  
696 (Fisher, Gibco™ 10500-064) and 1% penicillin/streptomycin ( $5000 \text{ U ml}^{-1}$ ). Cells were passaged every 2-3  
697 days. Prior to each experiment, cells were seeded on coverslips (Fisher, 10252961) in 24-well plates ( $50$   
698  $000 \text{ cells/ml}$ ). After 24 hours, cells were transiently transfected with a plasmid using the Jet prime kit  
699 (Ozyme, POL101000015) (Table 1). The medium was then replaced after 4 hours. Experiments were  
700 performed 48 hours post transfection.

701 Table 1: List of plasmids

Plasmid	Transfection ratio
pAAV_hSyn_JEDI-2P_GSS3_Kv2.1	0.75 µg DNA: 1.5 µl transfectant
pAAV_CamKIIa_ChRoME-ST_P2A_H2B_BFP	0.75 µg DNA: 1.5 µl transfectant

702 **Electrophysiology for scanless two-photon voltage imaging in CHO cells**

703 48 hours post transfection, whole-cell voltage clamp recordings of JEDI-2P-kv-expressing CHO cells were  
 704 performed at room temperature (21 - 23°C). An upright microscope (Scientifica, SliceScope) was equipped  
 705 with a far-red LED (Thorlabs, M660L4), oblique condenser, microscope objective (Nikon, CFI APO NIR,  
 706 40X, 0.8 NA), tube lens (Thorlabs, TTL200-A), and an sCMOS camera (Photometrics, Kinetix, or  
 707 Hamamatsu, Flash4.0) to collect light transmitted through the sample. Patch clamp recordings were  
 708 performed using an amplifier (Molecular Devices, Multiclamp 700B), a digitizer (Molecular Devices, Digidata  
 709 1550B) at a sampling rate of 10 kHz and controlled using pCLAMP11 (Molecular Devices). Cells were  
 710 continuously perfused with artificial cerebrospinal fluid (ACSF) comprised of 125 mM NaCl, 2.5 mM KCl,  
 711 1.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 0.3 mM ascorbic acid, 25 mM D-glucose, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>.  
 712 Continuous aeration of the recording solution with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, resulted in a final pH of 7.4  
 713 (measured). Borosilicate pipettes (with filament, OD: 1.5 mm, ID: 0.86 mm, 10 cm length, fire polished,  
 714 WPI) were pulled using a Sutter Instruments P1000 puller, to a tip resistance of 3.5–6 MΩ. Pipettes were  
 715 filled with an intracellular solution consisting of 135 mM K-gluconate, 4 mM KCl, 4 mM Mg-ATP, 0.3 mM  
 716 Na<sub>2</sub>-GTP, 10 mM Na<sub>2</sub>-phosphocreatine, and 10 mM HEPES (pH 7.35). All membrane potentials reported in  
 717 this manuscript are Liquid Junction Potential (LJP) corrected by -15 mV (measured). Recordings were  
 718 compensated for capacitance (Cm) and series resistance (Rs) to 70 % (Cm = 11.6 ± 4.7 pF; Rs = 16.1 ±  
 719 8.2 MΩ; mean ± s.d.). Only recordings with an access resistance below 35 MΩ were included in subsequent  
 720 analysis.

721  
 722 All experiments were performed with laser A, except data presented in Supplementary Figure 20 where  
 723 laser C was used.

724  
 725 For protocols 1 and 2 (Figure 2), JEDI-2P-kv-expressing CHO cells were patched and clamped at -55 mV  
 726 and 3, 100 mV steps were applied under either continuous (3 s, power density: 0.88 mW µm<sup>-2</sup>,  
 727 corresponding to 100 mW per cell) or strobed illumination (200 ms every 2.5 s, power densities ranging  
 728 from 0.66 to 1.55 mW µm<sup>-2</sup> (75 to 175 mW per cell), as specified in the main text). The fluorescent responses  
 729 to the depolarization steps were simultaneously recorded at 100 Hz. For protocol 3, JEDI-2P-kv-expressing  
 730 CHO cells were patched and clamped at -75 mV to mimic the resting potential of neurons in the dentate  
 731 gyrus of hippocampal organotypic slices. A 20 Hz train of 10, 3 ms, 100 mV steps was electrically induced,

732 and the fluorescent response to different scanless illumination methods was recorded simultaneously (500  
733 ms, power density:  $1.33 \text{ mW } \mu\text{m}^{-2}$ , corresponding to 150 mW per cell, 1 kHz acquisition).

734

735 The ability to record voltage responses using JEDI-2P-kv under 1030 nm illumination was assessed using  
736 protocol 2 with a holographic spot (12  $\mu\text{m}$  diameter, not temporally focused, power density:  $0.4 \text{ mW } \mu\text{m}^{-2}$ ,  
737 corresponding to 45 mW per cell, Supplementary Figure 19).

738

739 For data presented in Supplementary Figure 20, ChromE-ST expressing cells were patched in whole cell  
740 voltage clamp configuration at -55 mV and photocurrents in response to 17.5 ms pulses of light (power  
741 densities ranging from 0.02 to  $0.04 \text{ mW } \mu\text{m}^{-2}$ , corresponding to 2.5 to 5 mW per cell) were recorded.

742

#### 743 **Preparation of hippocampal organotypic slice cultures for validating scanless two-photon voltage** 744 **imaging of neuronal activity using JEDI-2P-kv**

745 All experimental procedures were conducted in accordance with guidelines from the European Union and  
746 institutional guidelines on the care and use of laboratory animals (Council Directive 2010/63/EU of the  
747 European Union). Hippocampal organotypic slices were prepared from mice (Janvier Labs, C57Bl6J) at  
748 postnatal day 8 (P8). Hippocampi were sliced with a tissue Chopper (McIlwain type 10180, Ted Pella) into  
749  $300 \mu\text{m}$  thick sections in a cold dissecting medium consisting of GBSS (Sigma, G9779) supplemented with  
750 25 mM D-glucose, 10 mM HEPES, 1 mM Na-Pyruvate, 0.5 mM  $\alpha$ -tocopherol, 20 nM ascorbic acid, and 0.4%  
751 penicillin/streptomycin ( $5000 \text{ U ml}^{-1}$ ).

752 After 30 - 45 min of incubation at  $4 \text{ }^\circ\text{C}$  in the dissecting medium, slices were placed onto a porous membrane  
753 (Millipore, Millicell CM PICM03050) and cultured at  $37 \text{ }^\circ\text{C}$ , 5%  $\text{CO}_2$  in a medium consisting of 50% Opti-  
754 MEM (Fisher 15392402), 25% heat-inactivated horse serum (Fisher 10368902), 24% HBSS, and 1%  
755 penicillin/streptomycin ( $5000 \text{ U ml}^{-1}$ ). This medium was supplemented with 25 mM D-glucose, 1 mM Na-  
756 Pyruvate, 20 nM ascorbic acid, and 0.5 mM  $\alpha$ -tocopherol. After three days in-vitro (DIV), the medium was  
757 replaced with one containing 82% neurobasal-A (Fisher 11570426), 15% heat-inactivated horse serum  
758 (Fisher 11570426), 2% B27 supplement (Fisher, 11530536), 1% penicillin/streptomycin ( $5000 \text{ U ml}^{-1}$ ),  
759 which was supplemented with 0.8 mM L-glutamine, 0.8 mM Na-Pyruvate, 10 nM ascorbic acid and 0.5 mM  
760  $\alpha$ -tocopherol. This medium was removed and replaced once every 2-3 days.

761 Slices were transduced with AAV9\_hSyn\_JEDI-2P\_Kv2.1 at DIV 3 by bulk application of  $1 \mu\text{l}$  of virus per  
762 slice (see Table 2). Experiments were performed between DIV 7 and 15.

763

764

765



766 **Electrophysiology for validating scanless two-photon voltage imaging of neuronal activity using**  
767 **JEDI-2P-kv in hippocampal organotypic slices**

768 At DIV 10-15, whole-cell patch clamp recordings of JEDI-2P-kv expressing granule cells in DG were  
769 performed at temperatures varying between 31-35°C. During experiments, slices were perfused with ACSF  
770 as previously described. This extracellular solution was supplemented with 1  $\mu$ M AP5 (Abcam, ab120003)  
771 and 1  $\mu$ M NBQX (Abcam, ab120046) in all experiments except for the spontaneous activity recordings  
772 (Figures 5-6, Supplementary Figures 14 and 18). Patch pipettes were filled with intracellular solution (see  
773 above).

774 Neurons were held at -75 mV in voltage clamp configuration and recordings were compensated for  
775 capacitance ( $C_m$ ) and series resistance ( $R_s$ ) to 70 % ( $C_m = 21 \pm 6.3$  pF;  $R_s = 19.2 \pm 8.5$  M $\Omega$ ; mean  $\pm$  s.d.).  
776 In current clamp configuration, neurons were injected with some current (less than 100 pA) if necessary to  
777 maintain their resting membrane potential to -75 mV. In the latter configuration, bridge potential was  
778 corrected (Bridge potential =  $13.9 \pm 4.2$  M $\Omega$ ; mean  $\pm$  s.d.).

779 Neurons were first patched in whole-cell voltage clamp configuration. Protocol 1 was then performed to  
780 confirm that the fluorescence of the patched cell was voltage responsive (Supplementary Figure 12).

781 The ability to record single action potentials in neurons (Figure 3c and d) was assessed by electrically  
782 triggering a 1 Hz train of 50 action potentials with short latency and jitter under strobed illumination (10 ms,  
783 power densities ranging from 0.66 to 1.55 mW  $\mu$ m<sup>-2</sup>, corresponding to 75 to 175 mW per cell) while  
784 recording at three different acquisition rates (500 Hz, 750 Hz and 1 kHz). Action potentials were triggered  
785 by injecting 700-900 pA currents for 2 ms.

786 Then, to assess the ability to record fast spike trains in neurons, trains of 10 action potentials from 25 to  
787 125 Hz were electrically induced under illumination at different power densities (from 0.66 to 1.55 mW  $\mu$ m<sup>-2</sup>  
788 <sup>2</sup>, corresponding to 75 to 175 mW per cell) and recorded at acquisition rates varying between 500 Hz, 750  
789 Hz and 1 kHz. The amount of current injected was one that was sufficient to evoke 10 action potentials at  
790 each of the different spike trains. Recordings where one action potential was missing in the  
791 electrophysiological trace were dismissed. 125 Hz was found to be the limit at which the granule cells could  
792 spike in our conditions (Figure 3e-f and Supplementary Figure 13).

793 For data presented in Figure 4, 6, 20 ms steps separated by 30 ms and ranging from 0 to 2.5 mV (in 0.5  
794 mV steps) were induced in JEDI-2P-kv expressing neurons under strobed illumination (40 ms centered  
795 around the steps, power density: 1.33 mW  $\mu$ m<sup>-2</sup>, corresponding to 150 mW per cell) while recording the  
796 fluorescence response at 1 kHz. This was repeated 50 - 75 times.

797 To record spontaneous activity (Figure 5), JEDI-2P-kv expressing neurons were patched and their  
798 membrane potential was monitored under continuous illumination for 30 s at a power density of 1.33 mW

799  $\mu\text{m}^2$  (150 mW per cell) while recording the fluorescent response at 1 kHz. Cells were not patched for the  
800 recordings presented in Supplementary Figure 14. The same protocol was repeated to perform long-term  
801 voltage imaging of JEDI-2P-kv expressing neurons for a maximum of 20 min with a dark period of <10 s in  
802 between each recording (due to limitations in data transfer rates). To overcome this issue, we performed  
803 longer recordings (1 min) at a lower acquisition rate (500 Hz) with the same power density (1.33 mW  $\mu\text{m}^2$ ,  
804 corresponding to 150 mW per cell). This allowed us to shorten the dark period in between recordings to  
805 < 5 s (Supplementary Figure 15).

806 The axial resolution of JEDI-2P-kv (Supplementary Figure 12) was measured by electrically triggering an  
807 action potential and measuring the fluorescence response while displacing the objective in the z axis (from  
808 +50 to -50  $\mu\text{m}$ , in 5  $\mu\text{m}$  steps). The lateral resolution was measured (from +20 to -20  $\mu\text{m}$ , in 2  $\mu\text{m}$  steps) by  
809 mechanically moving the sample in the x-y axis.

810 To measure the performances of JEDI-2P-kv under 1030 nm illumination, hippocampal organotypic slices  
811 were infected with a mixture of AAV1\_EF1a\_DIO\_JEDI-2P\_Kv2.1\_WPRE and  
812 AAV9\_hSyn\_Cre\_WPRE\_hGH (see Table 2) at DIV 3 in order to get a sparser expression. Isolated  
813 expressing cells in the dentate gyrus were then patched in whole cell current clamp configuration and  
814 illuminated with a holographic spot (12  $\mu\text{m}$  diameter, not temporally focused) at 1030 nm (power density:  
815 1.21 mW  $\mu\text{m}^2$ , corresponding to 137 mW per cell). Single action potentials and spontaneous activity  
816 recordings were obtained as described previously (Supplementary figure 19).

#### 817 **Preparation of hippocampal organotypic slices for two-photon actuation and imaging of neural** 818 **activity using ChROME-ST and JEDI-2P-kv**

819 All animal procedures followed national and European animal care guidelines (Directive 2010/63/EU) and  
820 institutional guidelines on animals used for research purposes. Hippocampal organotypic slice preparations  
821 were prepared as described in reference<sup>77</sup> with a few modifications. Briefly, hippocampi were extracted  
822 from P5-P8 C56Bl/6J mouse pups sacrificed by decapitation. The dissection was carried out in filter  
823 sterilized (0.2  $\mu\text{m}$  pore size) ice cold medium containing: 248 mM sucrose, 26 mM  $\text{NaHCO}_3$ , 10 mM glucose,  
824 4 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 2 mM kynurenic acid and 0.001 % phenol red saturated with 95 %  
825  $\text{O}_2$  / 5 %  $\text{CO}_2$ . Transverse slices of 300 – 400  $\mu\text{m}$  thickness were cut with Mcllwain Tissue Chopper using  
826 double edge stainless steel razor blades. Using a plastic transfer pipette, undamaged slices were  
827 individually transferred onto the small pieces of PTFE membrane (Millipore FHLP04700) placed on  
828 membrane inserts (Millicell PICMORG50) in the 6-well-plate containing 1 mL pre-warmed culture medium.  
829 The slices were cultured at 37 °C and 5 %  $\text{CO}_2$  in antibiotic free culture medium consisting of 80 % MEM  
830 and 20 % Heat-inactivated horse serum supplemented with 1 mM L-glutamine, 0.01 mg/ml Insulin, 14.5  
831 mM NaCl, 2 mM  $\text{MgSO}_4$ , 1.44 mM  $\text{CaCl}_2$ , 0.00125 % Ascorbic acid and 13mM D-glucose. The culture  
832 medium was partially replaced with fresh, 37 °C warmed culture medium every 3 days.

833 Various titrations were tested to achieve sufficient levels of expression of both sensor and actuator. When  
 834 slices were transduced with both viruses on the same day, we observed a reduction in the expression of  
 835 JEDI-2P-kv. Furthermore, overexpression-mediated apoptosis was observed in some cases when slices  
 836 were transduced with both viruses simultaneously. The best results were obtained by transducing slices  
 837 with JEDI-2P-kv first, followed a week later by ChroME-ST which resulted in strong co-expression of both  
 838 proteins (Figure 8a). However, in general we found that the expression levels of both proteins were more  
 839 variable when the two constructs were co-expressed than when either construct was expressed  
 840 independently.

841 Slices were transduced firstly with AAV9-hSyn-JEDI-2P-Kv2.1 at DIV 3 and secondly with  
 842 AAV9\_Camk2a\_ChroME-ST\_P2A\_H2B\_tagBFP2 (provided by H. Adesnik, University of California,  
 843 Berkeley, USA) at DIV 10 by bulk application of 1  $\mu$ l of virus per slice (Table 2). Channelrhodopsin-  
 844 expressing cells were visualized using stable expression of an H2B-BFP2 fusion, which resulted in nuclear  
 845 localized BFP2 fluorescence. Experiments were performed between DIV 13 and 17.

846 To characterize the performances of ChroME-ST (Figure 7), ChroME-ST and JEDI-2P-kv co-expressing  
 847 granule cells in DG were patched in whole-cell current clamp configuration. 5, 17.5 ms pulses of light at 5  
 848 Hz were applied at different power densities ranging from 0 to 0.09  $\text{mW } \mu\text{m}^{-2}$  (0 to 10 mW per cell) to photo-  
 849 evoke action potentials. The fluorescent responses were recorded at an acquisition rate of 1 kHz. The  
 850 latency and jitter of light-evoked action potentials, respectively defined as the mean and standard deviation  
 851 of the time between the onset of stimulation and the peak of the action potential, were measured using the  
 852 same protocol. The axial resolution of ChroME-ST was measured using a similar protocol, while displacing  
 853 the spot axially by mechanically moving the objective from +75 to -50  $\mu\text{m}$ , in 5  $\mu\text{m}$  steps in the vicinity of  
 854 the cell (from +55  $\mu\text{m}$  to -10  $\mu\text{m}$ ) and then in 10  $\mu\text{m}$  steps (Figure 7 and Supplementary Figure 20).

855 For each cell, the power density was increased until a spike was detected optically in at least one of five  
 856 repeats. The final set of power densities used was between 0.02 – 0.08  $\text{mW } \mu\text{m}^{-2}$  (2.5 – 9 mW) per cell.

857 Table 2: List and final titres of viruses

Virus	Final titer (vg ml <sup>-1</sup> )
AAV9_hSyn_JEDI-2P_GSS3_Kv2.1	$3.12 \times 10^{13}$
AAV9_CamKIIa_ChRoME-ST_P2A_H2B_BFP	$4.46 \times 10^{12}$
AAV1_EF1a_DIO_JEDI-2P_Kv2.1_WPRE	$2.36 \times 10^{13}$
AAV9_hSyn_Cre_WPRE_hGH	$2.3 \times 10^{11}$

858

859

860 **Immunostaining**

861 Immunostaining was performed on hippocampal organotypic slices to assess the potential non-linear  
862 photodamage induced by two different laser sources (A and C) during our experiments.

863 In the case of laser A, slices expressing JEDI-2P-kv were illuminated with a holographic spot (12  $\mu\text{m}$   
864 diameter, temporally focused, power densities between 0.66 – 1.55  $\text{mW } \mu\text{m}^{-2}$ , corresponding to 75 – 175  
865  $\text{mW}$  per cell) in the dentate gyrus. The illumination protocol consisted of 50, 10 ms pulses of light, using the  
866 same protocol used to record single action potentials (see previous section), repeated on > 15 cells per  
867 region illuminated. A negative control (no illumination) and a positive control where a whole region was  
868 continuously illuminated for 30 min (power density: 1.64  $\text{mW } \mu\text{m}^{-2}$ , corresponding to 185  $\text{mW}$  per cell) were  
869 also performed.

870 Slices expressing JEDI-2P-kv and ChromE-ST were illuminated with 5 holographic spots generated with  
871 laser source C (12  $\mu\text{m}$  diameter, power densities ranging between 0.02 – 0.09  $\text{mW } \mu\text{m}^{-2}$  (2.5 – 10  $\text{mW}$  per  
872 cell), 45  $\mu\text{m}$  separation), and moved laterally across 120  $\mu\text{m}$  in 20  $\mu\text{m}$  steps. The illumination protocol used  
873 to characterize the ChromE-ST (see previous section) was repeated 5 times at each position, and the  
874 hologram was recomputed each time.

875 After experiments, slices were immediately fixed in PFA 4% for 3-5 min. Permeabilization of the tissue was  
876 performed by incubation of the slice in a solution comprising of Triton X-100 in PBS (0.5 %) for 12 hours at  
877 4 °C. Non-specific sites were then blocked by incubation in a blocking solution (BSA 20 % in PBS) for 4  
878 hours at room temperature (21-23 °C).

879 Slices were incubated with primary antibodies diluted in a solution of BSA 5 % in PBS (Table 3) overnight  
880 at 4 °C and placed in a solution of BSA 5 % in PBS on a horizontal shaker for 10 minutes to wash off excess  
881 antibodies. This process was repeated three times.

882 Slices were then incubated with species-appropriate secondary antibodies conjugated to Alexa fluor 555 (to  
883 detect anti-activated-Caspase-3 immunostaining) and Alexa fluor 647 (to detect anti-HSP70/72  
884 immunostaining) diluted in the same solution as the primary ones, for 3-4 hours at room temperature (21-  
885 23 °C). They were then washed again following the same process, but in PBS only.

886 Slices were immediately mounted in Fluoromount-g mounting medium (Southern Biotech, 0100-01) to be  
887 imaged using confocal microscopy (Olympus FV3000, 20X magnification, 0.8 NA, pixel size 0.6214  $\mu\text{m}$ ,  $\lambda$   
888 488, 561, 640 nm). The same imaging parameters were used for all experimental conditions.

889

890 Table 3: List and dilutions of antibodies used for immunostaining

Antibody	Supplier	Reference	Species	Working dilution
HSP70 / HSP72	Enzo Life Science	ADI-SPA-810-D	Mouse	1:400
Cleaved Caspase-3 (Asp175)	Cell Signaling	9661	Rabbit	1:250
Anti-mouse, Alexa fluor 647	Thermofisher Scientific	A21235	Goat	1:500
Anti-rabbit, Alexa fluor 555	Thermofisher Scientific	A21429	Goat	1:500

891

892 **Statistics**

893 All experiments were repeated for at least two (and generally many more) independent passages of cells,  
 894 transfections or infections. The Shapiro test (`scipy.stats.shapiro`) was used to test whether data were  
 895 normally distributed. For normally distributed data, the paired or unpaired two-tailed students t-test was  
 896 used to compare two independent samples. The non-parametric Mann-Whitney *U*-test  
 897 (`scipy.stats.mannwhitneyu`) was used to compare two samples in the case when either or both samples  
 898 were found not to be normally distributed. 'n' refers to the number of independent biological replicates, as  
 899 stated in each figure caption and summarized in Supplementary Table 2. A statistical comparison was  
 900 deemed significant if the p-value was less than 0.05. For all figures \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$   
 901 and \*\*\* denotes  $p < 0.0001$ . All results reported in the manuscript are communicated as the mean value  $\pm$   
 902 standard deviation of at least three technical replicates unless otherwise stated. As specified, error bars in  
 903 plots denote either the standard deviation or the standard error. All biological replicates were included in  
 904 each estimate. Estimation stats were performed using the Python package `dabestr`<sup>78</sup>.

905

906 **References**

- 907 1. Scanziani, M. & Hausser, M. Electrophysiology in the age of light. *Nature* **461**, 930–939 (2009).
- 908 2. Emiliani, V. *et al.* Optogenetics for light control of biological systems. *Nat. Rev. Methods Prim.* **2**,  
909 55 (2022).
- 910 3. Abdelfattah, A. *et al.* Neurophotonic Tools for Microscopic Measurements and Manipulation:  
911 Status Report. *Neurophotonics* **9**, 1–86 (2022).
- 912 4. Clapham, D. E. Calcium Signaling. *Cell* **131**, 1047–1058 (2007).
- 913 5. Grienberger, C. & Konnerth, A. Imaging Calcium in Neurons. *Neuron* **73**, 862–885 (2012).
- 914 6. Tsien, R. Y. New calcium indicators and buffers with high selectivity against magnesium and  
915 protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404  
916 (1980).
- 917 7. Tian, L., Andrew Hires, S. & Looger, L. L. Imaging neuronal activity with genetically encoded  
918 calcium indicators. *Cold Spring Harb. Protoc.* **7**, 647–656 (2012).
- 919 8. Chen, Y. *et al.* Soma-Targeted Imaging of Neural Circuits by Ribosome Tethering. *Neuron* **107**,  
920 454-469.e6 (2020).
- 921 9. Shemesh, O. A. *et al.* Precision Calcium Imaging of Dense Neural Populations via a Cell-Body-  
922 Targeted Calcium Indicator. *Neuron* **107**, 470-486.e11 (2020).
- 923 10. Zhu, M. H., Jang, J., Milosevic, M. M. & Antic, S. D. Population imaging discrepancies between a  
924 genetically-encoded calcium indicator (GECI) versus a genetically-encoded voltage indicator  
925 (GEVI). *Sci. Rep.* **11**, 1–15 (2021).
- 926 11. Peterka, D. S., Takahashi, H. & Yuste, R. Imaging Voltage in Neurons. *Neuron* **69**, 9–21 (2011).
- 927 12. Davila, H. V, Salzberg, B. M., Cohen, L. B. & Waggoner, A. S. A Large Change in Axon  
928 Fluorescence that Provides a Promising Method for Measuring Membrane Potential. *Nat. New*  
929 *Biol.* **241**, 160–161 (1973).
- 930 13. Loew, L. M. *et al.* A naphthyl analog of the aminostyryl pyridinium class of potentiometric  
931 membrane dyes shows consistent sensitivity in a variety of tissue, cell, and model membrane  
932 preparations. *J. Membr. Biol.* **130**, 1–10 (1992).
- 933 14. Abdelfattah, A. S. *et al.* Bright and photostable chemigenetic indicators for extended in vivo  
934 voltage imaging. *Science (80-. )*. **365**, 699–704 (2019).
- 935 15. Knöpfel, T. & Song, C. Optical voltage imaging in neurons: moving from technology development

- 936 to practical tool. *Nat. Rev. Neurosci.* **20**, 719–727 (2019).
- 937 16. Lu, X. *et al.* Detecting rapid pan-cortical voltage dynamics in vivo with a brighter and faster voltage  
938 indicator Authors. *bioRxiv* (2022). doi:10.1101/2022.08.29.505018
- 939 17. Quicke, P. *et al.* Single-Neuron Level One-Photon Voltage Imaging With Sparsely Targeted  
940 Genetically Encoded Voltage Indicators. *Front. Cell. Neurosci.* **13**, 1–12 (2019).
- 941 18. Hochbaum, D. R. *et al.* All-optical electrophysiology in mammalian neurons using engineered  
942 microbial rhodopsins. *Nat. Methods* **11**, 825–833 (2014).
- 943 19. Parot, V. J. *et al.* Compressed Hadamard microscopy for high-speed optically sectioned neuronal  
944 activity recordings. *J. Phys. D. Appl. Phys.* **52**, (2019).
- 945 20. Fan, L. Z. *et al.* All-Optical Electrophysiology Reveals the Role of Lateral Inhibition in Sensory  
946 Processing in Cortical Layer 1. *Cell* **180**, 521-535.e18 (2020).
- 947 21. Xiao, S. *et al.* Large-scale voltage imaging in behaving mice using targeted illumination. *iScience*  
948 **24**, (2021).
- 949 22. Fisher, J. A. N., Salzberg, B. M. & Yodh, A. G. Near infrared two-photon excitation cross-sections  
950 of voltage-sensitive dyes. *J Neurosci Methods* **148**, 94–102 (2005).
- 951 23. Sims, R. R. *et al.* Optical manipulation and recording of neural activity with wavefront engineering.  
952 in *Neuromethods* (ed. Papagiakoumou, E.) **191**, (Humana Press New York, 2023).
- 953 24. Acker, C. D., Yan, P. & Loew, L. M. Single-voxel recording of voltage transients in dendritic spines.  
954 *Biophys. J.* **101**, L11–L13 (2011).
- 955 25. Roome, C. J. & Kuhn, B. Simultaneous dendritic voltage and calcium imaging and somatic  
956 recording from Purkinje neurons in awake mice. *Nat. Commun.* **9**, 1–14 (2018).
- 957 26. Brinks, D., Klein, A. J. & Cohen, A. E. Two-Photon Lifetime Imaging of Voltage Indicating Proteins  
958 as a Probe of Absolute Membrane Voltage. *Biophys. J.* **109**, 914–921 (2015).
- 959 27. Fisher, J. a N. *et al.* Two-photon excitation of potentiometric probes enables optical recording of  
960 action potentials from mammalian nerve terminals in situ. *J. Neurophysiol.* **99**, 1545–1553 (2008).
- 961 28. Wu, J. *et al.* Kilohertz two-photon fluorescence microscopy imaging of neural activity in vivo. *Nat.*  
962 *Methods* **17**, 287–290 (2020).
- 963 29. Villette, V. *et al.* Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake  
964 Behaving Mice. *Cell* **179**, 1590-1608.e23 (2019).
- 965 30. Platasa, J. *et al.* High-Speed Low-Light In Vivo Two-Photon Voltage Imaging of Large Neuronal

- 966 Populations. *bioRxiv* 2021.12.07.471668 (2021). doi:10.1101/2021.12.07.471668
- 967 31. Kulkarni, R. U. *et al.* In Vivo Two-Photon Voltage Imaging with Sulfonated Rhodamine Dyes. *ACS*  
968 *Cent. Sci.* **4**, 1371–1378 (2018).
- 969 32. Kazemipour, A. *et al.* Kilohertz frame-rate two-photon tomography. *Nat. Methods* **16**, 778–786  
970 (2019).
- 971 33. Bando, Y., Wenzel, M. & Yuste, R. Simultaneous two-photon imaging of action potentials and  
972 subthreshold inputs in vivo. *Nat. Commun.* **12**, 1–12 (2021).
- 973 34. Cornejo, V. H., Ofer, N. & Yuste, R. Voltage compartmentalization in dendritic spines in vivo.  
974 *Science (80-. ).* **375**, 82–86 (2022).
- 975 35. Li, B. *et al.* Two-Photon Voltage Imaging of Spontaneous Activity from Multiple Neurons Reveals  
976 Network Activity in Brain Tissue. *iScience* **23**, 101363 (2020).
- 977 36. Papagiakoumou, E. *et al.* Scanless two-photon excitation of channelrhodopsin-2. *Nat. Methods* **7**,  
978 848–854 (2010).
- 979 37. Papagiakoumou, E., de Sars, V., Oron, D. & Emiliani, V. Patterned two-photon illumination by  
980 spatiotemporal shaping of ultrashort pulses. *Opt. Express* **16**, 22039–22047 (2008).
- 981 38. Papagiakoumou, E. *et al.* Functional patterned multiphoton excitation deep inside scattering  
982 tissue. *Nat. Photonics* **7**, 274–278 (2013).
- 983 39. Pégard, N. M., Oldenburg, I., Sridharan, S., Waller, L. & Adesnik, H. 3D scanless holographic  
984 optogenetics with temporal focusing. *Nat. Commun.* **8**, 1228 (2017).
- 985 40. Liu, Z. *et al.* Sustained deep-tissue voltage recording using a fast indicator evolved for two-photon  
986 microscopy. *Cell* **185**, 3408–3425.e29 (2022).
- 987 41. Oron, D., Tal, E. & Silberberg, Y. Scanningless depth-resolved microscopy. *Opt. Express* **13**,  
988 1468–1476 (2005).
- 989 42. Zhu, G., van Howe, J., Durst, M., Zipfel, W. & Xu, C. Simultaneous spatial and temporal focusing  
990 of femtosecond pulses. *Opt. Express* **13**, 2153–2159 (2005).
- 991 43. Papagiakoumou, E., Ronzitti, E. & Emiliani, V. Scanless two-photon excitation with temporal  
992 focusing. *Nat. Methods* **17**, 571–581 (2020).
- 993 44. Mardinly, A. R. *et al.* Precise multimodal optical control of neural ensemble activity. *Nat. Neurosci.*  
994 **21**, 881–893 (2018).
- 995 45. Bègue, A. *et al.* Two-photon excitation in scattering media by spatiotemporally shaped beams and



- 996 their application in optogenetic stimulation. *Biomed. Opt. Express* **4**, 2869–2879 (2013).
- 997 46. Dal Maschio, M., Donovan, J. C., Helmbrecht, T. O. & Baier, H. Linking Neurons to Network  
998 Function and Behavior by Two-Photon Holographic Optogenetics and Volumetric Imaging. *Neuron*  
999 **94**, 774-789.e5 (2017).
- 1000 47. Golan, L., Reutsky, I., Farah, N. & Shoham, S. Design and characteristics of holographic neural  
1001 photo-stimulation systems. *J. Neural Eng.* **6**, 66004 (2009).
- 1002 48. Yang, S. *et al.* Three-dimensional holographic photostimulation of the dendritic arbor. *J. Neural*  
1003 *Eng.* **8**, 46002 (2011).
- 1004 49. Xie, M. E. *et al.* High-fidelity estimates of spikes and subthreshold waveforms from 1-photon  
1005 voltage imaging in vivo. *Cell Rep.* **35**, (2021).
- 1006 50. Cai, C. *et al.* VolPy: Automated and scalable analysis pipelines for voltage imaging datasets. *PLoS*  
1007 *Comput. Biol.* **17**, 1–27 (2021).
- 1008 51. Kralj, J. M., Douglass, A. D., Hochbaum, D. R., Maclaurin, D. & Cohen, A. E. Optical recording of  
1009 action potentials in mammalian neurons using a microbial rhodopsin. *Nat. Methods* **9**, 90–5  
1010 (2012).
- 1011 52. Hopt, A. & Neher, E. Highly nonlinear photodamage in two-photon fluorescence microscopy.  
1012 *Biophys. J.* **80**, 2029–36 (2001).
- 1013 53. Quicke, P., Howe, C. L. & Foust, A. J. Balancing the fluorescence imaging budget for all-optical  
1014 neurophysiology experiments. in *Neuromethods* (ed. Papagiakoumou, E.) **191**, (Humana Press  
1015 New York, 2023).
- 1016 54. Blankenship, A. G. & Feller, M. B. Mechanisms underlying spontaneous patterned activity in  
1017 developing neural circuits. *Nat. Rev. Neurosci.* **11**, 18–29 (2010).
- 1018 55. Ronzitti, E. *et al.* Sub-millisecond optogenetic control of neuronal firing with two-photon  
1019 holographic photoactivation of Chronos. *J. Neurosci.* **37**, 1246–17 (2017).
- 1020 56. Schrödel, T., Prevedel, R., Aumayr, K., Zimmer, M. & Vaziri, A. Brain-wide 3D imaging of neuronal  
1021 activity in *Caenorhabditis elegans* with sculpted light. *Nat. Methods* **10**, 1013–1020 (2013).
- 1022 57. Prevedel, R. *et al.* Fast volumetric calcium imaging across multiple cortical layers using sculpted  
1023 light. *Nat. Methods* **13**, 1021–1028 (2016).
- 1024 58. Demas, J. *et al.* High-speed, cortex-wide volumetric recording of neuroactivity at cellular resolution  
1025 using light beads microscopy. *Nature Methods* **18**, (2021).
- 1026 59. Song, A. *et al.* Volumetric Two-photon Imaging of Neurons Using Stereoscopy (vTwINS). *Nat.*

- 1027 *Methods* **14**, 420–426 (2017).
- 1028 60. Hernandez, O. *et al.* Three-dimensional spatiotemporal focusing of holographic patterns. *Nat.*  
1029 *Commun.* **7**, 11928 (2016).
- 1030 61. Accanto, N. *et al.* Multiplexed temporally focused light shaping for high-resolution multi-cell  
1031 targeting. *Optica* **5**, 1478–1491 (2018).
- 1032 62. Botcherby, E. J. *et al.* Aberration-free three-dimensional multiphoton imaging of neuronal activity  
1033 at kHz rates. *Proc. Natl. Acad. Sci.* **109**, 2919–2924 (2012).
- 1034 63. Botcherby, E. J., Juskaitis, R., Booth, M. J., Wilson, T. & Juškaitis, R. An optical technique for  
1035 remote focusing in microscopy. *Opt. Commun.* **281**, 880–887 (2008).
- 1036 64. Nöbauer, T. *et al.* Video rate volumetric Ca<sup>2+</sup> imaging across cortex using seeded iterative  
1037 demixing (SID) microscopy. *Nat. Methods* **14**, 811–818 (2017).
- 1038 65. Bovetti, S. *et al.* Simultaneous high-speed imaging and optogenetic inhibition in the intact mouse  
1039 brain. *Sci. Rep.* **7**, 40041 (2017).
- 1040 66. Zhang, T. *et al.* Kilohertz two-photon brain imaging in awake mice. *Nat. Methods* **16**, 1119–1122  
1041 (2019).
- 1042 67. Moretti, C., Antonini, A., Bovetti, S., Liberale, C. & Fellin, T. Scanless functional imaging of  
1043 hippocampal networks using patterned two-photon illumination through GRIN lenses. *Biomed.*  
1044 *Opt. Express* **7**, 3958 (2016).
- 1045 68. Accanto, N. *et al.* A flexible two-photon fiberscope for fast activity imaging and precise optogenetic  
1046 photostimulation of neurons in freely moving mice. *Neuron* **111**, 1–14 (2023).
- 1047 69. Moretti, C. & Gigan, S. Readout of fluorescence functional signals through highly scattering tissue.  
1048 *Nat. Photonics* **14**, 361–364 (2020).
- 1049 70. Printz, Y. *et al.* Determinants of functional synaptic connectivity among amygdala-projecting  
1050 prefrontal cortical neurons. (2021).
- 1051 71. Hage, T. A. *et al.* Synaptic connectivity to L2/3 of primary visual cortex measured by two-photon  
1052 optogenetic stimulation. *eLife* **11**, (2022).
- 1053 72. Sridharan, S. *et al.* High-performance microbial opsins for spatially and temporally precise  
1054 perturbations of large neuronal networks. *Neuron* 1–17 (2022). doi:10.1016/j.neuron.2022.01.008
- 1055 73. Di Leonardo, R., Ianni, F. & Ruocco, G. Computer generation of optimal holograms for optical trap  
1056 arrays. *Opt. Express* **15**, 1913–22 (2007).

- 1057 74. Gerchberg, R. W. & Saxton, W. O. A practical algorithm for the determination of the phase from  
1058 image and diffraction pictures. *Optik (Stuttg)*. **35**, 237–246 (1972).
- 1059 75. Edelstein, A. D. *et al.* Advanced methods of microscope control using  $\mu$ Manager software. *J. Biol.*  
1060 *Methods* **1**, e10 (2014).
- 1061 76. Pinkard, H. *et al.* Pycro-Manager: open-source software for customized and reproducible  
1062 microscope control. *Nat. Methods* **18**, 226–228 (2021).
- 1063 77. Gee, C. E., Ohmert, I., Wiegert, J. S. & Oertner, T. G. Preparation of slice cultures from rodent  
1064 hippocampus. *Cold Spring Harb. Protoc.* **2017**, 126–130 (2017).
- 1065 78. Ho, J., Tumkaya, T., Aryal, S., Choi, H. & Claridge-Chang, A. Moving beyond P values: data  
1066 analysis with estimation graphics. *Nat. Methods* **16**, 565–566 (2019).
- 1067
- 1068

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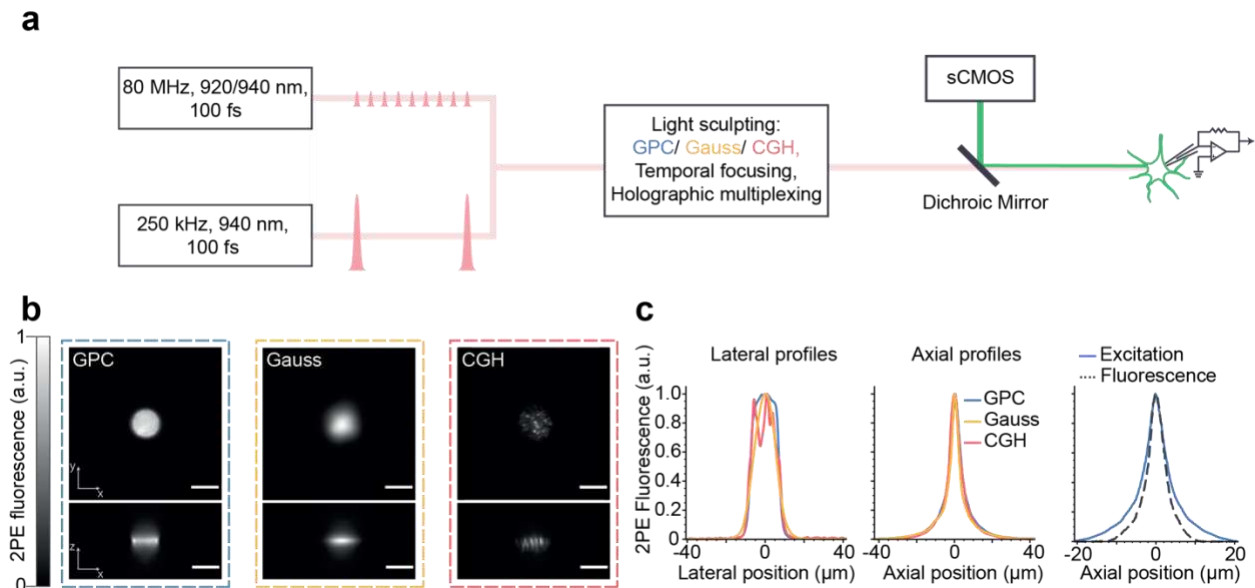
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1088

1089 **Figure 1**



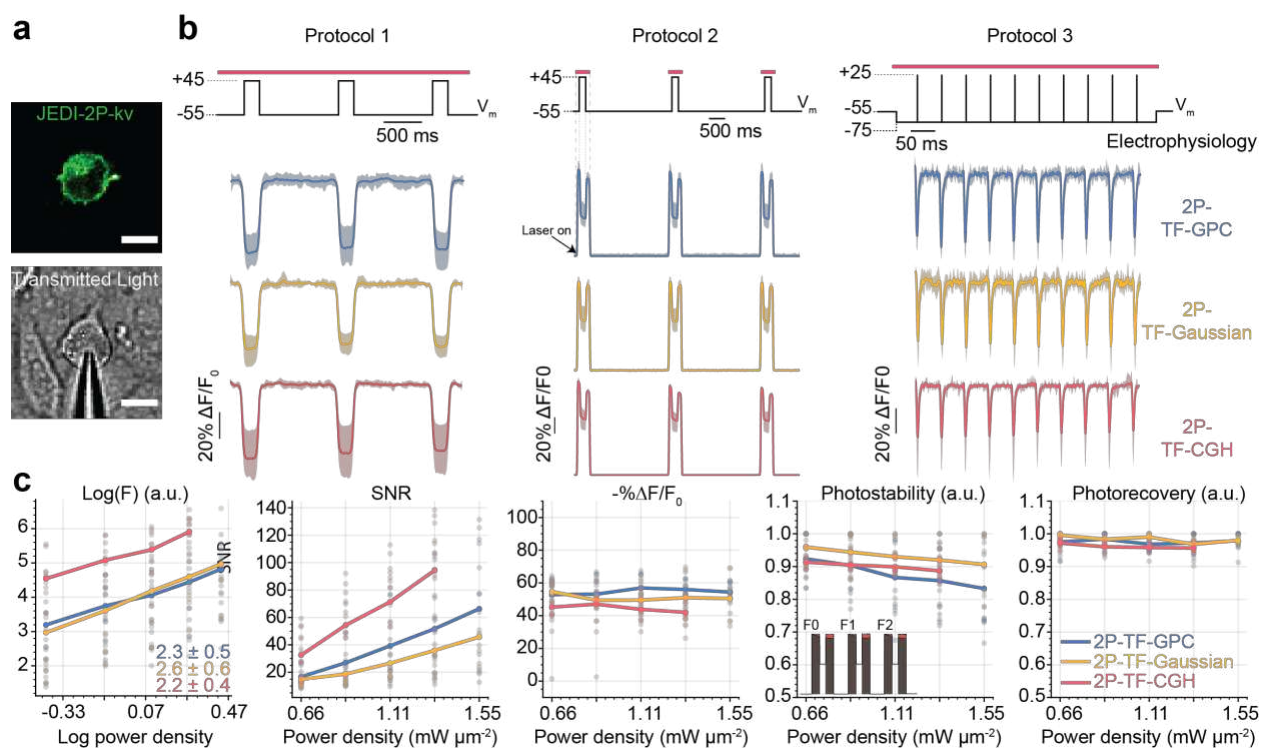
1090

1091 **Schematic and characterization of the optical setup developed for scanless two-photon voltage**  
 1092 **imaging**

1093 **(a)** Summary of the optical setup designed to generate 12  $\mu\text{m}$  (Full Width Half Maximum), temporally  
 1094 focused, Gaussian, Generalised Phase Contrast (GPC) and holographic (CGH) spots. The setup was  
 1095 equipped with three lasers, two of them delivering nJ-pulse energies at 80 MHz (Coherent Discovery, 1 W,  
 1096 80 MHz, 100 fs tuned to 920, 940 or 1030 nm; Spark Alcor, 4 W, 80 MHz, 100 fs, 920 nm) and the third a  
 1097 custom Optical Parametric Amplifier (OPA) pumped by an amplified fibre laser, with fixed wavelength output  
 1098 (Amplitude Satsuma Niji, 0.5-0.6 W, 250 kHz, 100 fs, 940 nm). Fluorescence signals were acquired using  
 1099 an sCMOS camera. The microscope was equipped for electrophysiology patch-clamp recordings. **(b)**  
 1100 Lateral and axial cross sections of two-photon excited fluorescence generated with Gaussian (yellow), GPC  
 1101 (blue) and GCH (red) beams, as indicated in the legend. Scale bars represent 10  $\mu\text{m}$ . **(c)** Lateral and axial  
 1102 profiles of two-photon excited fluorescence generated with each excitation modality, and the corresponding  
 1103 system response, demonstrating single-cell resolution.

1104

1105 **Figure 2**

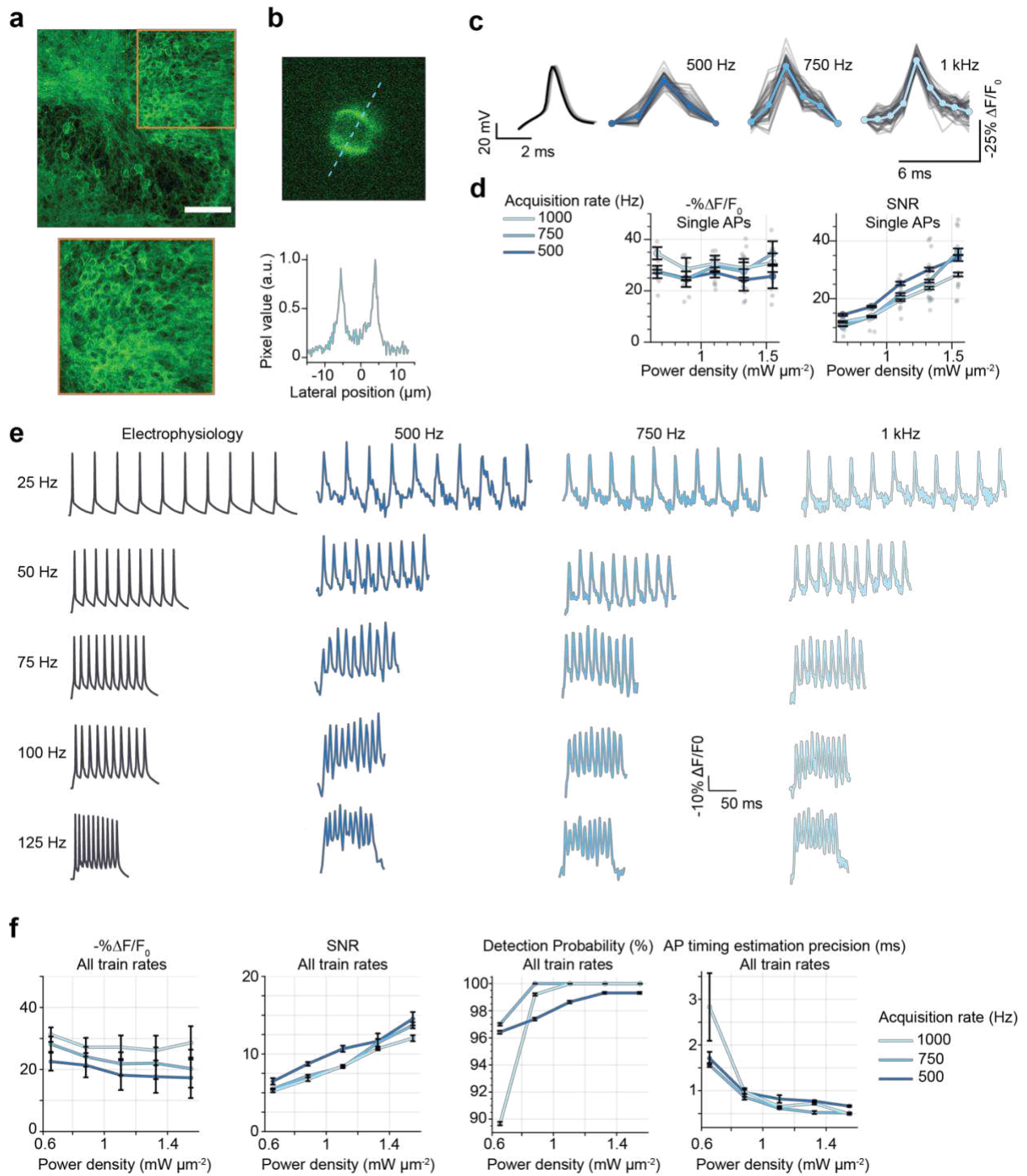


1106

1107 **In-vitro electrophysiological characterisation of scanless two-photon voltage imaging in cultured**  
 1108 **CHO cells.**

1109 **(a)** Confocal image of a JEDI-2P-kv expressing CHO cell (upper) and transmitted light image of a patched  
 1110 CHO cell (lower). Scale bars represent 10  $\mu m$ . **(b)** Data from three protocols used to test the performance  
 1111 of each of the three different parallel illumination modalities for two-photon voltage imaging. Responses are  
 1112 reported as the fluorescence change ( $\Delta F$ ) normalized by the baseline fluorescence ( $F_0$ ), expressed as a  
 1113 percentage of the baseline fluorescence ( $\% \Delta F/F_0$ ). The average trace and 95 percent confidence interval  
 1114 from all cells imaged with each modality are plotted (blue – GPC, yellow – Gaussian, red – CGH). The  
 1115 corresponding electrophysiology control signals are plotted in black. The red bar above the  
 1116 electrophysiology trace indicates the illumination epoch. **(c)** Quantification of data for all cells from protocol  
 1117 2. Log(F), SNR,  $-\Delta F/F_0$ , photobleaching and photorecovery are plotted as a function of power density  
 1118 (power density: 0.66 – 1.55  $mW \mu m^{-2}$ , 75 – 175 mW per cell,  $n = 8 - 13$ ), see also Supplementary Figure 9.  
 1119 Each point represents a measurement from an individual cell. The mean is plotted for each condition.  
 1120 Photostability is defined as the ratio between the integral of the baseline fluorescent trace to  $F_0 \cdot n_t$  where  $F_0$   
 1121 represents the fluorescence in the first frame and  $n_t$  the number of baseline fluorescence timepoints (see  
 1122 schematic diagram, fourth panel, inset). Photorecovery is defined as the average ratio of the fluorescence  
 1123 prior to the 100-mV depolarization in each illumination epoch (for instance  $F_1/F_0$  as defined in the schematic  
 1124 diagram, fourth panel, inset). All data was acquired with laser A tuned to 940 nm and camera A (See  
 1125 Supplementary Figure 1 and Supplementary Tables 1 and 2).

1126

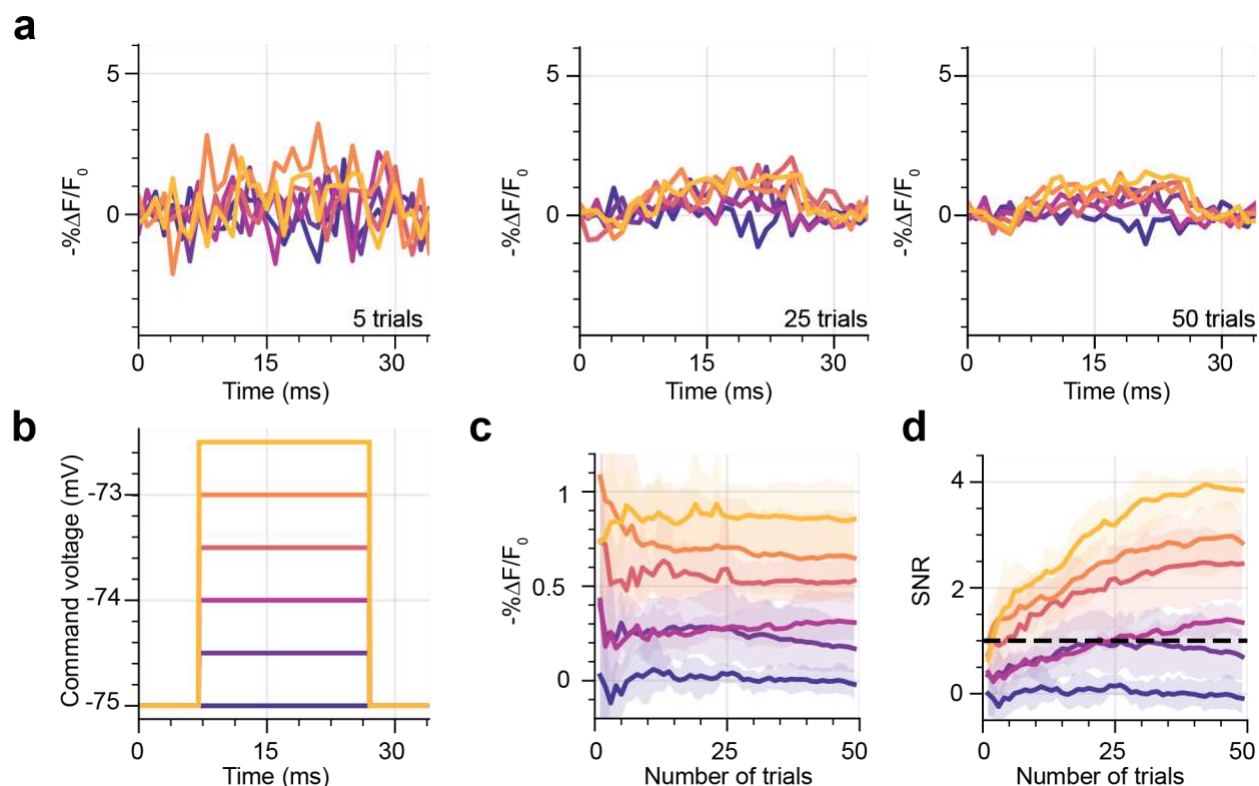


1129 **Recording electrically evoked single action potentials and high-frequency spike trains in JEDI-2P-**  
1130 **kv expressing hippocampal organotypic slices with 2P-TF-GPC.**

1131 **(a)** Upper: confocal image of a representative organotypic slice bulk-infected with JEDI-2P-kv. Scale bar  
1132 represents 75  $\mu\text{m}$ . Lower: zoom (x2) of densely expressing region where data was recorded. **(b)** Upper:  
1133 representative single frame from data acquired with TF-GPC (1 ms exposure time), Lower: line-profile  
1134 through the image (indicated by the dashed line) demonstrating that single cells are imaged with high-  
1135 contrast in densely labelled samples with 2P-TF-GPC. **(c)** Electrically induced and recorded action  
1136 potentials (left) and optically recorded (right) were resolved in single trials using 2P-TF-GPC at different  
1137 acquisition rates. Individual trials are plotted in grey. The average trace across all trials is plotted in a  
1138 different shade of blue corresponding to each acquisition rate (500 Hz, 750 Hz and 1 kHz, as labelled).  
1139 Power density:  $1.1 \text{ mW } \mu\text{m}^{-2}$  (125 mW per cell). **(d)**  $-\% \Delta F/F_0$  and SNR plotted as a function of power density  
1140 in different shades of blue for different acquisition rates (see legend). Error bars represent the standard  
1141 error of measurements across all cells ( $n = 4-6$ ). Individual points represent the average value over 50  
1142 action potentials for individual cells. All data were acquired using laser A tuned at 940 nm, and camera A  
1143 (See Supplementary Figure 1 and Supplementary Tables 1 and 2). **(e)** Representative fluorescence traces  
1144 recorded from individual cells to different rates of electrically evoked spike trains recorded at the different  
1145 acquisition rates of 500 Hz, 750 Hz and 1 kHz corresponding to 2 ms, 1,33 ms and 1 ms exposure time  
1146 (power density:  $1.1 \text{ mW } \mu\text{m}^{-2}$ , 125 mW per cell). A representative trace of electrically evoked spike trains is  
1147 also plotted in black (left). **(f)**  $-\% \Delta F/F_0$ , SNR, action potential detection probability and precision of action  
1148 potential timing estimation (defined as the jitter in timing estimation for all identified action potentials relative  
1149 to the corresponding electrophysiological recordings) plotted as a function of power density for different  
1150 acquisition rates (500 Hz, 750 Hz, and 1 kHz, see legend). A lower value indicates superior timing  
1151 estimation. Data plotted for all train rates ( $n = 2-5$ ). All data were acquired using laser B fixed at 920 nm,  
1152 and camera B (See Supplementary Figure 1 and Supplementary Tables 1 and 2).



1153 **Figure 4**

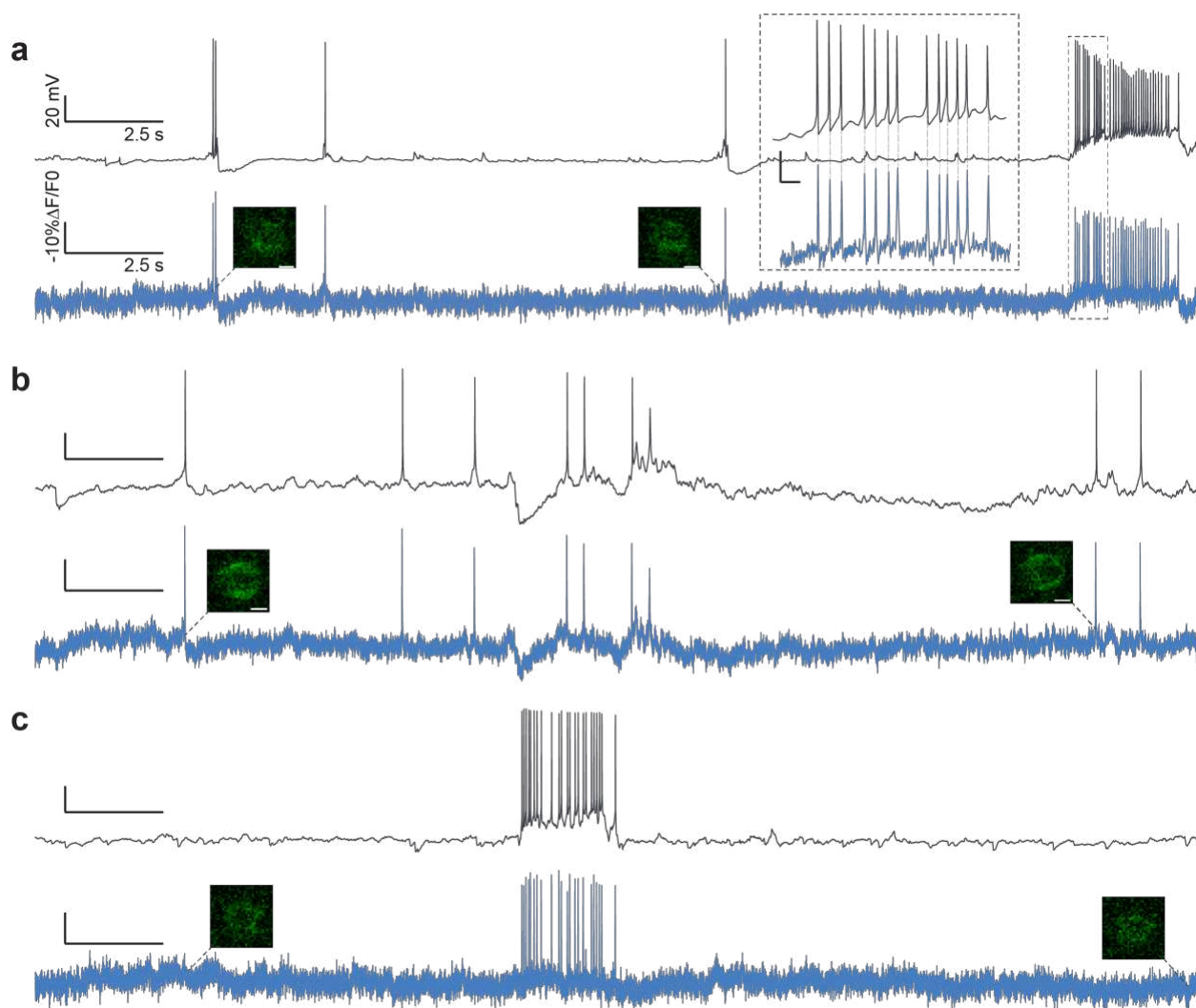


1154

1155 **Recording sub-threshold depolarizations in JEDI-2P-kv expressing hippocampal organotypic slices**  
1156 **using 2P-TF-GPC.**

1157 **(a)** Average fluorescence traces recorded from neurons after 5, 25 and 50 trials for different magnitudes of  
1158 sub-threshold depolarizations ranging between 0 and 2.5 mV. Sub-threshold depolarisations < 2.5 mV  
1159 cannot be reliably resolved in single trials using 2P-TF-GPC and JEDI-2P-kv, however after 25 trials  
1160 depolarisations greater than or equal to 1 mV can be resolved. Traces were recorded with a 1 ms exposure  
1161 time and 1.1 mW μm<sup>-2</sup> (125 mW per cell). **(b)** Command voltage steps used to change the membrane  
1162 potential of patched neurons. **(c)** Average -%ΔF/F<sub>0</sub> and **(d)** SNR of the fluorescence response to different  
1163 sub-threshold changes of membrane potential plotted as a function of number of repeats. The 95%  
1164 confidence interval is also plotted (shaded region). All data (n = 6) were acquired using laser B fixed at 920  
1165 nm and camera B (See Supplementary Figure 1 and Supplementary Tables 1 and 2).

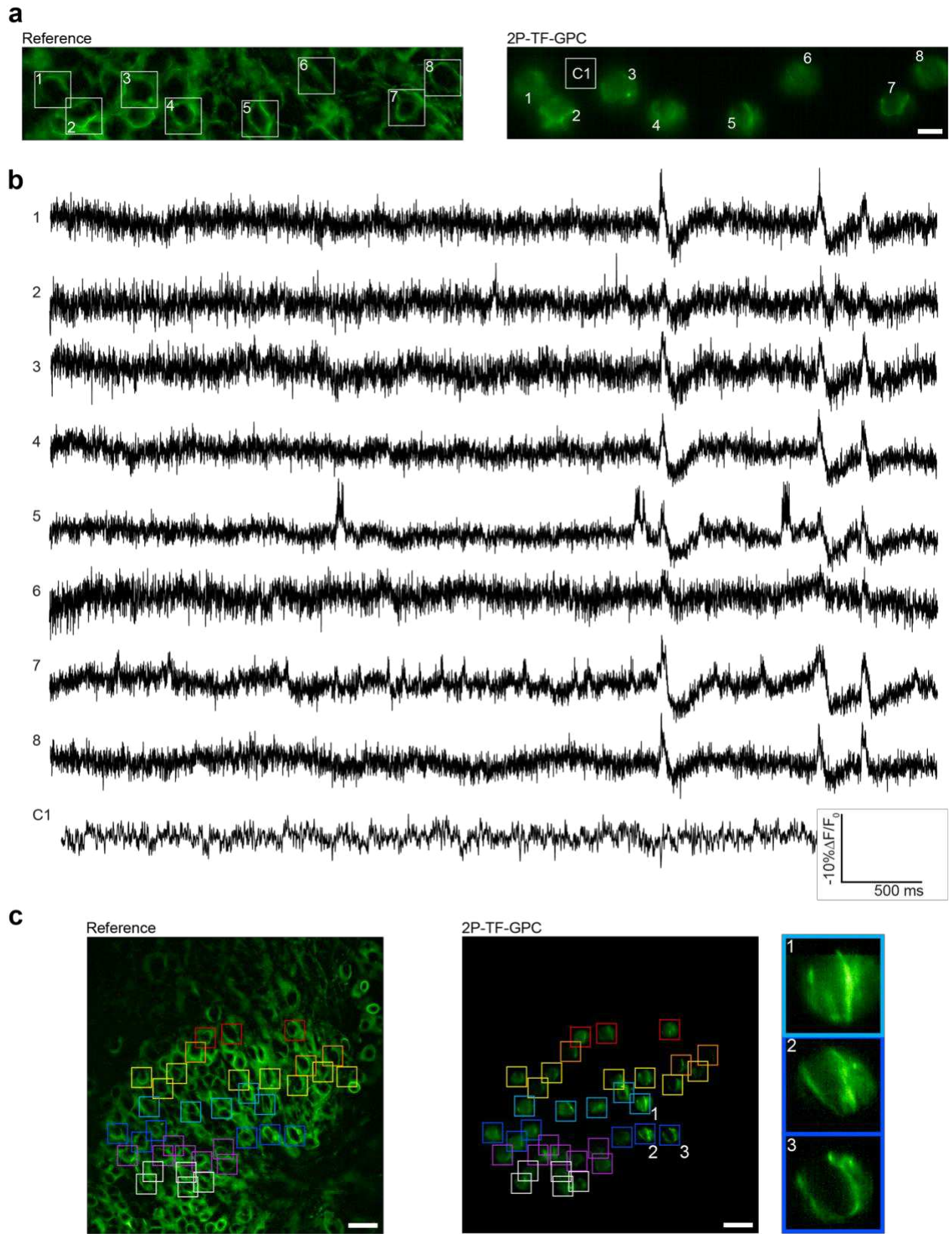
1166 **Figure 5**



1167  
1168 **Recording spontaneous neural activity in JEDI-2P-kv expressing hippocampal organotypic slices**  
1169 **using 2P-TF-GPC**

1170 **(a-c)** Simultaneous current clamp (upper, black) and fluorescence recordings (lower, blue) of spontaneous  
1171 activity in neurons from hippocampal organotypic slices over a continuous 30 s recording period. Single  
1172 imaging frames are shown close to the beginning and end of each recording. Scale bars represent 5  $\mu\text{m}$ .  
1173 Inset, **(a)** zoomed in portion of the electrophysiological and fluorescence traces. Corresponding action  
1174 potentials in the electrophysiological and fluorescence traces (average rate: 17 Hz) is indicated by the  
1175 dashed lines. (Power density:  $1.33 \text{ mW } \mu\text{m}^{-2}$ , 150 mW per cell, 1 kHz acquisition rate). All data was acquired  
1176 using laser A tuned to 940 nm and camera A (See Supplementary Figure 1 and Supplementary Tables 1  
1177 and 2).

1178

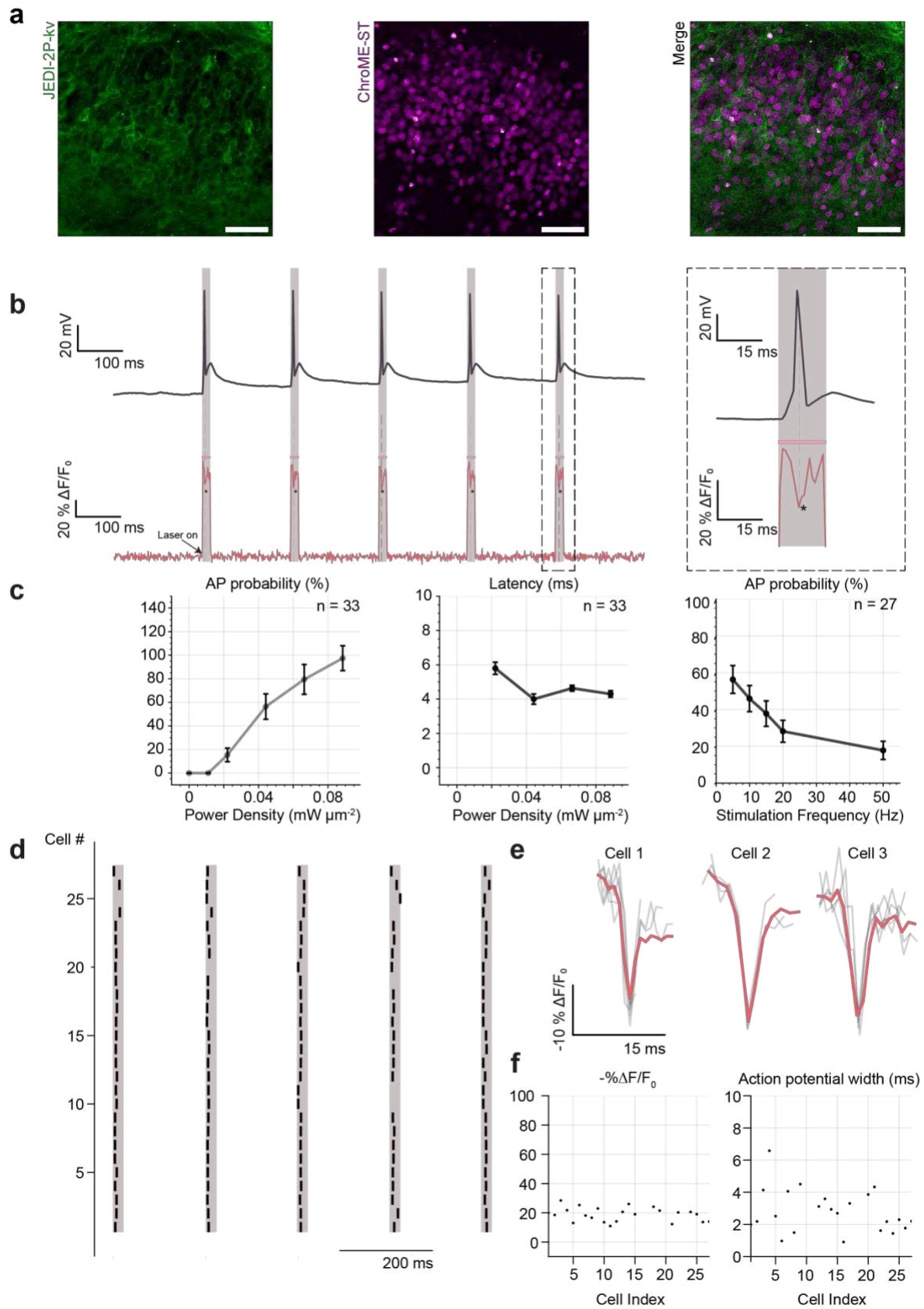


1181 **Multi-cell recordings of spontaneous neural activity in JEDI-2P-kv expressing hippocampal**  
1182 **organotypic slices using multiplexed 2P-TF-GPC**

1183 **(a)** Reference image of a hippocampal organotypic slice expressing JEDI-2P-kv in the dentate gyrus (left  
1184 panel) and average projection of the corresponding voltage imaging dataset (right panel). 8 neurons  
1185 targeted simultaneously with 8, 12- $\mu\text{m}$  2P-TF-GPC spots can be identified (as numbered and highlighted  
1186 by the square boxes). The scale bar represents 10  $\mu\text{m}$ . C1 refers to the area used to generate the control  
1187 trace plotted in (b). This ROI was not targeted with a GPC spot during experiments. **(b)** Fluorescent traces  
1188 plotted for each of the neurons indicated in (a), including the control trace. **(c)** Voltage imaging throughout  
1189 a large field of view using multiplexed 2P-TF-GPC. Left panel: cross-section of a hippocampal organotypic  
1190 slice expressing JEDI-2P-kv in the dentate gyrus. Middle panel: combined maximum projections of data  
1191 from 7 consecutive acquisitions (indicated by the coloured squares), spanning a total area of 200 x 150  
1192  $\mu\text{m}^2$ . Zoom in for best viewing. Scale bars represent 25  $\mu\text{m}$ . Right panel: zoomed in regions of the central  
1193 panel (indicated by numbering and coloured boxes) showing maximum projections of data acquired from  
1194 individual cells targeted with multiplexed 2P-TF-GPC. All data was acquired using laser C (940 nm, power  
1195 density: 0.02 – 0.09  $\text{mW } \mu\text{m}^{-2}$ , 2.5 – 10  $\text{mW}$  per cell) and camera A with an acquisition rate of 1 kHz (See  
1196 Supplementary Figure 1 and Supplementary Tables 1 and 2).

1197

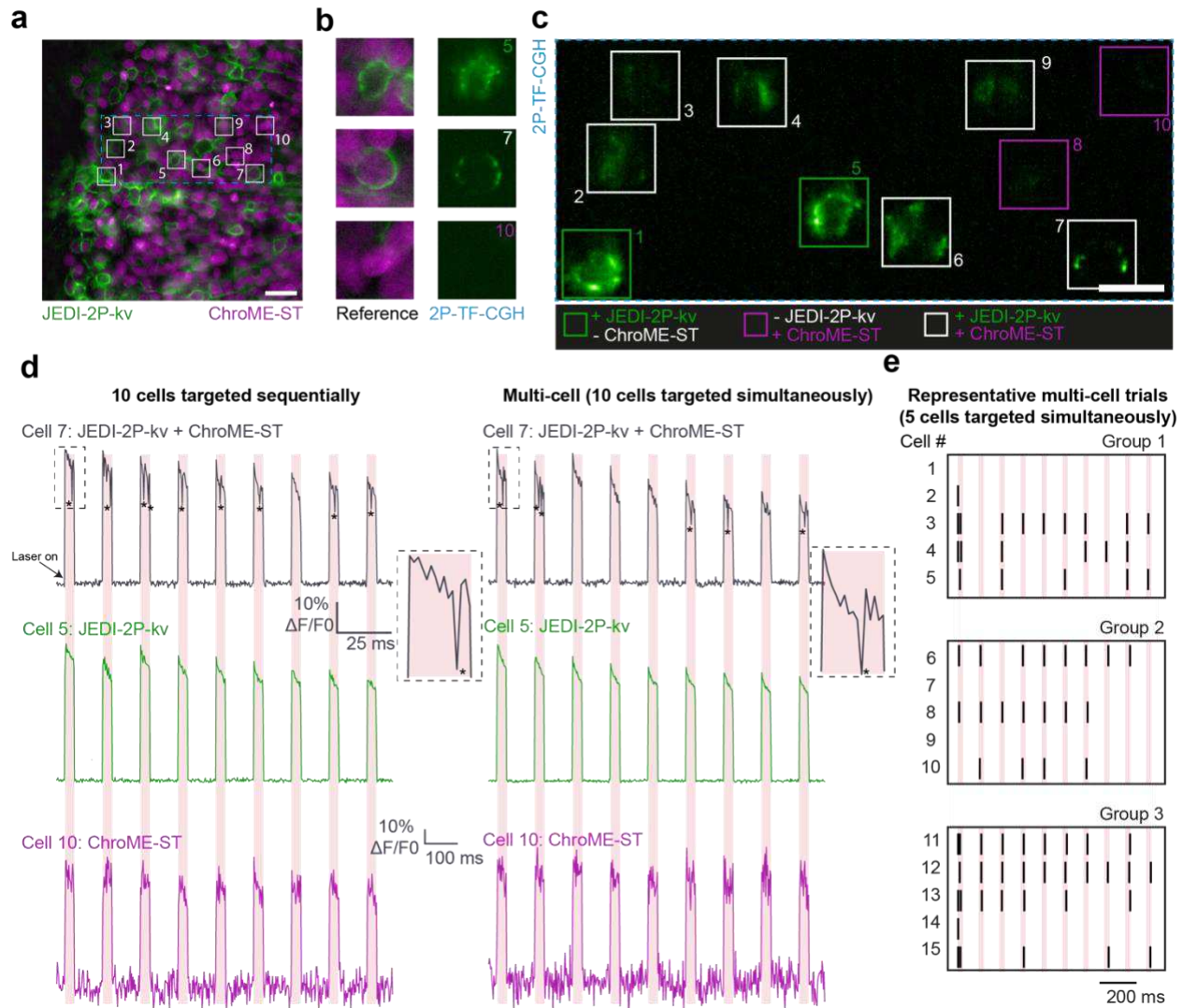
Figure 7



1199 **Fluorescence recordings of photo-evoked spikes in hippocampal organotypic slices co-expressing**  
1200 **JEDI-2P-kv and ChroME-ST, using 2P-TF-CGH.**

1201 **(a)** Cross-sections of hippocampal organotypic slices co-expressing the genetically encoded voltage  
1202 indicator JEDI-2P-kv and the soma-targeted channelrhodopsin ChroME-ST in the dentate gyrus.  
1203 Channelrhodopsin-expressing cells were identified according to their nuclear-localized fluorescence (see  
1204 Methods). Scale bar represents 50  $\mu\text{m}$ . **(b)** (left) Simultaneous optical and electrophysiological recordings  
1205 demonstrating that action potentials can be evoked and imaged using a single excitation spot (12  $\mu\text{m}$   
1206 diameter, power density 0.02  $\text{mW } \mu\text{m}^{-2}$  (2.5  $\text{mW}$  per cell), 15 ms strobed illumination at 5 Hz). (right) Zoom  
1207 on simultaneous optical and electrophysiological recordings of one action potential. **(c)** All-optical in-situ  
1208 characterisation of photo evoked action potentials. Error bars represent the standard error of recordings  
1209 obtained for 33 cells. The probability of evoking and recording action potentials is plotted as a function of  
1210 power density. Only cells in which at least one optically evoked action potential was detected are included.  
1211 The latency of optically evoked action potentials is plotted as a function of power density. The average  
1212 latency measured all-optically matches that obtained using electrophysiology (Supplementary Figure 20c).  
1213 The action potential probability is plotted as a function of stimulation frequency. Action potential probability  
1214 is calculated as the number of action potentials evoked and recorded over five trials (power density: 0.01 –  
1215 0.09  $\text{mW } \mu\text{m}^{-2}$ , 1.5 – 10  $\text{mW}$  per cell). Error bars represent the standard error of recordings obtained for 33  
1216 cells. **(d)** Raster plot of 27 cells showing the number and timing of optically evoked action potentials (black)  
1217 relative to the imaging/photostimulation epoch (red) (power density: 0.02 – 0.08  $\text{mW } \mu\text{m}^{-2}$ , 2.5 – 9  $\text{mW}$  per  
1218 cell). **(e)** Examples of fluorescence recordings of optically evoked action potentials for three representative  
1219 cells. Individual trials are plotted in grey. The average trace across all trials is plotted in red (power density:  
1220 0.02 – 0.08  $\text{mW } \mu\text{m}^{-2}$ , 2.5 – 9  $\text{mW}$  per cell). **(f)** Summary statistics for the amplitude ( $-\% \Delta F/F_0$ ) and width of  
1221 the optically evoked action potentials from (d). All data were acquired using laser C fixed at 940 nm and  
1222 camera A (See Supplementary Figure 1 and Supplementary Tables 1 and 2).

1223



1225

1226 **Characterisation of simultaneous multi-target photostimulation and voltage imaging using a single**  
 1227 **beam scanless two-photon excitation.**

1228 **(a)** Cross-sections of hippocampal organotypic slices co-expressing JEDI-2P-kv and ChroME-ST in the  
 1229 dentate gyrus. The boxes indicate cells that were targeted simultaneously during a representative  
 1230 experiment (as numbered). Scale bar represents 25  $\mu$ m. **(b)** Reference widefield images of individual  
 1231 targeted cells (left) and corresponding images obtained using 2P-TF-CGH. Upper: a cell exclusively  
 1232 expressing JEDI-2P-kv. Middle: a cell co-expressing JEDI-2P-kv and ChroME-ST. Lower: a cell exclusively  
 1233 expressing ChroME-ST. **(c)** Data acquired when the 10 cells identified in (a) were targeted simultaneously  
 1234 using 2P-TF-CGH and imaged at 500 Hz. Scale bar represents 15  $\mu$ m. **(d)** Traces from the three cells  
 1235 highlighted in (b) when targeted sequentially (left) or simultaneously (right). Of the three selected cells,  
 1236 as expected, no action potentials were detected for cell 5 (green) or cell 10 (purple), which did not co-express  
 1237 the two constructs. In both the sequential and multi-cell acquisitions, action potentials were only evoked/  
 1238 recorded in the cells co-expressing JEDI-2P-kv and ChroME-ST (black). **(e)** Raster plots from 3 further  
 1239 experiments in which 5 cells were targeted simultaneously. Black lines indicate the time at which a cell  
 1240 fired; the red lines indicate the imaging/photostimulation laser. All data were acquired using laser C fixed  
 1241 at 940 nm and camera A (See Supplementary Figure 1 and Supplementary Tables 1 and 2).

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