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2 **Implantable photonic neural probes for**
3 **light-sheet fluorescence brain imaging**
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30 **ABSTRACT**

31 **Significance:** Light-sheet fluorescence microscopy is a powerful technique for high-speed
32 volumetric functional imaging. However, in typical light-sheet microscopes, the illumination and
33 collection optics impose significant constraints upon the imaging of non-transparent brain tissues.
34 Here, we demonstrate that these constraints can be surmounted using a new class of implantable
35 *photonic neural probes*.

36 **Aim:** Mass manufacturable, silicon-based light-sheet photonic neural probes can generate planar
37 patterned illumination at arbitrary depths in brain tissues without any additional micro-optic
38 components.

39 **Approach:** We develop implantable photonic neural probes that generate light sheets in tissue.
40 The probes were fabricated in a photonics foundry on 200 mm diameter silicon wafers. The light
41 sheets were characterized in fluorescein and in free space. The probe-enabled imaging approach
42 was tested in fixed and *in vitro* mouse brain tissues. Imaging tests were also performed using
43 fluorescent beads suspended in agarose.

44 **Results:** The probes had 5 to 10 addressable sheets and average sheet thicknesses $< 16 \mu\text{m}$ for
45 propagation distances up to $300 \mu\text{m}$ in free space. Imaging areas were as large as $\approx 240 \mu\text{m} \times 490$
46 μm in brain tissue. Image contrast was enhanced relative to epifluorescence microscopy.

47 **Conclusions:** The neural probes can lead to new variants of light-sheet fluorescence microscopy
48 for deep brain imaging and experiments in freely-moving animals.

49

50 **Keywords:** Neurophotonics, integrated optics, functional imaging, microscopy, biophotonics

51

52 **I. INTRODUCTION**

53 New methods in optogenetics [1–3] and, especially, the advent of fluorescent reporters of
54 neuronal activity, have opened many novel approaches for actuating and recording neural activity
55 *en masse*, through the use of powerful *free-space* single-photon and multi-photon microscopy
56 methods [4–8]. However, existing approaches to functional imaging of the brain have significant
57 limitations. Single-photon (1P) epifluorescence imaging readily lends itself to high frame-rate
58 wide-field microscopy, but, in its simplest implementations, image contrast is hampered by out-
59 of-focus background fluorescence, and the depth of imaging is restricted by the optical attenuation

60 in the tissue. Confocal imaging improves the contrast by optical sectioning, and out-of-focus light
61 is rejected using a pinhole; however, a laser beam must be scanned across each point of the tissue
62 and this significantly slows the image acquisition rate [9]. Multiphoton microscopy is also
63 inherently a point or line scanning method, but because it uses infrared excitation (which provides
64 a longer optical attenuation length [5]), the imaging depth in brain tissue can be extended to ~1
65 mm and the focus of the light beam can be rastered in three-dimensions to achieve volumetric
66 imaging [5, 10–12].

67 Light-sheet fluorescence microscopy (LSFM), which is also known as selective-plane
68 illumination microscopy, combines the benefits of fast wide-field imaging, volumetric imaging,
69 and optical sectioning [13]. In conventional LSFM, a thin sheet of excitation light is generated
70 either by cylindrical focusing elements or digitally scanning a Gaussian or Bessel beam [14–16].
71 The sheet is translated in one dimension across the sample; the fluorescence images are then
72 sequentially collected in the direction perpendicular to the illumination plane to form a volumetric
73 image [17]. With digitally scanned two-photon (2P) LSFM, it is also possible to increase the
74 optical penetration depth [16]. Non-digitally scanned 1P-LSFM is inherently faster than point- or
75 line-scan methods; and since the illumination is restricted to a plane, photobleaching,
76 phototoxicity, and out-of-focus background fluorescence are reduced compared to epifluorescence
77 microscopy. However, conventional LSFM requires two orthogonal objective lenses, and
78 appropriately positioning these largely limits the imaging modality to quasi-transparent organisms
79 (*e.g.*, *C. elegans*, *Drosophila* embryos, larval zebrafish), chemically cleared mammalian brains
80 [17], and brain slices [18]. An LSFM variant called swept confocally-aligned planar excitation
81 (SCAPE) microscopy, which requires only a single objective, removes these constraints [6, 19].
82 While *in vivo* calcium neural imaging has been demonstrated using SCAPE in mice [6],
83 miniaturization of the system to be compatible with freely moving animal experiments remains
84 challenging due to the additional optics required.

85 To make LSFM compatible with non-transparent tissues such as mammalian brains and,
86 eventually, behavioral experiments with freely moving animals necessitates drastic miniaturization
87 of the light-sheet generation and fluorescence imaging compared to today's archetypical table-top
88 systems. The feasibility of fluorescence microscopy in small and lightweight form factors has
89 already been established by way of head-mounted microscopes for 1P and 2P calcium imaging in

90 mice [4, 20–23], though the endoscopic implantation of the requisite gradient index (GRIN) lenses,
91 with typical diameters of 0.5 - 2 mm, displaces a significant amount of brain tissue.

92 On the other hand, it remains a formidable and unsolved challenge to generate light sheets by
93 implantable elements at arbitrary brain depths, while minimizing tissue displacement and
94 remaining compatible with a sheet-normal imaging system. For example, in [24], to generate a
95 light sheet perpendicular to the imaging GRIN lens required implantation of a millimeter-scale
96 prism coupled to a second GRIN lens. In another example, in [25], a single light sheet was
97 produced from a microchip using a grating coupler, a glass spacer block, and a metallic slit lens.
98 The overall device was $> 100 \mu\text{m}$ thick and $> 600 \mu\text{m}$ wide, which would displace a significant
99 amount of tissue upon implantation.

100 Here, we solve these challenges by using wafer-scale nanophotonic technology to realize
101 implantable, silicon-based, light-sheet photonic neural probes that require *no additional* micro-
102 optics. They are fully compatible with free space fluorescence imaging (light collection) outside
103 the brain, where the axis of collection is oriented perpendicular to the light sheets. These silicon
104 (Si) probes synthesize light sheets in tissue using sets of nanophotonic grating couplers (GCs)
105 integrated onto thin, implantable, 3 mm long Si shanks with 50 - 92 μm thickness, widths that
106 taper from 82 - 60 μm along their length, and sharp tips at the distal ends. These prototype photonic
107 neural probes (Fig. 1) are capable of generating and sequentially addressing up to 5 illumination
108 planes with a pitch of $\approx 70 \mu\text{m}$. Additionally, the form factor and illumination geometry of the
109 probes open an avenue toward their integration with GRIN lens endoscopes and miniature
110 microscopes, as shown conceptually in Fig. 2(b); offering a singular pathway to rapid, optically
111 sectioned functional imaging at arbitrary depths in the brain.

112 The probes were fabricated on 200 mm Si wafers in a Si photonics foundry for manufacturing
113 scalability and mass-producibility. Elsewhere, we have used this technology to realize photonic
114 neural probes that emit dynamically-reconfigurable, patterned light with cellular-scale beam
115 widths [26] and steerable beams without moving parts [27], adding to a growing number of
116 photonic neural probe demonstrations with increasing levels of integration and sophistication [28–
117 30]. In this work, we employ this integrated nanophotonics technology to realize implantable,
118 microscale probes that form light sheets for imaging over areas as large as $\approx 240 \mu\text{m} \times 490 \mu\text{m}$ in
119 brain tissue. Our preliminary results were reported in [31]. Here, we report in detail the imaging

120 properties of the light-sheet neural probes – characterizing their performance by means of
121 suspended fluorescent beads in phantoms as well as in adult mouse brain slices. A first
122 demonstration of *in vivo* calcium imaging is also reported in the Supplementary Materials.

123

124 II. RESULTS

125 A. Photonic neural probes on 200 mm silicon wafers

126 To ensure that fabrication of our photonic neural probes can be scaled up for dissemination to
127 the neuroscience community, we have adapted from the outset foundry Si photonics manufacturing
128 processes. The neural probes described herein were fabricated in a 200 mm Si photonic line; silicon
129 nitride (SiN) waveguides (135 nm nominal thickness) with SiO₂ cladding were patterned onto Si
130 wafers, deep trenches were etched in the wafers to define the probe shapes, and the wafers were
131 thinned to thicknesses of 50 - 92 μm . The shank thickness can be reduced in future iterations to 18
132 μm , as in [26, 31]. The fabrication is more fully detailed in Methods.

133 The light-sheet neural probe design is shown in Figs. 1(a)-(c). Light is coupled onto the probe
134 chip using fiber-to-chip edge couplers that taper from 5.2 μm in width at the chip facet to single-
135 mode waveguides with widths of 270 – 330 nm. The waveguide-coupled optical power is divided
136 between four to eight waveguides using a routing network consisting of 1×2 multimode
137 interference (MMI) splitters [32] and in-plane waveguide crossings [33]. The light is then guided
138 along the implantable shanks via 1 μm wide, multimode waveguides, and subsequently emitted
139 near the distal end of the probe by a row of GCs. Light sheets are synthesized by overlapping the
140 emission from an array of simultaneously-fed GCs. Each row of GCs generates a separate light
141 sheet. The width, period, and duty cycle of the GCs are designed to achieve a large output
142 divergence angle along the width-axis of the sheet, and only a small divergence along the
143 thickness-axis. Nominal lateral GC widths, periods, and duty cycles are 1.5 μm , 440 to 480 nm,
144 and 50%, respectively.

145 The waveguide routing network is detailed in Fig. S1 in the Supplementary Materials. The
146 photonic components were designed for a wavelength of 488 nm to enable excitation of common
147 fluorophores such as green fluorescent protein (GFP) and green calcium dyes; however, these
148 components can also be designed for green, yellow, and red wavelengths, as we show in [34] for
149 excitation of other fluorophores. The probe shanks are 3 mm in length and separated with a 141

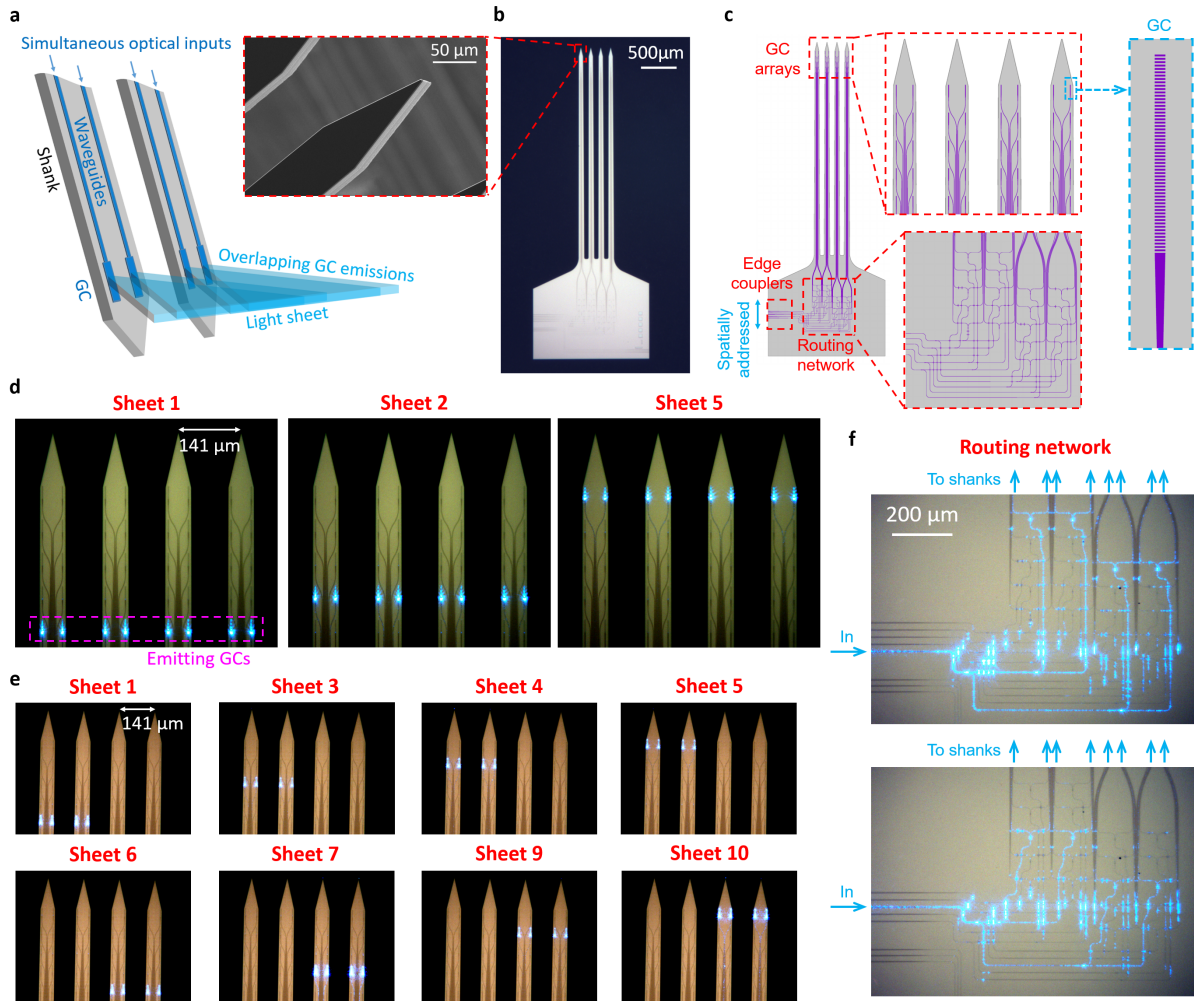


FIG. 1: Light-sheet photonic neural probes. (a) Illustration of the light-sheet synthesis method (adapted from [31]). A series of simultaneously fed optical waveguides emits light via a row of grating couplers (GCs) designed for large divergences along the sheet-axis and small divergences along the GC-axis. (b) Optical micrograph of a fabricated neural probe, (inset) scanning electron micrograph (SEM) of the tip of a shank. (c) Top-down schematics of the neural probe. (d-e) Annotated optical micrographs of 2 neural probes with various GC rows emitting light sheets. (d) Neural probe design with sheets generated from 4 shanks. (e) Probe design with sheets generated from 2 shanks (“half-sheet design”). (f) Optical micrographs showing the routing network from the probe in (d) guiding light for optical inputs to 2 different edge couplers. (d-e) have been contrast and brightness adjusted to enhance the visibility of the waveguides.

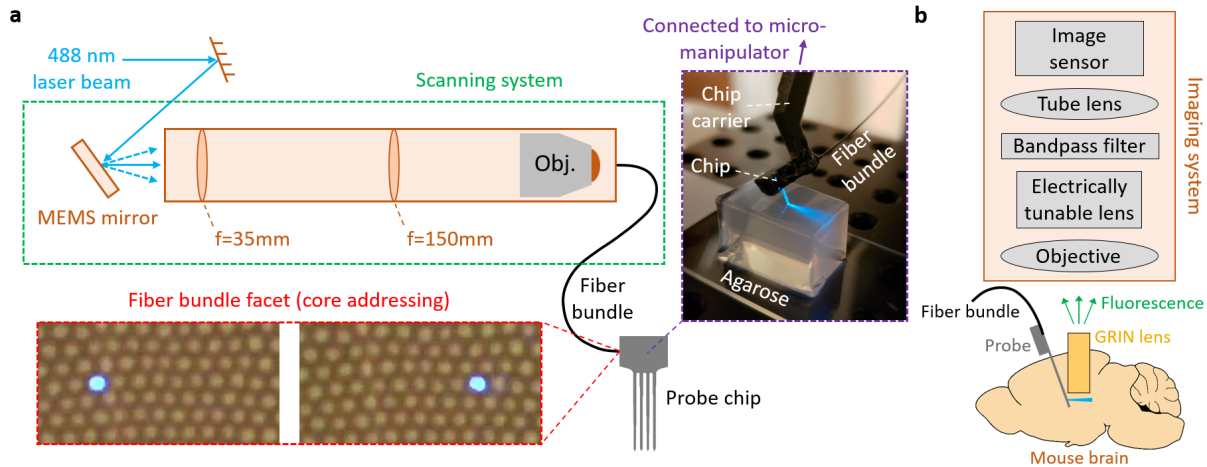


FIG. 2: Optical addressing method and proposal for deep-brain photonic-probe-enabled LSFM. (a) Schematic of the optical addressing method (not to scale). The scanning system addresses on-chip edge couplers via spatial addressing of the cores of an image fiber bundle. Bottom inset: micrographs of the distal facet of a fiber bundle connected to the scanning system with different cores addressed (adapted from [31]). Top inset: annotated photograph of a packaged light-sheet neural probe inserted into an agarose block. (b) Illustration of the proposed use of the light-sheet neural probe with a GRIN lens endoscope for deep brain LSFM (not to scale). In this first investigation of the probe functionality, the configuration in (b) has not been demonstrated, and instead, the results here focus on a simpler imaging configuration where light-sheet probe illuminated samples are directly imaged with a fluorescence microscope without a GRIN lens (see Results).

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152 μm pitch; the rows of GCs integrated onto the shanks, each row corresponding to a different sheet,
153 are separated by a $75 \mu\text{m}$ pitch along the shanks. The shanks taper in width from 82 to $60 \mu\text{m}$ over
154 their length and each converges to a sharp tip at its distal end.

155 To rapidly switch between different sheets, we used a spatial addressing approach similar to
156 [35] and as illustrated in Fig. 2(a). An image fiber bundle was epoxied to the probe chip on a
157 common carrier, with each edge coupler on the probe aligned to a different core of the fiber bundle.
158 By actuating the microelectromechanical systems (MEMS) mirror, light was input to a selected
159 core of the fiber bundle and the corresponding input waveguide for a light sheet. The light-sheet
160 switching speed was limited to $\approx 5 \text{ ms}$ (0.2 kHz) in the following demonstrations, a constraint

161 arising from the MEMS mirror. Future designs will employ optimized MEMS mirrors operating
162 in resonance mode that can yield switching frequencies > 30 kHz [36]. Videos S1 and S2
163 demonstrate rapid switching between different light sheets from packaged probes. The fiber bundle
164 used in these first experiments did not maintain polarization, whereas the photonic circuitry was
165 polarization dependent. Therefore, in these first probe prototypes, the fiber bundle must be held
166 still during imaging. This limitation can be overcome in future designs with use of polarization-
167 maintaining multi-core fibers.

168 Table I summarizes three light-sheet photonic neural probes we have carefully evaluated and
169 report upon in this article. Beam profiles for the three probes are characterized. Probe 1 is used for
170 imaging fluorescent beads and fixed tissue, and Probe 2 is used for *in vitro* imaging. In the table,
171 the “emission angle” refers to the angle of the sheet relative to the normal of the shanks. It is
172 noteworthy that the sheets were designed to emit at an angle of $\sim 20^\circ$ in tissue; this permits
173 implanting the probe next to an imaging lens such that the light sheets can be generated beneath
174 the lens parallel to the focal plane.

175 Two probe designs were investigated: a first, in which each light sheet is generated by a row of
176 eight GCs spanning four adjacent shanks (Probe 1, with 5 independent sheets), and a second based
177 on a “half-sheet design”, in which each sheet is generated by a row of four GCs spanning two
178 adjacent shanks (Probes 2 and 3, with 10 independent half-sheets). In principle, the half-sheet
179 design roughly doubles the sheet intensity for a given input optical power to the probe at the
180 expense of a smaller sheet width. More advanced designs can achieve even larger sheet widths by
181 distributing GCs along > 4 shanks at the expense of: 1) a more complex routing network with
182 higher optical losses, and 2) higher input optical powers to the probe chip to achieve a given light-
183 sheet intensity.

TABLE I: **Light-sheet photonic neural probes described in this work**

Probe name	Nominal probe thickness (μm)	Shanks per sheet	Number of sheets	Emission angle in fluorescein ($^\circ$)	Sheet pitch in fluorescein (μm)
Probe 1	92	4	5	23.5	69
Probe 2	92	2	10	19.8	71
Probe 3	50	2	10	22.3	69

184 **B. Light-sheet generation**

185 The photonic circuitry employed in these devices is designed to provide lower loss for
186 transverse-electric (TE) polarized light. In the following characterization and imaging work, we
187 use TE-polarized optical inputs to the probe chips. The insertion loss of the neural probes (defined
188 here as the ratio of emitted power from the GCs and the input laser power to the scanning system)
189 is summarized in the histograms for Probes 1 and 2 in Fig. S2 in the Supplementary Materials.
190 Probe 3 broke before sheet transmissions were able to be characterized.

191 Transmission ranged from -38 dB to -20 dB, with a median of about -30 dB. This large variation
192 in transmission was due both to the irregularly positioned individual cores within the fiber bundles
193 and alignment drift during attachment of the fiber to the probe chip. Sheet transmissions measured
194 with a single-mode fiber with optimized alignment typically varied by < 3 dB. In future designs,
195 such transmission variations can be reduced by implementing optimal fiber-to-chip packaging and
196 by employing custom multi-core fibers with a constant core pitch that closely matches that of the
197 on-chip edge couplers. Nonetheless, by modulating the input laser power while switching between
198 sheets or adjusting the MEMS mirror positions for each sheet, these variations can be compensated
199 with the present devices.

200 We have measured the intrinsic properties of light sheets generated both in free space and in
201 non-scattering fluorescein solutions – characterizing the light-sheet thicknesses, their intensity
202 uniformities, and the magnitudes of associated, higher-order GC diffraction. We determine the in-
203 plane sheet intensity profile by imaging top-down while the probes are immersed in fluorescein
204 solution, Fig. 3(a). When imaging from the side, the sheet thickness is overestimated since out-of-
205 focus light can also be captured. In the free-space method, Fig. 4(a), a coverslip coated on one side
206 with a fluorescent thin film is placed above the probe parallel to the shanks, and a cross-section of
207 the beam profile is imaged on the coverslip. The light sheet intensities were volumetrically profiled
208 versus propagation distance by translating the probe relative to the coverslip.

209 Figures 3(b)-(c) show top-down fluorescence light-sheet profiles from the probes in
210 fluorescein. The GC emissions diverge and overlap to form regions of moderately uniform
211 illumination. For Probe 1, the semi-uniform region, which we define to be the region where the
212 maximum intensity variations were $< 2.5\times$, is the green region in the binned-color-scale sheet
213 profile of Fig. 3(b). The semi-uniform illumination region forms a continuous sheet at a

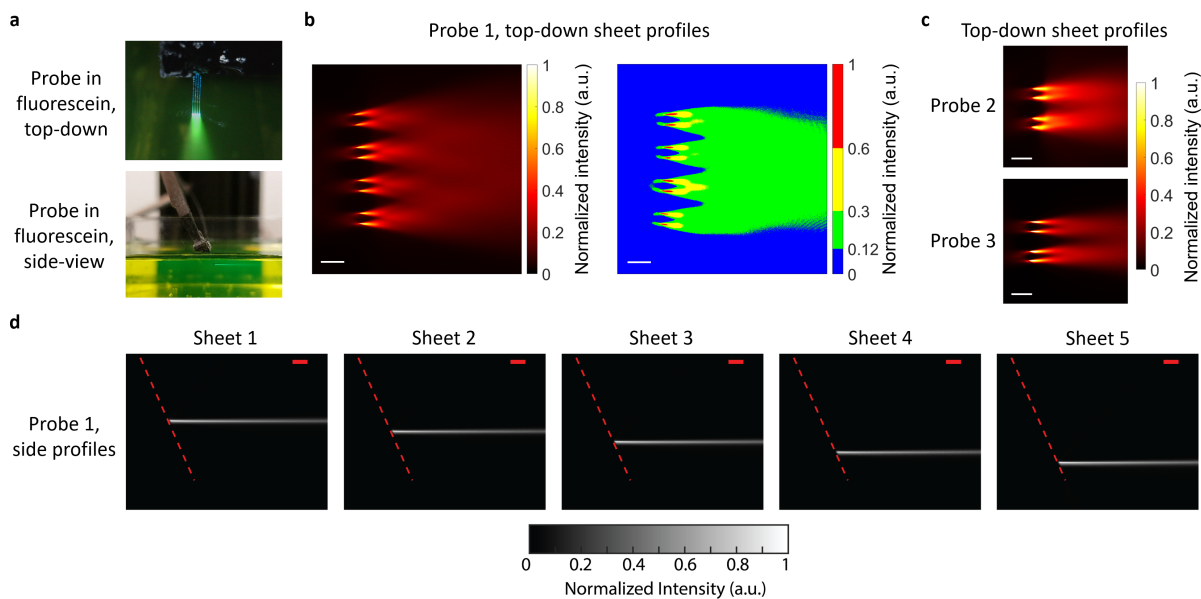


FIG. 3: Light-sheet characterization in fluorescein. (a) Top-down and side-view photographs of a light-sheet neural probe immersed in a fluorescein solution. (b) Top-down light-sheet intensity profile for Probe 1 - Sheet 5 imaged with an epifluorescence microscope above the probe. The plot on the right is the sheet profile with a binned color scale to show a semi-uniform sheet region (green) over which the intensity varies by at most $2.5\times$. (c) Top-down light-sheet intensity profiles for Probe 2 - Sheet 10 and Probe 3 - Sheet 7. (d) Side profile measurements of the light sheets from Probe 1 captured with a second microscope aligned to the side of the fluorescein chamber. The dashed red lines delineate the top surface of the shanks. The scale bars are $100\ \mu\text{m}$.

214
215 propagation distance of about $180\ \mu\text{m}$ and spans an area $> 0.22\ \text{mm}^2$. In scattering media such as
216 brain tissue, the semi-uniform illumination region will form at shorter propagation distances away
217 from the probe. The side profiles of the Probe 1 light sheets in fluorescein are shown in Fig. 3(d),
218 and side profiles from Probes 2 and 3 are shown in Fig. S3 in the Supplementary Materials. Weak
219 second-order diffraction results in an additional, upward-pointing beam for each sheet; this is not
220 visible in Fig. 3(d) due to the low second-order diffraction of Probe 1, but it is visible for Probe 2
221 in Fig. S3. The second-order diffraction profiles were similar to the light sheets, forming “second-
222 order light sheets.” The light sheet optical powers were $> 15\times$, $3\times$, and $16\times$ larger than the second-
223 order diffraction powers for Probes 1 to 3, respectively.

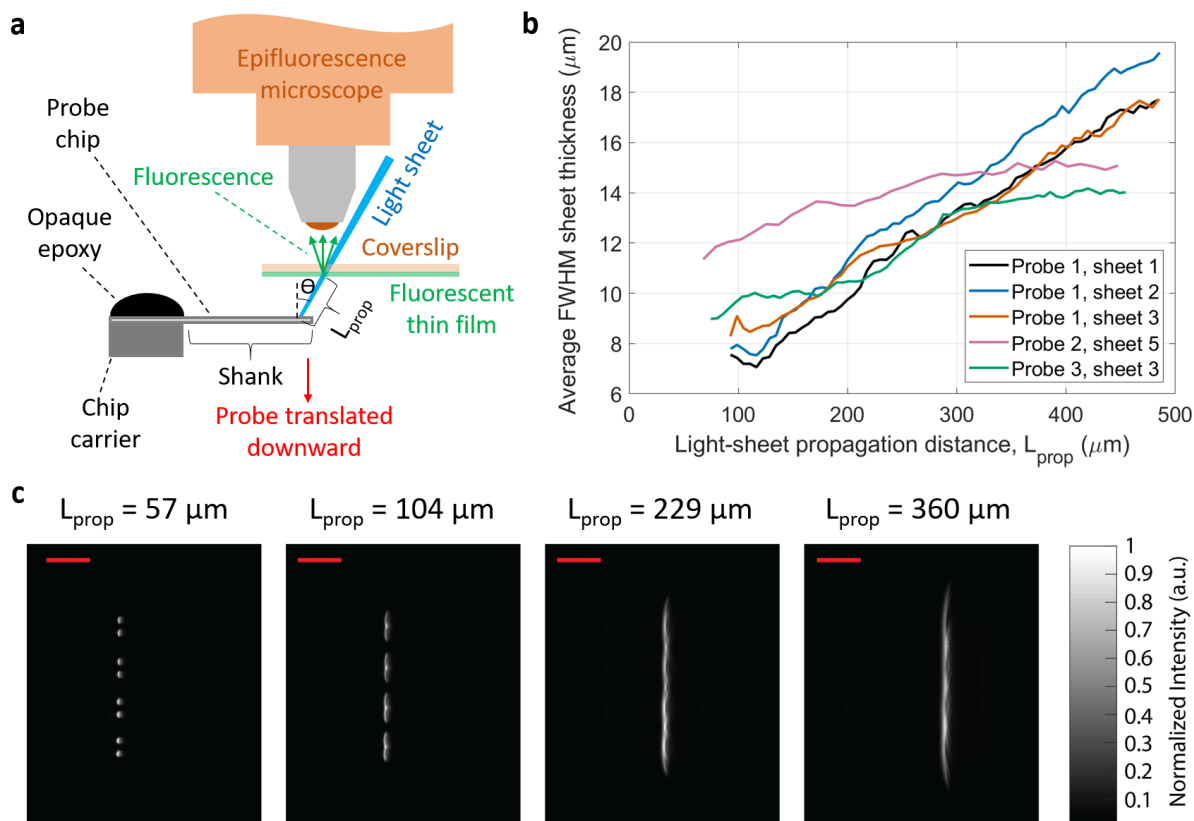


FIG. 4: Free-space light-sheet profile measurements. (a) Illustration of the light-sheet profile measurement protocol using a coverslip coated with a fluorescent thin film (not to scale). Fluorescence images of the coverslip provide cross-sectional profiles of the incident light sheet, and vertical translation of the probe enables volumetric profiling. (b) Average full width at half maximum (FWHM) light-sheet thicknesses versus propagation distance for sheets from Probes 1 to 3. The sheet thicknesses are averaged over the sheet width [vertical axis in (c)] for each sheet cross-section. (c) Light-sheet cross-sections imaged at various light-sheet propagation distances, L_{prop} , for Probe 1. The scale bars are $150 \mu\text{m}$.

224

225 Figure 4(c) shows light-sheet cross-sections at several propagation distances for Probe 1 imaged
 226 with the free-space beam profiling method. The extracted average full width at half maximum
 227 (FWHM) light-sheet thickness versus propagation distance for sheets from Probes 1 to 3 are shown
 228 in Fig. 4(b). The sheet thicknesses, averaged over the width of each sheet cross-section, are < 16
 229 μm for propagation distances up to $300 \mu\text{m}$. Since the coverslip was not perpendicular to the sheet

230 propagation axis, the propagation angle of each sheet is used to convert the thickness of the sheet
231 projection on the coverslip to a sheet thickness corrected for alignment perpendicular to the
232 propagation axis. The apparent reduction of FWHM divergence for Probes 2 and 3 sheets at
233 propagation distances above 300 μm in Fig. 4(b) is a consequence of the evolution of the sheet
234 shape. The full width at $1/e^2$ of maximum thickness (Fig. S4 in Supplementary Materials), in
235 general, increases linearly with propagation distance.

236 Small amplitude fringes are visible in the sheet cross-sections in Fig. 4(c) and the top-down
237 profiles in Fig. 3. These fringes are caused by multi-path interference from the multiple GCs that
238 contribute to each sheet. The interference pattern is related to the differing waveguide path lengths
239 connected to each GC and the coherence length of the laser. In brain tissue, we expect these fringes
240 will be smoothed (suppressed) by the scattering properties of the medium.

241 C. Light-sheet fluorescence imaging

242 We investigate the efficacy of the probes for LSFM by first imaging fluorescent beads
243 suspended in agarose, and then by imaging fixed and *in vitro* brain tissues. Since most miniaturized
244 microscopes today use wide-field IP fluorescence imaging, we compare the images obtained with
245 the light-sheet probe illumination against those with epi-illumination using the same microscope.
246 Figures 5(a)-(c) illustrate the imaging setup. An electrically tunable lens was attached to the back
247 of the objective to provide fast focus adjustment to the different light-sheet depth planes. When
248 the epi-illumination was on, the input to the probe was off, and vice versa. The comparisons are
249 performed at the same image plane, *i.e.*, the tunable lens and microscope objective are not adjusted
250 when switching between light-sheet and epi-illumination. The probe insertion angle was set to
251 orient the light sheets parallel to the top surface of the sample (sheet-normal imaging).

252 To demonstrate optical sectioning, Probe 1 was inserted into an agarose block containing 3 μm
253 diameter fluorescent beads. Figure 5(d) shows the fluorescence images captured using 3 of the
254 sheets of Probe 1 compared with epifluorescence images. Significantly out-of-focus beads and
255 fluorescence are not present with light-sheet probe illumination. This yields a dramatic reduction
256 of the background intensity in comparison with epi-illumination. We quantify the gain in contrast
257 in imaging experiments with tissue slices, discussed next. Video S3 shows a simple proof-of-
258 concept volumetric imaging example. The video demonstrates fluorescence imaging of fluorescent
259 beads in an agarose block with switching between three of the probe-generated sheets, and

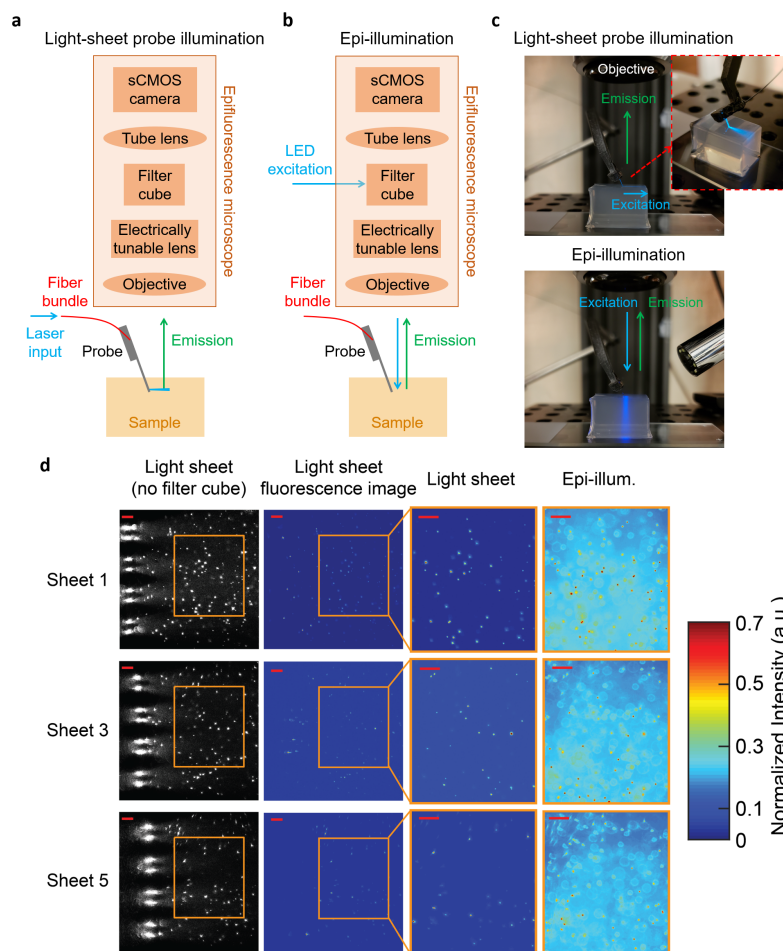


FIG. 5: Imaging of fluorescent beads suspended in agarose. (a-b) Illustrations of the imaging apparatus for (a) light-sheet probe illumination, and (b) microscope epi-illumination (not to scale). (c) Photographs of Probe 1 inserted into an agarose block during light-sheet probe illumination and microscope epi-illumination. (d) Imaging of fluorescent beads suspended in an agarose block using light-sheet illumination from Probe 1 (Sheets 1, 3, and 5) and epi-illumination. First column: light-sheet illumination images with no filter cube in the microscope path to show both scattered excitation light and fluorescence. The 8 large bright spots at the left of the images are the emitting GCs on the shanks. Second column: fluorescence images with light-sheet illumination and the filter cube in the microscope path. Third and fourth columns: fluorescence images of the regions of interest delineated by the orange boxes with light-sheet and epi-illumination, respectively. The epi-illumination images were captured at the same focal planes as the corresponding light-sheet images. The second to fourth columns are normalized to the maximum intensity in each image and the color scale is truncated at 0.7 to enhance bead visibility. The scale bars are $50 \mu\text{m}$.

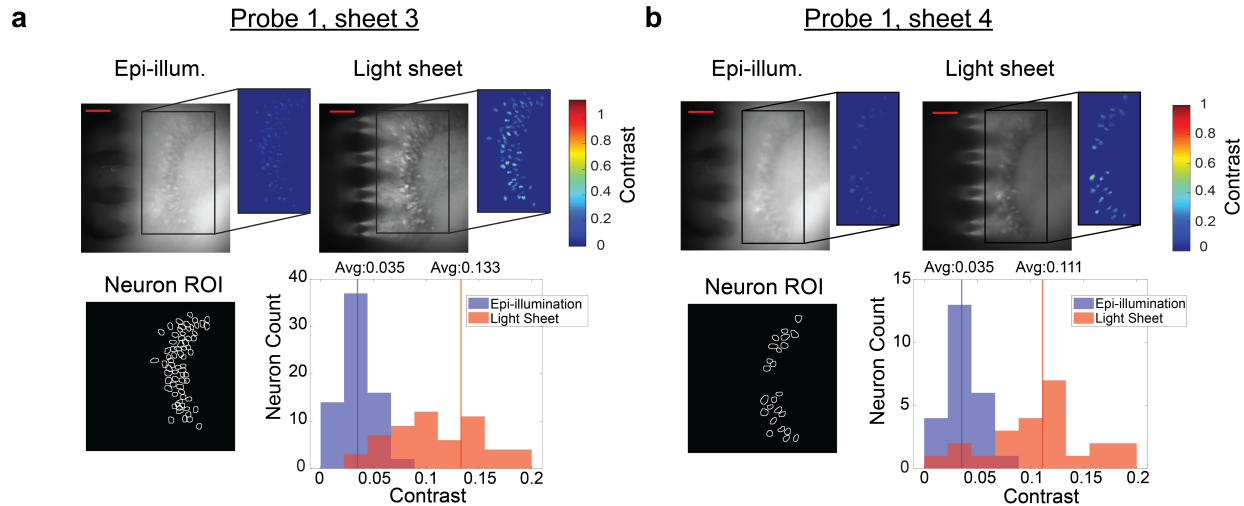


FIG. 6: Comparison of light-sheet neural probe illumination and microscope epi-illumination for fluorescence imaging of fixed brain tissue (dentate gyrus) from a Thy1-GCaMP6s mouse. Two adjacent light sheets from Probe 1 were used: (a) Sheet 3, and (b), Sheet 4. Sheet 3 was $\approx 60 \mu\text{m}$ in depth from the surface of the brain tissue, and Sheet 4 was $69 \mu\text{m}$ deeper than Sheet 3. Top row: fluorescence images for epi- and light-sheet illumination with insets indicating the contrast of neurons within a region of interest of high neuron density. The scale bars are $100 \mu\text{m}$. Bottom row: regions of interest (ROIs) of identified neurons and corresponding histograms of image contrast for the identified neurons; the contrast of each neuron is the average over its ROI.

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262 synchronized focus switching enabled by the electrically tunable lens.

263

264 We subsequently imaged the hippocampus in a fixed brain slice obtained from a Thy1-
265 GCaMP6s mouse. Figure 6 shows the probe- and epi-illuminated fluorescence images captured
266 following insertion of Probe 1 into the fixed tissue. The tissue was about 1 mm thick, and imaging
267 was performed with Sheets 3 and 4. Again, the probe-illuminated images showed remarkably less
268 background fluorescence than epi-illumination. Neurons are observable over a sheet area of ≈ 240
269 $\mu\text{m} \times 490 \mu\text{m}$ for Sheet 3, and different neurons are visible with Sheet 3 versus Sheet 4
270 illumination. The neurons in the image from Sheet 4, which was $69 \mu\text{m}$ deeper in the tissue than
271 Sheet 3, appear less in focus; this is due to the scattering of the fluorescence emission in the tissue.

271

272

To quantify the difference in contrast between probe- and epi-illumination, an algorithm described in Supplementary Note 1 (see Supplementary Materials) is applied to identify the

273 neurons in each image, and the neurons found in both images are selected (Fig. 6 “Neuron ROI”)
274 for contrast analysis using the definition of contrast in Supplementary Note 1. Figure 6 shows the
275 distributions of the image contrasts of the identified neurons. The contrast distributions of the two
276 illumination methods are statistically different ($p < 0.001$, two-tailed Wilcoxon signed-rank test),
277 with the average contrast for light-sheet illumination higher than that of epi-illumination by $3.8\times$
278 for Sheet 3 and $3.2\times$ for Sheet 4. 98.6% and 100% of the neurons for Sheets 3 and 4, respectively,
279 exhibit higher contrast using light-sheet illumination compared to epi-illumination. The color
280 insets in Fig. 6 show the contrast of each pixel within each neuron ROI, while the histograms show
281 neuron contrasts that are averaged over each neuron ROI. The illumination intensities for the fixed
282 tissue and *in vitro* imaging are discussed in Supplementary Note 2.

283 Photonic neural probe tests were also performed for *in vitro* functional calcium imaging using
284 a $450\ \mu\text{m}$ thick brain slice, prominently featuring the auditory cortex, from a Thy1-GCaMP6s
285 mouse. Preparation of the tissue is described in Methods. For increased neuronal activity, the brain
286 slice was perfused with an artificial cerebral spinal fluid (aCSF) solution containing 4-
287 aminopyridine (4-AP) [37]. Figure 7(a) shows maximum projection images over time from the
288 probe- and epi-illumination videos of the auditory cortex region of the brain slice. Sheet 5 from
289 Probe 2 was used, and the probe was inserted into the brain slice such that Sheet 5 was $\approx 60\ \mu\text{m}$ in
290 depth from the surface of the slice. The data analysis procedure for neuron identification and
291 extraction of the fluorescence change, $\Delta F/F$, is described in Supplementary Note 3. Figure 7(c)
292 shows the $\Delta F/F$ time traces of the 16 identified active neurons using probe-illumination, and $\Delta F/F$
293 values as large as 5.5 were observed. Figure 7(d) shows the image contrast of 5 of the neurons at
294 the peaks of all observed events; the neurons were selected with the criterion that at least 5 events
295 were recorded for both light-sheet and epi-illumination. Higher image contrast is observed for
296 light-sheet compared to epi-illumination for 4 of the 5 neurons ($p < 0.01$, two-tailed Wilcoxon
297 rank-sum test); a possible explanation for the lower light-sheet contrast of the one neuron is that
298 its depth may have been outside or on the periphery of the sheet. The ratios of the median light-
299 sheet- and epi-illumination neuron contrasts were 6.71, 0.77, 2.04, 2.46, and 3.39. Samples of
300 calcium imaging video with both probe- and epi-illumination are shown in Videos 1 and 2.

301 During calcium imaging experiments, illumination was alternated between probe- and epi-
302 illumination. The full time-traces are shown in Fig. S6 in the Supplementary Materials. Due to the

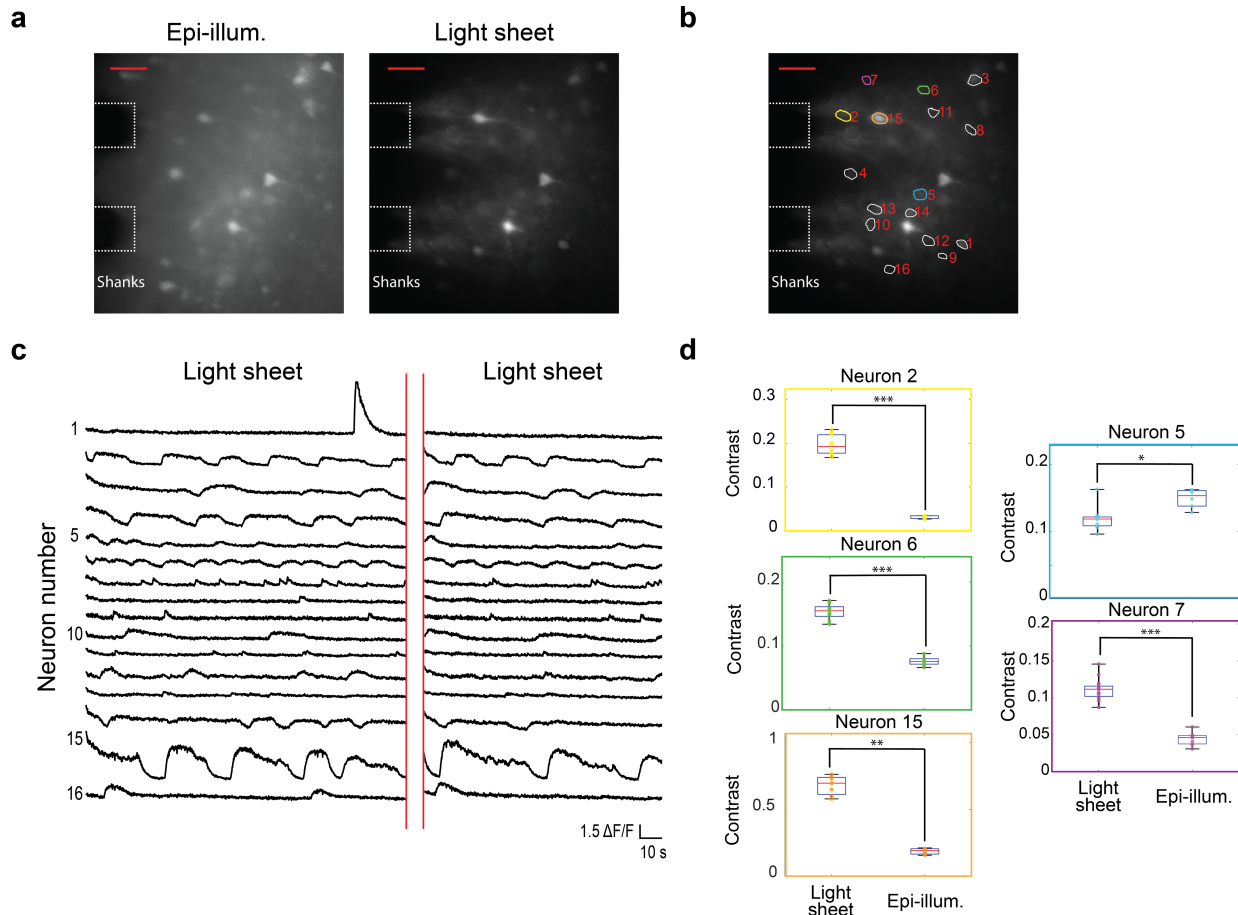


FIG. 7: *In vitro* functional calcium imaging of a brain slice from a Thy1-GCaMP6s mouse.

(a) Maximum projection images of 142 s and 92 s segments of the recorded video for light-sheet and epi-illumination, respectively, with annotations showing the approximate positions of the shanks in the image plane. The scale bars are $50 \mu\text{m}$. (b) Light-sheet maximum projection image with ROIs for identified active neurons shown. (c) Fluorescence change, $\Delta F/F$, time traces of all active neurons identified in (b). (d) Box plots showing the image contrast of 5 neurons at the peaks of all events recorded for light-sheet and epi-illumination; the numbers and colors of the box plots correspond to the ROIs in (b). Asterisks indicate significant group differences. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$, two-tailed Wilcoxon rank-sum test. A sample of the calcium imaging video with light-sheet probe illumination is presented (Video 1, 12.9 MB, mp4). A sample of the calcium imaging video with epi-illumination is also presented (Video 2, 29.8 MB, mp4). The videos are accelerated $5\times$. The $\Delta F/F$ time traces for epi-illumination are shown in Fig. S6 in the Supplementary Materials.

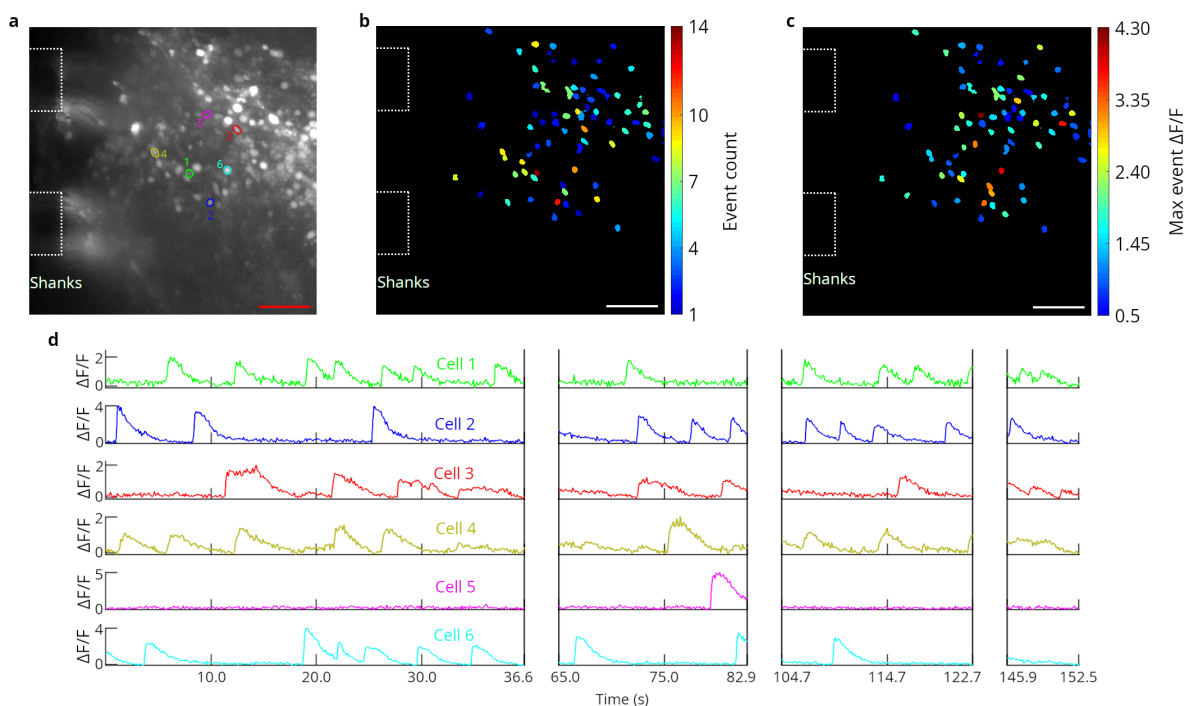


FIG. 8: *In vitro* functional calcium imaging of a Cal-520 AM loaded brain slice from a wild type mouse using light-sheet neural probe illumination. (a) Maximum projection fluorescence image of 36.6 s of the recorded video with annotations showing the approximate positions of the shanks in the image plane. (b) Maximum observed $\Delta F/F$ and (c) number of calcium events observed for all identified cells. (b-c) are of the same scale as (a), and the scale bars are $50 \mu\text{m}$. (d) Fluorescence change, $\Delta F/F$, time traces of 6 cells; the first 4 cells had the highest number of events and the last 2 had the highest peak $\Delta F/F$ among the remaining cells. The ROIs for these cells are shown in (a) with colors and numbers corresponding to the time traces in (d). Breaks in the time traces correspond to times when the illumination was switched to epi-illumination. A sample of the calcium imaging video with light-sheet probe illumination is presented (Video 3, 19.6 MB, mp4). The video is real-time.

304
305 larger background fluorescence of epi-illumination, the apparently larger $\Delta F/F$ for light-sheet
306 compared to epi-illumination does not necessarily represent a larger signal-to-noise ratio of the
307 calcium events. A direct comparison of signal-to-noise ratio for calcium events under these two
308 illumination conditions is beyond the scope of this work.

309 To investigate the operation of the probes in tissues with a higher density of labeled neurons,

310 tests were also performed on a green calcium dye loaded (Cal-520 AM, AAT Bioquest), 450 μm
311 thick, cerebellum brain slice from a wild type mouse. The tissue preparation is described in
312 Methods, and 4-AP was added to the aCSF perfusion solution. Probe 2 was inserted into the brain
313 slice, and light-sheet illumination was applied from Sheet 10, which was positioned $< 50 \mu\text{m}$ in
314 depth from the brain slice surface (Fig. 8). Full fluorescence time-traces with the illumination
315 cycled between probe- and epi-illumination are shown in Fig. S8 in the Supplementary Materials.
316 Samples of the calcium imaging video are shown in Videos 3 and S4. The labeled cells are likely
317 a combination of neurons and glial cells. The data analysis procedure is described in
318 Supplementary Note 3. For the probe illumination in Fig. 8, $\Delta F/F$ values as high as 4.3 were
319 observed, and 73 cells were identified. The variation in maximal $\Delta F/F$ values in Fig. 8(c) may
320 arise from a combination of the position of the cell within the sheet (both laterally and in depth)
321 as well as the magnitude of the calcium events. A complication in this experiment arises from the
322 penetration depth of the dye into the slice during bath-loading; this limits the thickness of labeled
323 tissue available to contribute to background fluorescence during epi-illumination. As a result, the
324 image contrast enhancement of light-sheet versus epi-illumination is expected to be less than our
325 results with Thy1-GCaMP6s mouse brain tissue (Figs. 6 – 7), where the labeling is more uniform
326 in depth. This is confirmed by the minor contrast difference between light-sheet and epi-
327 illumination observed for the Cal-520 AM loaded brain slice (Videos 3 and S4, Fig. S8 in the
328 Supplementary Materials), relative to the significant contrast enhancement of light-sheet
329 illumination in Figs. 6 – 7.

330 We have carried out initial *in vivo* tests as shown in Fig. S11 and discussed in Supplementary
331 Note 4 (see Supplementary Materials). For these experiments, a light-sheet probe was inserted
332 approximately $< 200 \mu\text{m}$ deep into the parieto-temporal lobe of an anesthetized Thy1-GCaMP6s
333 mouse at the approximate location of the somatosensory cortex. Time-dependent fluorescence
334 using probe-illumination was observed with a maximum $\Delta F/F$ of 0.12, and contrast enhancements
335 were observed compared to epi-illumination. In this case, it proved difficult to establish statistical
336 comparisons due to the low number of neurons exhibiting activity in the anesthetized mouse. The
337 probe used for this test was an earlier prototype, which pre-dated our foundry-fabricated probes;
338 details of these earlier devices are listed in Supplementary Note 5.

339 III. DISCUSSION AND CONCLUSION

340 We have conceived of and demonstrated a new paradigm for implantable photonic neural
341 probes that enables lensless delivery of multiple addressable light sheets. These can facilitate 1P-
342 LSFM at arbitrary depths in mammalian brains and other non-transparent tissues. The light-sheet
343 profiles were experimentally characterized, and the probes were validated by fluorescence imaging
344 in fixed tissue and by functional imaging *in vitro*. This imaging approach requires *no active*
345 *components* on the probe, which can otherwise induce deleterious tissue heating. By contrast,
346 miniaturized forms of digitally scanned 1P- or 2P-LSFM would likely require actuators on the
347 probe or in close proximity thereof. As the light-sheet neural probes are passive, the ultimate
348 volumetric imaging rate is similar to that of conventional light-sheet imaging systems. In our
349 probe-based implementation, it is limited by external components that include the electrically
350 tunable lens, MEMS mirror, and the image sensor. The apparatus we employ here is not yet fully
351 optimized to achieve maximum volumetric imaging rates and is primarily limited by the tunable
352 lens, which has a response time of 25 ms. Other system components are faster; the MEMS mirror
353 step time is ~ 5 ms, and the maximum full frame rate of the camera is 101 frames per second.
354 Accordingly, with optimized component choices and engineering of the imaging system,
355 volumetric imaging rates ≥ 30 volumetric scans per second will be attainable [38]. Although
356 continuous-wave light was used in our experiments, future implementations can employ pulsed
357 light to mitigate any potential phototoxicity and photodamage.

358 The light sheets created by our probes are synthesized from an incoherent sum of multiple GC
359 optical emissions. We expect sheets generated by neural probes or by conventional light-sheet
360 microscopes to be similarly affected by scattering within brain tissue. Optical scattering has been
361 evaluated for conventional light-sheet microscopes in [39] and for nanophotonic GC emissions in
362 [26, 29].

363 In future iterations of our probe designs, their photonic circuits can be further optimized by
364 leveraging state-of-the-art integrated photonic technology. For example, the sheet density may be
365 increased by integrating multiple photonic layers [40]. Also, the optical transmission of the probes
366 can be increased by roughly an order of magnitude with efficient fiber-to-chip edge couplers [41]
367 and optimized low-loss components; the fiber-to-chip coupling efficiency of the edge couplers in
368 this work was limited to $\approx 14\%$ with optimal alignment [34]. Optimized packaging solutions can

369 also mitigate transmission variations amongst light sheets and improve the thermal stability of the
370 packaged probes. (The latter may eliminate the turn-on-transient documented in Fig. S10 in the
371 Supplementary Materials.) Optimization of probe transmission and packaging can also minimize
372 potential tissue heating arising from the packaged probes absorbing extraneous scattered light from
373 on-chip photonic circuitry and edge couplers.

374 With their micro-scale form factors, ultrathin profiles, and their compatibility with sheet-
375 normal imaging using implantable GRIN lens endoscopes, the light-sheet photonic neural probes
376 we have demonstrated herein can engender exciting and powerful new variants of LSFM, both for
377 deep brain imaging and for behavioral experiments with freely-moving animals. Beyond LSFM
378 imaging, these neural probes can also be used for laminar optogenetic neural stimulation, *e.g.*, for
379 addressing *individual* cortical layers. When combined with a new class of implantable neural
380 probes containing photodetector arrays that is now emerging [42], they can enable complex image
381 reconstruction realized by means of a complete, implantable lensless imaging system.

382

383 IV. METHODS

384 **Photonic neural probe fabrication.** The neural probes were fabricated on 200 mm diameter
385 Si wafers at Advanced Micro Foundry (AMF). First, the 1.48 μm and 135 nm thick SiO_2 bottom
386 cladding and SiN waveguide layer were deposited by plasma enhanced chemical vapor deposition
387 (PECVD). Fully etched SiN waveguides were formed using deep ultraviolet (DUV) lithography
388 followed by reactive-ion etching (RIE), and the 1.55 μm thick PECVD SiO_2 top cladding layer
389 was then deposited. Deep trenches were etched to define the probe shape and form facets for edge
390 couplers. Finally, as in [43], backgrinding was used to thin the wafers to $\approx 50 - 92 \mu\text{m}$, which
391 exposed the deep trenches and separated the probes on the grinding tape (autodicing). Chemical
392 mechanical planarization (CMP) was used for layer planarization during the fabrication. The
393 fabrication process and waveguide characteristics are described in more detail in [34].

394 **Neural probe packaging.** The probe chip was first epoxied to a 3D-printed chip carrier. The
395 image fiber bundle (Fujikura FIGH-06-300S) was connected and aligned to the scanning optical
396 system, Fig. 2(a). The fiber bundle was aligned and then UV-epoxied to the probe chip and the
397 chip carrier; the emitted optical power from the probe was monitored during the process. The probe
398 chip (excluding the shanks) and the fiber bundle were then coated with optically opaque epoxy to
399 block stray light not coupled to the on-chip waveguides. The chip carrier had a steel rod attached
400 to the proximal end, and this steel rod was connected to additional rods to mount the packaged
401 probe on a 4-axis micro-manipulator (QUAD, Sutter Instrument Company, Novato, CA, USA).

402 **Spatial addressing of the neural probes.** The 2-axis MEMS mirror in the external scanning
403 optical system, Fig. 2(a), had a nominal maximum mechanical tilt angle of $\pm 5.5^\circ$ and a mirror
404 diameter of 3.6 mm (A7B2.1-3600AL, Mirrorcle Technologies Inc., Richmond, CA, USA). The
405 scanning system used bi-convex lenses with 35 mm and 150 mm focal lengths and a 20 \times objective
406 lens (Plan Apochromat, 20 mm working distance, 0.42 numerical aperture, Mitutoyo Corporation,
407 Kawasaki, Japan). The loss of the scanning system (from the input laser beam to the distal facet of
408 the image bundle) was typically 40 - 60%. The 488 nm laser (OBIS 488 nm LS 150 mW, Coherent
409 Inc., Santa Clara, CA, USA) was fiber-coupled to a single-mode fiber (460-HP, Nufern Inc., East
410 Granby, CT, USA), which was connected to a fiber collimator that formed the free-space laser
411 beam input to the scanning system. The input beam was gated by a mechanical shutter. The input
412 polarization to the scanning system was set via an inline fiber polarization controller.

413 **Fluorescein beam profiling.** The neural probes were dipped into 10 μmol fluorescein
414 solutions ($\text{pH} > 9$), Fig. 3(a). Top-down sheet profiles were measured using an epifluorescence
415 microscope above the probe. Side profiles showing the sheet thicknesses were measured using an
416 additional microscope positioned at the side of the chamber containing the fluorescein. One of the
417 walls of the chamber was removed and replaced with a coverslip to create a viewing port with low
418 optical distortion for the side microscope. Bandpass optical filters on both microscopes rejected
419 excitation light from the probe and transmitted the emission light from the fluorescein. The
420 insertion axis of the micro-manipulator holding the probe was angled such that the sheets
421 propagated parallel to the surface of the fluorescein solution with the probe immersed.

422 **Free-space beam profiling.** Coverslips with a fluorescent thin film were fabricated by mixing
423 fluorescein (free acid) powder with SU-8 photoresist [44] and spin coating it onto $\approx 170 \mu\text{m}$ thick
424 coverslips. After curing, an $\approx 8 \mu\text{m}$ thick fluorescent thin film was formed on one side of the
425 coverslips, which were then cleaved in half to prevent the edge bead from limiting the probe-to-
426 coverslip distance. The coverslip was fixed above the probe with the shanks, coverslip, and optical
427 table parallel (Fig. 4). The fluorescent film was on the bottom side of the coverslip, closest to the
428 probe. An epifluorescence microscope above the coverslip imaged the fluorescent patterns created
429 by the intersection of the probe light sheets and the thin film. Vertical translation of the probe
430 enabled volumetric profiling of each sheet for measurements of the sheet thickness and
431 propagation angle. The sheet propagation angles were used to convert the micro-manipulator
432 vertical translation step size into sheet propagation distance step sizes, and the angles were also
433 used to calculate the sheet thicknesses from the angled projections on the coverslip. To verify the
434 uniformity and linearity of the thin film's fluorescence, volumetric profiles were captured over
435 input optical powers spanning roughly an order of magnitude and at multiple positions on the
436 coverslip. Measured average sheet thicknesses varied by $< 2 \mu\text{m}$ throughout the trials.

437 **Fluorescent beads in agarose.** To prepare the agarose blocks with fluorescent beads, 100 mg
438 of agarose powder was mixed with 10 mL of Milli-Q water to form a 1% agarose solution. The
439 solution was heated until boiled, and after cooling, 50 μL of yellow-green fluorescent microbeads
440 (3 μm bead diameter, 2.5% concentration, Magsphere, Pasadena, CA, USA) were mixed into the
441 solution. The solution was placed on a rocker to evenly distribute the beads, and then, poured into
442 a plastic mold and stored in a refrigerator until solidified. The intensity scales of the grayscale

443 images of fluorescent beads in Fig. 5(d) were set with the bottom and top 1% of all pixel intensities
444 saturated.

445 **Imaging apparatus.** The fluorescence imaging apparatus, Figs. 5(a) and (b), includes an
446 epifluorescence microscope (Eclipse FN1, Nikon, Tokyo, Japan) with an sCMOS camera (Zyla
447 4.2 PLUS, Andor Technology Ltd., Belfast, UK) and an EGFP filter cube (49002, Chroma
448 Technology Corporation, Bellows Falls, VT, USA). A 10× objective lens (Mitutoyo Plan
449 Apochromat, 34 mm working distance, 0.28 numerical aperture) was used for the beam
450 characterization and *in vitro* Cal-520 AM brain slice imaging. A 20× objective (Mitutoyo Plan
451 Apochromat, 20 mm working distance, 0.42 numerical aperture) was used for the fluorescent
452 beads, fixed tissue, and *in vitro* GCaMP6s brain slice imaging. An electrically tunable lens
453 (Optotune, Dietikon, Switzerland) attached to the back of the objective was used for fast focus
454 adjustments in the fluorescence imaging experiments but not for the beam characterization. The
455 fluorescent beads, fixed tissue, and *in vitro* imaging used 200 ms, 500 ms, and 100 ms camera
456 exposure times, respectively. The packaged probe was attached to a 4-axis micro-manipulator for
457 positioning the probe in the characterization and imaging experiments. The shanks were aligned
458 to the insertion axis of the micro-manipulator, and in the imaging experiments, the insertion angle
459 was selected for sheet-normal imaging. Since the fiber bundle was not polarization-maintaining
460 and the probe was polarization-sensitive, the bundle was fixed in position during imaging
461 experiments to minimize polarization fluctuations.

462 **Animals.** All experimental procedures described here were reviewed and approved by the
463 animal care committees of the University Health Network in accordance with the guidelines of the
464 Canadian Council on Animal Care. Adult Thy1-GCaMP6s mice [2] (The Jackson Laboratory, Bar
465 Harbor, ME, USA, stock number 025776) and C57BL/6 mice (Charles River Laboratories,
466 Wilmington, MA, USA) were kept in a vivarium maintained at 22°C with 12-h light on/off cycle.
467 Food and water were available ad libitum.

468 **Fixed tissue preparation.** Fixed tissue was prepared from a Thy1-GCaMP6s mouse, postnatal
469 day 172, as ≈ 1 mm thick transverse slices from the hippocampus (dentate gyrus). Briefly, the
470 animal was anesthetized via an intra-peritoneal injection of sodium pentobarbital (75 mg/kg,
471 Somnotol, WTC Pharmaceuticals, Cambridge, Ontario, Canada) and transcardially perfused with
472 1× phosphate-buffered saline (PBS) followed with paraformaldehyde (PFA) (4%). Then the

473 extracted brain was kept in PFA at 4°C for 12 hours. After fixation, the hippocampal slices were
474 prepared in 1× PBS with a vibratome (VT1200S, Leica Biosystems, Wetzlar, Germany).

475 ***in vitro* imaging brain slice preparation.** Brain slices were prepared from 30-60 day old Thy1-
476 GCaMP6s and C57BL/6 mice for the *in vitro* GCaMP6s and calcium dye imaging experiments,
477 respectively. The animals were anesthetized with an intra-peritoneal injection of sodium
478 pentobarbital (75 mg/kg) and transcardially perfused with cold (4°C) N-methyl-D-glucamine
479 (NMDG) recovery solution [45] prior to decapitation. The brain was quickly dissected, brain
480 tissues were glued on a vibratome stage, and 450 μm thick slices were prepared with the vibratome
481 using iced NMDG solution. The brain slices were then stabilized in NMDG solution at 34°C for
482 12 min while being aerated with carbogen (95% O₂, 5% CO₂). Only for experiments with the Cal-
483 520 AM calcium dye, following a 12-minute recovery period, slices were rinsed and then bathed
484 in a Cal-520 AM solution (AAT Bioquest, Sunnyvale, CA, USA) for 60 - 90 minutes at 37°C. For
485 all *in vitro* experiments, the slices were then maintained in room temperature incubation solution
486 [45] for 1-8 hours prior to imaging. The Cal-520 AM solution was aerated with carbogen and
487 consisted of 50 μg of Cal-520 AM mixed with 20 μL of 20% Pluronic F-127 in dimethyl sulfoxide
488 (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and then diluted in 4 - 6 mL of incubation solution
489 to a final concentration of 7 - 10 μMol. During imaging of a slice, the slice was mounted in a
490 perfusion chamber with a constant flow of rodent artificial cerebrospinal fluid (aCSF) solution
491 [45] aerated with carbogen. A 100 - 200 μMol solution of 4-aminopyridine (4-AP) was added to
492 the aCSF bath to put the neurons in a hyperexcitable state for increased neuronal activity [37]. For
493 the Thy1-GCaMP6s imaging, a transverse slice prominently featuring the auditory cortex was
494 chosen, and for the Cal-520 AM imaging, a sagittal slice prominently featuring the cerebellum was
495 chosen. The cerebellum was chosen since we observed that it had high neuron activity density.
496 The cerebellum could not be chosen for the Thy1-GCaMP6s experiment due to the low labeling
497 density in the cerebellum for this strain [2].

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503 V. Appendix

504 Four supplementary videos are included in this work, and descriptions are below.

505

506 **Video S1:** Top-down microscope imaging of Probe 3 with switching between light sheets. The
507 video is real-time.

508

509 **Video S2:** Side microscope fluorescence imaging of Probe 1 immersed in a fluorescein solution
510 with switching between the 5 light sheets. The power of the sheets was roughly equalized here by
511 not optimally aligning the MEMS mirror for the sheets with relatively high transmission.
512 Additional ambient illumination was applied to make the shanks visible. The video is real-time.

513

514 **Video S3:** Fluorescence imaging of 3 μm diameter fluorescent beads suspended in an agarose
515 block with light-sheet illumination from Probe 1 and imaging using the epifluorescence micro-
516 scope above the sample. At about 17 s, the illumination is switched from light-sheet probe
517 illumination to epi-illumination from the microscope. During light-sheet probe illumination,
518 switching between Sheets 1, 3, and 5 is performed. The tunable lens is synchronized to the sheet
519 switching to focus the collection optics on the depth planes corresponding to each sheet. After
520 switching to epi-illumination, the tunable lens focus switching continues and shows
521 epifluorescence imaging of the same depth planes imaged with light-sheet probe illumination.
522 The video is real-time.

523

524 **Video S4:** *In vitro* calcium imaging with epi-illumination of a Cal-520 AM loaded brain slice
525 from a wild type mouse. This is a sample of the calcium imaging video corresponding to Fig. S8
526 in the Supplementary Materials. The video is from the same experiment as Video 3 but with epi-
527 illumination instead of light-sheet probe illumination. The video is real-time.

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543 GCaMP6 functional imaging.

544

545

546 **Data availability.** The data are available from the corresponding authors upon reasonable request.

547

548

549 **Competing interests.** The authors declare no competing interests.

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552 **Additional information.** Supplementary information is available for this paper.

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