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Non-telecentric two-photon microscopy for 3D random access mesoscale imaging

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Diffraction-limited two-photon microscopy permits minimally invasive optical monitoring of neuronal activity. However, most conventional two-photon microscopes impose significant constraints on the size of the imaging field-of-view and the specific shape of the effective excitation volume, thus limiting the scope of biological questions that can be addressed and the information obtainable. Here, employing a non-telecentric optical design, we present a low-cost, easily implemented and flexible solution to address these limitations, offering a several-fold expanded three-dimensional field of view. Moreover, rapid laser-focus control via an electrically tunable lens allows near-simultaneous imaging of remote regions separated in three dimensions and permits the bending of imaging planes to follow natural curvatures in biological structures. Crucially, our core design is readily implemented (and reversed) within a matter of hours, making it highly suitable as a base platform for further development. We demonstrate the application of our system for imaging neuronal activity in a variety of examples in zebrafish, mice and fruit flies.
Laser scanning two-photon (2P) microscopy allows the imaging of live cellular processes deep inside intact tissue with high signal-to-noise, temporal fidelity and spatial resolution\(^1\). Nonetheless, standard diffraction-limited 2P setups with a collimated laser excitation beam have several key characteristics that constrain their broad applicability; namely, a typically small field of view (FOV), a fixed-size excitation spot and restricted options for rapid random access 3-dimensional scans. These are significant limitations because the biological samples that are interrogated with 2P microscopy can exhibit substantial variations in size and spatial structure. For example, the volume of an adult mouse brain is approximately four orders of magnitude larger than that of a larval zebrafish, and seven orders of magnitude larger than a first instar larval fruit fly (Fig. 1a). Similarly, neuronal sub-structures are also highly variable in density and size, ranging from sub-micron levels for some synapses up to 20 µm or more for some somata. Additionally, neural densities vary by more than an order of magnitude across different animal brains\(^2\). As such, 2P microscopy tends to reveal very different levels of detail and organization across its diverse experimental applications. To maximize biological information, upgrades for 2P microscopy should enable the imaging of

![Diagram of laser scanning two-photon microscopy setup](image)

- **DL** (Diaphragm Lens) setup
- **nTC1** (Near-Infrared Tunable Coherent) setup
- **nTC2** (Near-Infrared Tunable Coherent) setup

**FOV (mm)**
- 0.7 mm
- 1.2 mm
- 1.8 mm
- 2.5 mm
- 3.5 mm

**Power at sample (%)**
- Mouse somata
- Zebrafish
- Fruit fly

**xy-PSF (µm)**
- 0.5 µm
- 1.0 µm
- 2.0 µm
- 2.5 µm

**z-PSF (µm)**
- 0 µm
- 5 µm
- 10 µm
- 15 µm

**Power at sample (%)**
- 0%
- 10%
- 20%
- 30%
- 40%
- 50%
neuronal activity from many neural structures of a given size and density across a sufficiently large 3D volume of tissue at sufficiently high frame rates for the chosen neuronal process and biosensor.

In response to this demand, a profusion of custom modifications to 2P microscopes have been developed to expand the spatial and temporal boundaries over which neural structures can be optically interrogated. For example, the maximal planar field of view (FOV) has been increased from typically 0.7 mm to between 3.1 and 10 mm diameter by the exchange (3.1 mm: ref. 3, 7 mm: ref. 4) or size-increase of optical components (10 mm)5, custom built objectives (3.1 mm)6, enhanced scan engines (5 mm)7 and a mesoscope configuration (5 mm)8 to allow ‘mesoscale’ interrogation of neural circuits. In parallel, customizations using multiple beams have allowed simultaneous scanning of different brain regions6,9,10. Likewise, higher temporal resolutions have been achieved by tailoring the point spread function (PSF) to the geometry and distribution of the neuronal structures of interest, thus increasing signal-to-noise ratio (SNR) and, in turn, decreasing the minimally-required dwell time per pixel11,12. Moreover, the imaging plane has been expanded by engineering an excitation spot with Bessel focus13,14 or by elongated Gaussian focal stereocopy15. These customizations provide efficient ways to merge image structures that are located at different depths into a single volumetric plane. Furthermore, in recent years, systems integrating acousto-optic deflectors16,17, electrical tunable lenses18-21 and remote focusing units22,23 have enabled quasi-simultaneous multiplane volumetric scans.

These types of extensions have been essential in driving the field forward, yet many are expensive, require custom-produced optical elements, complex optical alignment and/or introduce new limitations. The latter can include limitations in both excitation (e.g. power loss8, wavefront dispersion17) and collection4,5. Here we introduce an alternative design for 2P microscopy that overcomes many of these limitations while simultaneously approaching the capabilities of a wide range of state-of-the-art performance customisations, and being ultra-low-cost, simple and flexible.

Our non-telecentric (nTC) design, implemented for ~£1000 on an existing Sutter MOM-type 2P setup equipped with a standard x20 objective, allows the expansion of the planar FOV from typically ~0.7 mm in diameter to anywhere up to 3.5 mm to flexibly suit experimental needs (Fig. 1). This expansion is accompanied by a moderate increase in the system’s 3D PSF.

For example, unlike a standard diffraction limited (DL) setup (left in Fig. 1b, c), our nTC setup (right in Fig. 1b, c) allows simultaneous imaging of three entire zebrafish brains (Fig. 1c), or about a third of the width of a mouse’s brain (Fig. 1f, Supplementary Movie S1). The addition of an electrically tunable lens (ETL) then allows near-simultaneous sampling in distant brain regions separated in 3 dimensions. Crucially, our solution is both comparatively low-cost and easy to implement without the need for complex optical calibration, thus facilitating its widespread adoption in the community. We anticipate that others will be able to build on our core optical design using existing and new modifications to further increase its capability in the future. We demonstrate the current performance of our system with a range of examples from zebrafish, mice and fruit flies.

Results
Non-telecentric optics for field of view expansion. In traditional laser scanning 2P microscopy (left in Fig. 1b, c, g), a diffraction limited (DL) PSF is generated to excite fluorophores in a typically sub-micron volume of tissue. Here, xy-scanning mirrors reflect the laser beam into a collimation system comprised of a scan and a tube lens. The collimated beam then enters the back aperture of a high numerical aperture (N.A.) objective22,23 to converge at parallel rays into a DL spot at focal distance24. The Gaussian shape of the excitation beam dictates that it is not possible to perfectly match beam width to the objective’s back aperture. Instead, the back aperture is typically overfilled with a factor of 1/e2 as a compromise between maximising spatial resolution (i.e. small PSF size) and power transmission25.

In contrast, our nTC design (middle and right in Fig. 1b, c, g) illuminates the objective’s back-aperture with a decollimated and divergent beam. This leads to an increased angle of view as the light exits the objective’s front aperture, such that the same angular scan-mirror movement leads to a larger absolute shift in the image plane—thereby greatly increasing the FOV. In parallel, this also alters the effective excitation numerical aperture (N.A.) to yield a larger-than-DL excitation spot (i.e. an elongated PSF) at greater focal distance. The magnitudes of each of these effects scale with the angle of divergence as the beam enters the back aperture of the objective. Accordingly, simply shifting the plano-convex lenses up or down the laser path, or switching between different refractive power lenses, provides for easy control over the system’s optical properties.
A simple scan-lens modification yields up to 7-fold FOV expansion. An off-the-shelf infinity-corrected galvo-galvo Sutter-MOM setup equipped with a ×20 objective (Zeiss Objective W "Plan-Apochromat" ×20/1.0) offers a square FOV diameter of ~0.5 mm (left in Fig. 1g). This can be principally extended to a round ~0.7 mm diameter usable diffraction limited (DL) FOV of the objective (see below), for example by redesigning the native beam path to allow a greater range of laser travel. Alternatively, however, when underfilling the back aperture of the objective with a diverging laser (middle and right in Fig. 1g), the beam exits the objective front aperture at increasingly obtuse angles at an effectively decreased N.A. (Fig. 1i, Fig. S1a) and comes into focus at a greater distance (Fig. 1g, Fig. S1b). Together, this expands the effective excitation FOV in both xy (increased angle and decreased PSF) and z (elongated and laterally expanded PSF). To achieve this effect, it is necessary to bring the collimated laser beam, having passed the scan mirrors, to an "early" intermediary focal point (IFP) prior to reaching the objective, thus setting up the diverging beam thereafter (Fig. 1g, arrowheads). The specific divergence angle as the beam enters the back-aperture of the objective, which depends on IFP, defines the magnitude of the above-mentioned effects. We present two simple optical solutions (nTC1 and nTC2) to set-up an early IFP and thus expand the effective FOV to varying degrees.

In the standard DL configuration, the scan-lens (SL) and tube lens (TL) are separated from each other at a distance that is equal to their combined focal lengths (50mm + 200mm = 250 mm) to collimate the beam (left, Fig. 1g). In nTC1, we removed SL and instead inserted two off-the-shelf plano-convex lenses (L1, modified VISIR 15345PR136, Leica; L2, LA1229 Thorlabs) with focal lengths 190 and 175 mm, respectively (middle, Fig. 1g, ‘Methods’). L1 was fixed 190 mm in front of TL to set up an IFP exactly at the TL. Next, L2 was positioned between L1 and TL to further increase laser convergence and thus shift the exact position of IFP away from the TL. Accordingly, IFP is always in front of the TL, with L2 determining its exact position: Simply shifting L2 along the laser path between 100 and 5 mm distance from the TL expanded the effective FOV diameter to anywhere between 1.2 and 1.8 mm, respectively (compare Fig. 1g, middle).

In nTC2 (Fig. 1g, right), we replaced SL with a single lens (L3) of 200 mm focal length (LA1708, Thorlabs). L3 operated in much the same way as L2 in the previous modification M1, however now the IFP was behind rather than in front of TL. Depending on the position of L3, this yielded effective FOV diameters anywhere between 2.5 and 3.5 mm. Importantly, in each case effective image brightness remained approximately constant across the full FOV (Fig. S1c–f, Methods). Here, the marginal brightness increase towards the edges is related to the slight upwards bend in the imaging plane as commonly seen for large FOV 2P microscopes—see also below. The axial difference between the edge and centre of the imaging plane was 20, 45, 87 and 170 µm for 1.2, 1.8, 2.5, 3.5 mm FOV, respectively.

Our design’s full optical path and control logic are shown in Fig. 1h. All functions are executed from the scan software, which directly controls the xy-scan path as usual. To synchronize an electronically tunable lens (ETL, see below) and/or a Pockels cell to this xy-scan, a copy of the fast-mirror command is sent to two microcontrollers. Each of these then executes preloaded line-synchronized code that are defined using a stand-alone graphical user interface (GUI). In this way, this stand-alone z-control system only requires a copy of the scan mirror command, meaning that it can be directly added to a 2P microscope setup without the need for software modifications.

Increased effective laser power. Because our nTC design avoids overfilling of the objective’s back aperture and uses fewer optical elements in the laser path (by replacing the native scan lens consisting of two doublets with one doublet and one singlet in nTC1, or one singlet in nTC2), total achievable laser power at the sample was increased approximately 4-fold compared to all configurations of the DL setup (Fig. 1i). This additional power could, for example, be used to facilitate imaging deep in the brain, or alternatively to drive additional setups from the same laser source. For instance, when imaging the small brains of larval zebrafish or fruit flies, there is rarely a need to exceed 50 mW, meaning that it is theoretically possible to drive ten such nTC setups from a single standard laser (e.g. Coherent Chameleon Vision-S Laser, average power ~1.5 W at 930–960 nm, assuming 50% loss through the setup).

Spatial resolution under nTC. To establish how our nTC approach affected the excitation PSF, we first imaged 175 nm fluorescent beads across all configurations at 927 nm wavelength and 15–20 mW laser power at the sample ‘Methods’, Supplementary discussion). Starting from a DL spot-volume of 0.56 and 3.15 µm (xy and z, respectively), our different modifications elongated and laterally expanded the PSF to varying degrees, from 0.77 (xy) and 9.94 (z) µm for the 1.2 mm FOV configuration to 2.21 (xy) and 41.49 (z) µm at 3.5 mm FOV (Fig. 1k–m). Accordingly, increasing the FOV using nTC mainly elongated the PSF, while restricting its lateral expansion. However, PSF expansions were generally stronger than in other large FOV 2P-approaches which, for example, reported ~15 µm at the edge of a 10 mm FOV or <10 µm at the edge of a ~5 mm FOV, see also refs. 5, 6. These approaches achieve their optical results through custom made, large-diameter optics, which are generally more expensive and more difficult to retrofit to existing setups. Notwithstanding, the possibility of optically merging adjacent image structures strongly depends on the size and spatial distribution of labelled biological structures—a general limitation in optical microscopy, rather than a specific limitation to our nTC approach (discussed e.g. in ref. 26).

The systematic effects on PSF shape across configurations also meant that our nTC approach could be used to flexibly match...
PSF dimensions to specific experimental needs. For example, the sub-micron DL PSF offered by typical collimated 2P-setups maximises spatial resolution which is invaluable for resolving small synaptic processes or the somata of larval fruit flies (typically < 5 µm). However, many species’ cell bodies are much larger. For example, in the brain of larval zebrafish a very small DL PSF spatially typically oversamples the “mid-sized” ~5–10 µm somata at the expense of a potentially substantial loss in SNR. This limitation can be avoided by nTC-mediated adjustment of the PSF (cf. Figs. 2, 3). Similarly, for picking up somatic signals from cortical neurons in the mouse, a “10-fold expanded” ~5 µm PSF yields the best SNR\textsuperscript{12}. 
Imaging across the full FOV. To further assess how the different optical configurations impacted PSF-shapes across the whole FOV, we next visualised excitation volumes using a camera (Fig. 2a, b)\textsuperscript{27}. Specifically, we positioned a fluorescein-solution-filled cuvette below the objective and filmed it from the side (Fig. 2a). Compared to imaging beads (Fig. 1k–m) this approach had the advantage that excitation volumes could be visualised much more directly, as well as across different positions in space in rapid succession (Supplementary Movie S2). However, the resultant scan profiles overestimated PSF sizes and were thus not suitable to determine their absolute dimensions – rather, the goal was to observe relative variations in PSF shapes, positions, and orientations over the full FOV. Figure 2b shows a direct, scale-matched visualisation of effective scan profiles for all optical configurations. This confirmed that the DL configuration had the smallest profiles, followed by increasing-FOV variations of nTC1,2. Moreover, scan-profiles were curved to different degrees, with correspondingly tilted excitation volumes towards the edge\textsuperscript{5,8}. If required, this can be part-corrected via the ETL. However, biological structures are rarely perfectly flat either. As described further below, often a more useful solution might be to instead fit the scan-plane curvature to the 3D curvature of the interrogated sample.

To quantify absolute PSF dimensions across the full FOV, we next returned to measuring fluorescent beads (cf. Fig. 1k), however, this time imaging both at the FOV centre (C) and at the scan edges (top left: TL, bottom right: BR, etc., cf. Fig. 2c) in each of the five optical configurations (Fig. 2d, e). This confirmed that while as expected PSFs slightly tilted outwards at the edges (Fig. 2d, cf. Fig. 2c), their traverse and axial dimensions did not change significantly (except in two cases where there was a slight xy-expansion as indicated, likely due to imperfect laser centring, Fig. 2e). Moreover, no noticeable image distortion across the FOV was detected, illustrated by imaging a fixed sample under nTC2 2.5 mm configuration at three different lateral translations, followed by direct superposition of the same selected structure in each image (Fig S2). Together, this implied that in practice, the full FOV was usable for imaging, as further confirmed in an nTC2 3.5 mm FOV example scan of a fixed mouse brain slice labelled with SM131 for phosphorylated Neurofilament-H (NFH). Figure 2f–h, staining the basket cells that wrap around Purkinje neurons (Fig. 2f–h). Here, thin neuronal processes could be followed essentially all the way to the FOV-edge, where it clipped due to the objective back aperture (e.g. Fig. 2h).

Effective image resolution and brightness. Next, we directly compared the different effective spatial resolutions and signal integration by imaging the same in vivo zebrafish sample in each configuration. For this, we sparsely expressed GCaMP6f under the pan-neuronal promoter HuC in larval zebrafish\textsuperscript{28} and imaged one animal that randomly exhibited isolated and easily recognizable expression in neurons of the upper spinal cord, including one cell body (~7 μm diameter) and several individual synapses (~1 μm diameter, arrowheads indicate matching position of 2 such synapses across scans, Fig. 3a). These image structures were consistently recognisable across all optical configurations. This notion was further confirmed in functional scans during visual stimulation that was time-interweaved with the scanner retrace to avoid crosstalk (Fig. 3b–d, ‘Methods’). For example, full-field UV-flashes elicited different responses in different image structures on a trial-to-trial basis (e.g. see highlighted traces of nTC2 condition). The different configurations also achieved notably different overall brightness in the imaged structures and allowed “optically merging” the spinal tract in larger FOV (and thus more elongated PSF) scans to different degrees (see also Supplementary Movie S3). Accordingly, the same set of neuronal elements could be usefully imaged across all optical configurations in this sample, allowing experimenter-controlled trade-off between FOV, 3D spatial resolution, and consequent signal integration.

Rapid axial scans. In addition to expanding the FOV, our de-collimated design also shifts the excitation point beyond the objective’s nominal focal distance (Fig. S1b). The same optical effect can be exploited to drive rapid axial shifts in the excitation plane by the introduction of an electrically tunable lens (ETL) early in the laser path (Fig. 4, Fig. S3, c.f. Fig. 1h)\textsuperscript{18,21}. Specifically, we positioned an off-the-shelf ETL (EL-16-40-TC-20D, Opto-tune) 200 mm in front of the first scan mirror and controlled it with a custom driver board (see user manual). In this position, already a minor deviation from the perfectly flat curvature at zero input current slightly converged the laser which, in turn, strongly shifted the effective z-focus below the objective. For example, in both nTC1 and nTC2, stepping the input current from zero to 25% (50 mA) gave rise to a ~600 μm z-shift of the excitation plane (Fig. 4a, b). The use of only a small fraction of the ETL’s full dynamic range enabled short turnaround times (1–10 ms, depending on distance jumped, Fig. 4c, d, Fig. S3) and prevented overheating\textsuperscript{18,29}. An overview of possible scan-paths is presented in Supplementary Movie S4. If required, rapid synchronization of the ETL curvature with a Pockels cell for controlling effective laser power at the sample plane can compensate for any systematic variations in image brightness associated with increased penetration depth.

Taken together, our design, therefore, presents a low-cost (~£1000, cf. user manual) and easily implemented solution to expand the FOV of any 2P microscope in three dimensions. In the following, we demonstrate how these capabilities can be exploited in a range of neurophysiological applications in larval zebrafish, as well as the mouse cortex and fruit fly brain.

Imaging zebrafish under 2P. Owing to their small size and transparent larval stage, zebrafish have become a valuable model for interrogating brain-wide neural circuit function\textsuperscript{30,31}. However, from tip to tail, the brain and spinal cord of a 7–9 dpf larval

Fig. 2 Spatial resolution over the full field of view. a Schematic (top) and photograph (bottom) of the setup used to directly film excitation volumes. IR, infrared. b effective scan-planes directly visualised as indicated in (a) for all optical configurations as indicated, in each case with scan-points spaced to facilitate inspection of individual PSFs. c–e PSF measurements using fluorescent beads (cf. Fig. 1m) across the full FOV for all optical configurations as indicated in (c). Shown are exemplary scan profiles (pairs of xz and yz projections) from (d) and (e) quantification of full width half maxima (FWHM) in lateral (xy) and axial dimensions (z). For statistical evaluation we compared each “lateral” PSF estimates to the respective centre estimates using 1-way ANOVA. No significant differences were detected, except in two top-right (TR) xy values for nTC2 conditions, as indicated. These small differences are likely explained by slightly imperfect laser centring. Error bars in mean ± s.d., n = 20 experimentally independent samples per measurement. TL, top left, TR, top right, BL, bottom left, BR, bottom right. f–h, example scan under nTC2. 3.5 mm configuration of a mouse brain slice labelled with SM131 for phosphorylated Neurofilament-H (NFH). Full usable FOV (f) and two zoomed in regions (g, h) as indicated in (f), without moving the objective, to illustrate image quality at the FOV edges (f–h, 1024 × 1024 px, 0.49 Hz). Data leading to e in Source data file.
zebrafish reaches about 3.5–4.5 mm, with the central brain occupying approximately 1.2 mm in length and 0.7 mm in width. This is too large to fit into the FOV of a typical DL 2P setup. As a consequence, studies routinely “tile-scan” the brain in sequential stages to provide brain-wide data32,33. On the other hand, the transparent body wall of larval zebrafish makes them well-suited for 1-photon selective-plane-illumination microscopy (1p-SPIM / “lightsheet microscopy”), which is not FOV-limited in the same way as 2P microscopy29,30,34. However, 1p-SPIM and related techniques35 have a number of drawbacks, including constraints on achieving a homogenous image due to scattering and divergence of the excitation light with increasing lateral depth2, limited access to tissues that are shadowed by strongly-scattering tissue such as the eyes36,37 and, critically, a direct and bidirectional interference between the imaging system itself and any light stimuli applied for studying zebrafish vision38.

Fig. 3 In vivo calcium imaging across different optical configurations. a The same set of neurons of the 7 dpf larval zebrafish upper spinal cord (HuC:GCaMP6f, random sparse expression, see overview scan and schematic on the left) was imaged in all optical configurations as indicated at 512 × 512 px (1 Hz). Arrowheads highlight the same synaptic structures in each scan. b–d 64 × 64 px (7.81 Hz) activity scan from fields of view shown in (a) for all five configurations during presentation of full-field flashes of UV-light which stochastic elicited activity in these imaged neural structures. In each case the average scan projection (b) and neighbour-correlation based activity projection (c) are shown (hereafter referred to as “activity-correlation”). Darker shadings, equalised for visibility, denotes increased local activity (for details, see ref. 70). Black traces in (d) show time-traces for the same structure in all cases. For the nTC2 2.5 mm FOV condition, time-traces from different neural structures are extracted to illustrate different responses in different structures. All activity traces in this and the following figures are shown in z-scores relative to their own baseline (hence y-scale in s.d.). We choose this metric over dF/F as it emphasises detectability of events rather than the relative change from the indicator’s baseline fluorescence, which differs between biosensors.
These specific challenges could be readily addressed by our nTC 2P setup. To demonstrate this, we imaged larval zebrafish in a range of optical configurations.

Mesoscale whole-zebrafish 2P imaging. First, we used the 3.5 mm configuration of nTC1 to capture the largest-possible FOV of two larval zebrafish facing each other. This configuration comfortably allowed simultaneous mesoscale imaging of two entire zebrafish brains, here responding to full-field flashes of UV-light (Fig. 5a–e, Supplementary Movie S5). Alternatively, the same configuration could be used to capture the entire central nervous system of one fish in a single frame, including the brain and nearly up to the tip of the spinal cord (Fig. 5f). Zooming in throughout the sample enabled resolving cellular details (Fig. 5g).

Next, beyond mesoscale imaging, many studies of zebrafish neuronal function focus on either the brain or the spinal cord (rather than both). In this case, using the more highly resolved nTC1 configuration with 1.2 mm FOV may be preferable; this just about fits one full zebrafish brain at a time.

3D random access scanning across the zebrafish eye and brain. In the nervous system, key functionally linked circuits are often separated in 3D space, representing a general problem for systems neuroscience. For example, the retinal ganglion cells of the zebrafish eye project to the contralateral tectum and pretectum, which are both axially and laterally displaced by several 100s of microns. Accordingly, it has been difficult to simultaneously record at both sites, for example, to study how the output of the eye is linked to the visual input to the brain. To address this problem, we used our nTC1 configuration in synchronisation with the ETL to establish quasi-simultaneous 3D random access scanning of the zebrafish’s retinal ganglion cells across both the eye and brain (Fig. 6a–c). For this we used an Isl2bmGCaMP6f line which labels the majority of retinal ganglion cells in larval zebrafish. We first defined a slow, high-spatial resolution scan (512 x 512 px, 0.98 Hz) that captured the entire front of the head, however with a single z-jump at the centre of the frame to set-up a “staircase-shaped” scan-path (Fig. 6b, c). Here, empirical adjustment of the magnitude of the z-jump allowed us to identify the axonal processes of retinal ganglion cells in the brain, and their dendritic processes in the contralateral eye in the top and bottom of the same imaging frame, respectively. Based on this image, we next defined two scan regions for 3D random access scanning, one capturing a single plane across the tectum, while the other captured a smaller area of a subset of RGC dendrites and somata in the eye (Fig. 6d–f). Finally, we decreased the spatial resolution to 64 x 64 px to quasi-simultaneously image both regions at 7.81 Hz. This configuration allowed reliable recording of light-driven signals from individual RGC neurites across the eye and brain (Fig. 6g–j). Next, we repeated this experiment, however, this time in zebrafish larvae that were transiently injected with Isl2bmGCaMP6f plasmid. These animals stochastically express mGCaMP6f in only a very small number of RGCs, making it possible in principle to identify the processes belonging to the same RGC in both the eye and brain. As a proof of principle, we present one such experiment where we could clearly image the processes of single RGCs at both sites (Fig. 6k–o). For this type of application, it will be important to optimise the genetic protocol to improve expression levels and thereby facilitate the identification of the same RGC’s processes at both sites.

3D plane-bending for imaging activity across the zebrafish brain. During standard planar scans of the larval zebrafish brain, the powerful optical sectioning afforded by the 2P approach highlights the 3D curvature of distinct brain regions by cutting right across them (Fig. 7a–d). While it was possible to quasi-simultaneously image anywhere within the brain at high spatial resolution using nTC1, a planar scan grossly misrepresented the real 3D structure of the zebrafish brain (Fig. 7d, top panel). For example, the tectum in larval zebrafish is tilted upwards ~30°, meaning that rather than either cleanly sampling across its retinotopically organized surface, or perpendicularly across its stacked functional layers, the planar image instead cut the tectum at an effective 30° angle to yield a mixture of both, thus confounding interpretation. To ameliorate these issues, we used a 3D curved scan plane by driving the ETL as a sqrt(cosine) function of the slow y-mirror command (‘Methods’). This enabled z-curved “halfpipe” scans that could be empirically fitted to follow the natural curvature of the brain, thereby closely
Fig. 5 Mesoscale imaging of zebrafish larvae. a Photograph of two 9 dpf zebrafish larvae mounted head-to-head in a microscope chamber with mm-scale ruler in background. b The same 2 fish (HuC:GCaMP6f) as in (a) imaged under 2-photon with nTC₂ 3.5 mm FOV configuration, at 512 × 128 px (3.91 Hz). c–i Activity-correlation (cf. Fig. 3c) of the scan in (b) during presentation of full-field flashes of ultraviolet (UV)-light, with hand-selected exemplary ROIs, extracted time-traces (d) and light-stimulus-aligned averages (e). f–i The same fish as shown on the left in (b, fish 1), now shown at full 3.5 mm field of view (f, 512 × 128 px, 3.91 Hz) and increased spatial resolution scans of regions as indicated to reveal cellular detail (g–i, 1024 × 1024 px, 0.49 Hz). RGC, retinal ganglion cell.
**Fig. 6** 3D random access scanning of the zebrafish eye and brain. **a, b** Schematic of zebrafish larva from top (**a**) and front (**b**) with scan configurations indicated. **c** Direct x-z visualisation of the scan-profile used in the below. **d** nTC1 1024 × 1024 px scan across an Islet2b:mGCaMP6f 6 dpf larval zebrafish eye and brain. At the centre of the scan, the axial focus is shifted upwards such that the axonal processes of retinal ganglion cells (RGCs) in the tectum (top) and their somata and dendritic processes in the eye (bottom) can be quasi-simultaneously captured. **e, f** 1024 × 1024 px split-plane random access jump between tectum (**e**) and eye (**f**) and **g–j** 2 times 64 × 128 px (15.6 Hz) random access scan of the same scan regions with raw (**g**) and event-averaged (**h**) fluorescence traces, mean image (**i**) and activity-correlation (**j**, cf. Fig. 3c). The stimulus was a series of full-field broadband flashes of light as indicated. **k–o** as (**d–j**), with individual RGCs transiently expressing GCaMP6f under the same promoter.
Fig. 7 2P plane-bending to image the in vivo larval zebrafish brain. a–c Schematic of HuC:GCaMP6f larval zebrafish brain viewed from top (a) and front (b) with scan planes indicated, and (c) example-scan-profiles. d nTC, 512 × 1024 scans of a 6 dpf zebrafish brain with different plane curvatures, with peak axial displacement at scan centre as indicated. At curvatures ~100–150 µm peak displacement the scan approximately traverses the surface of the tectum. e–h Mean (e), activity-correlation (f, cf. Fig. 3c) and fluorescence traces (g, raw and h, event-triggered mean) from a 170 × 340 px scan (5.88 Hz) of the 100 µm peak displacement configuration (image 3 in (d)). The fish was presented with full-field and spectrally broad (~360–650 nm) series of light-flashes. See also Fig. S4.
capturing the functional anatomical organisation of the zebrafish brain (Fig. 7b–d, Supplementary Movie S6). From here, we chose a single halfpipe plane that best followed the curvature of the two tecta and imaged this plane at 7.81 Hz (256 × 128 px, 1 ms/line, Fig. 7e). We then presented spectrally broad full-field light stimulation. This allowed us to interrogate brain-wide visual function in response to arbitrary wavelength light (Fig. 7f–h). As required, the halfpipe scans could also be staggered for multiplane imaging at correspondingly lower image rates, including negative bends that surveyed the difficult-to-reach bottom of the brain between the eyes (Fig. S4, Supplementary Movie S7).

Mesoscale and 3D random access imaging of the mouse brain. The width of the adult mouse’s brain is ~10 mm 40 which makes it too large to be comprehensively captured by conventional 2P microscopy. Here, an experimental goal might be to reliably resolve the ~20±µm somata of major cortical or subcortical neurons across a 10 mm FOV. At the Nyquist detection limit, this would “only” require ~1000 pixels across, which is well within the range of standard high-resolution scan-configurations. Accordingly, currently the main limitation in achieving this goal is the microscope’s maximal FOV. Our nTC design makes important steps to address this limitation.

When configured for a 3.5 mm FOV (nTC2), our setup captures about a third of the width of a mouse’s brain. In this configuration, a scan of a transverse section from a Thy1-GCaMP6f mouse (Fig. 8a, b) illustrates how the objective’s back aperture casts a shadow at the image edge, thus limiting the spatial extent of the scan (Fig. 8c). Within this maximal window, a high-resolution 1024 × 1024 px scan allowed us to resolve the somata of major cortical and hippocampal neurons (Fig. 8d, Supplementary Movie S8). Accordingly, at this largest FOV configuration, effective signal detection largely sufficed to capture the mouse brain’s major neuron populations. However, with our galvo-galvo setup, scan rates at this level of spatial detail were slow (0.49 Hz, 2 ms/line). Accordingly, we used a mesoscopic imaging approach with reduced spatial sampling (256 × 256 px, 1 ms/line) to capture the entire image at 3.91 Hz. This permitted simultaneous population-level “brain-wide” recording of seizure-like activity across the cortex and underlying hippocampus following bath application of an epileptogenic (high K+, zero Mg2+) solution (Fig. 8e–g). To demonstrate the value of the system for more detailed readout of neuronal activity, we also used random access scans to simultaneously capture distant smaller scan-fields at high resolution, both spatially and temporally (two times 256 × 128 px at 3.91 Hz, Fig. 8d, h–l, Supplementary Movie S8). In the example provided, the laser travelled between the two scan fields separated by ~1 mm within two 1 ms scan lines. This allowed us to record quasi-simultaneous neural high activity across both the cortex and hippocampus. The generally high SNR in these recordings also suggested that additional temporal or spatial resolution could be gained by the use of resonance scanners in place of our galvoscanners 41.

The large FOV nTC2 configuration also lends itself to imaging mouse cortical neurons activity in vivo (Fig. 9), an increasingly common demand in neuroscience. Here, the maximal 3.5 mm FOV captured an entire cranial window of a Thy1-GCaMP6f mouse prepared for optical interrogation of the somatosensory cortex, comprising an estimated 10,000+ neurons in a given image plane (Fig. 9a–c, Supplementary Movie S9). Even in an intermediate nTC1 configuration (in this case a 1.5 mm FOV) the full image still comprised 1000s of neurons (Fig. 9d), many more than could be simultaneously captured at scan-rates suitable for functional circuit interrogation with a galvo-galvo setup. In an example scan we again used a random-access approach to quasi-simultaneously record two 330 × 210 µm regions separated by ~1.2 mm (two times 128 × 64 px at 7.81 Hz). As in the brain slice preparation (Fig. 8), this reliably resolved individual neurons in spatially distinct regions of the mouse brain (Fig. 9d–i). Finally, we also recruited the ETL to set up an axially tilted scan plane. This allowed quasi-simultaneous recording from neurons separated several hundreds of µm in depth across layers 1–4 of the mouse cortex in vivo (Fig. 9j–l).

Multi-plane circuit mapping with optogenetics in Drosophila. Despite the generally enlarged FOV and concomitant increase in the PSF, our setup was still capable of resolving details of small neural processes in the <0.1 mm diameter nervous system of a first instar larval fruit fly. To assess the difference in image-resolution between our nTC setup and a DL-configuration, we first obtained anatomical scans from a third instar VGlut:GCaMP6f larva which expressed GCaMP6f in structurally well-defined neurons of the ventral nerve cord (Fig. 10a, b). This revealed that while the DL image was clearly sharper (Fig. 10a), the nTC1 system nevertheless comfortably delineated individual somata (Fig. 10b).

Drosophila was an ideal preparation to demonstrate our system’s capacity for multi-plane imaging for optogenetic functional circuit mapping (Fig. 10c–k). At the first larval stage, the height of the brain excluding the ventral nerve cord is in the order of ~60–70 µm. Assuming an axial capture of ~3 µm per plane in a DL configuration (cf. Fig. 10o), comprehensively sampling from the whole brain would therefore require upwards of 20 planes (Fig. 10c). Here, the slightly elongated PSF of the nTC 1.2 mm configuration served as a useful compromise between spatial resolution and sampling density (Fig. 10d). To demonstrate the sampling that can be achieved under these conditions, we used a transgenic first instar larva that expressed the red-shifted optogenetic effector Chrimson in all olfactory sensory neurons (OSNs) on a background of pan-neuronal GCaMP6s (elav:GCaMP6s) (Fig. 10e, f). To reveal any potential bilateral crosstalk of olfactory signal processing across the brain’s two hemispheres, one of the olfactory nerves was cut. We set up six image planes (six times 340 × 170 px), each separated by ~15 µm which together captured the entire brain across both hemispheres at ~1 Hz (Fig. 10d, f). In this configuration, presentation of 2 s flashes of red light from a scanline-synchronized 590 nm LED activated olfactory sensory neurons (OSNs). These in turn propagated the signal to higher processing centres, which we visualised as regionally restricted GCaMP6s responses in the brain (Fig. 10g–k, Supplementary Movie S10). The most strongly activated region was the ipsilateral antennal lobe (AL) (see also Fig. S5) which is directly innervated by the still-intact OSNs. Similarly, the olfactory second order processing centres, the mushroom body and the lateral horn, showed clear ipsilateral activation. In addition to these three major olfactory centres and their connecting tracts (e.g. plane 3), further processes and somata across both the ipsi- and contralateral lobe were also activated. Taken together, despite the slight expansion of the DL excitation spot, our nTC setup nevertheless allowed us to delineate key structural and functional information in this small insect brain.

Discussion

The ongoing development of sophisticated optical probes to report on key biophysical events has increasingly raised the demand in neuroscience for high SNR and large FOV 2P microscopes. To date, however, these characteristics are almost exclusively limited to high-end and, inevitably, high-cost platforms. Here, we exploit the fact that in 2P microscopy there is no “traditional” collection plane, allowing us to deviate from the diffraction limited regime that is typically used in systems where the planes of excitation and collection must superimpose to avoid image blur. Instead, we propose a simple core modification of the
laser path that allows upgrading an out-of-the-box Sutter MOM DL 2P microscope into a system capable of performing high SNR and large-FOV volumetric scans. We demonstrate the capabilities of this system for interrogating dynamic events in the brains of a range of key model species that are already widely used in neuroscience research. Since the core modification only requires the user to swap the scan lens for one or two off-the-shelf lenses, it can be tested (and fully reversed) within a matter of hours without the need for optical re-alignment or calibration. We anticipate that the simplicity and cost-effectiveness of this
solution and the significant enhancement in 2P imaging capabilities that it permits, will lead to its wide adoption by the neuroscience community.

Combining an nTC approach with existing custom 2P designs. The estimation of metrics that meaningfully compare the capability of our nTC design with other custom solutions is difficult, as these can depend strongly on the specific objective (N.A., back aperture size, working distance (focus)), its distance from the tube lens, and indeed the nature of the interrogated sample and the biological question itself. Rather, because our nTC approach fundamentally differs from traditional DL optics, it opens the possibility to further enhance the capabilities of existing custom 2P microscope designs.

A key benefit of our nTC approach is the flexibility that it offers. It can be seamlessly implemented on setups with galvanometric or resonant mirrors to work with a wide range of scan-strategies. Here, the “extra” optical magnification afforded by the FOV expansion means that scan-mirror and ETL movements translate to relatively larger xy or z-translations, respectively, making it easy to rapidly execute complex and large-scale 3D scan-paths. Our approach could principally also be combined with existing setups that use rapid piezo-positioning of the objective for axial scans, although in this case the objective movement relative to the tube lens will generate small but systematic variations in FOV and PSF shape. Accordingly, the use of remote focussing before the scan-mirrors is likely to be preferable in most applications.

Like in most 2P designs, our use of a Gaussian beam does not permit the generation of a truly arbitrary PSF shape. Nevertheless, if used in combination with temporal focussing,12 it would, in principle, be possible to modulate axial PSF expansion without strongly affecting lateral expansion, thus facilitating a greater range of PSF shapes. Similarly, an optimized design of the objective lens43 and other optical elements4 including the use of large diameter lenses to minimize aberrations5, could all be combined with our optical design to further enhance the quality of 2P excitation.

nTC and optical aberrations. In general, beyond the PSF expansion that results from bypassing the objective’s infinity correction (Figs. 1, 2, Fig. S1), and the increased field curvature that inevitably follows from FOV expansion, the change from a standard 2P DL-setup to an nTC configuration does not notably worsen other optical aberrations. In short, chromatic aberrations (which necessitate complex optical corrections in 1P microscopy) do not apply in 2P microscopy, because the excitation laser is essentially monochromatic and collection is spatially invariant. Instead, spherical aberrations tend to be dominant in 2P microscopy, i.e. when peripheral and axial rays do not converge to a point44–49. Here, the optical element that has the largest impact is the objective, which is not changed under nTC. Further monochromatic aberrations are mainly related to the sample structure and surrounding (immersion) medium itself. In the future, it will be useful to explore how adaptive optics can address many of the above points, including spherical aberrations as well as coma and astigmatism46—see also Supplementary Discussion.

Axial signal integration. Beyond matching the PSF to a given biological application (by correspondingly switching between nTC configurations), the use of a non-DL excitation spot can also bring about additional benefits. First, the lower effective excitation N.A. produces a narrower light cone which is less likely to be scattered by tissue inhomogeneities50. Second, objects that are smaller than the focal excitation volume become dimmer, while objects that are similar in size or larger remain bright51. Third, PSF expansion also reduces photobleaching and photodamage which can have a more-than-quadratic intensity dependence52,53. For example, when using the large PSF of the 3.5 mm FOV configuration, it was possible to use up to 250 mW laser power without causing notable damage when imaging deep in the mouse cortex54. Here, calculations and experimental experience suggest that in general, our strategy of underfilling the objective’s back aperture will greatly ameliorate photodamage54,52,53. Notwithstanding, any axial expansion in the PSF must be balanced with potentially undesirable merging of distinct image structures separated in depth.

Taken together, our nTC approach offers key advantages over traditional DL 2P microscopy, including the capacity for an increased FOV, rapid z-travel through minimal ETL commands, and overall increased laser power at the sample plane48,55,56. Moreover, it could principally be combined with a wide range of existing customisations to further push the capabilities of 2P microscopy in general. At the same time, our nTC approach is cost effective and can be readily implemented with minimal need for optical alignments and calibration.

Methods
Animal experiments. All animal experiments presented in this work were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986 and institutional regulations at the University of Sussex. All procedures were carried out in accordance with institutional, national (UK Home Office PPL/70–8400 (mice), PPL/PE082AD2AD2 (zebrafish)) and international (EU Directive 2010/63/EU) regulations for the care and use of animals in research.

Zebrafish larvae preparation and in vivo imaging. Zebrafish were housed under a standard 14:10 day/night rhythm and fed 3 times a day. A group of animals were grown in 200 mM 1-phenyl-2-thiourea (Sigma) from 1 day post fertilization (dpf) to prevent melanogenesis. Preparation and mounting of zebrafish larvae was carried out as described previously37. In brief, we used 6–7 dpf zebrafish (Danio rerio) larvae that were immobilised in 2% low melting point agarose (Fisher Scientific, Cat: BP1360–100), placed on the side on a glass coverslip and submerged in fish water. For eye-brain imaging, eye movements were prevented by injection of a-bungarotoxin (1 nL of 2 mg/ml; Tocris, Cat: 2133) into the ocular muscles behind the eye. Transgenic lines used were Islet2b:mGCaMP6f (eye-brain imaging) and HuC:GCaMP6f28 (image of 3 zebrafish in same FOV). Zebrafish were imaged at 930 nm and 30–60 or 50–100 mW for brain and eye imaging, respectively.

Creation of Islet2b:mGCaMP6f transgenic line. Tg(Islet2b:mGCaMP6f) was generated by co-injecting pTol2-isl2b-hlsTrpR-pA and pBH-tUAS-memGCaMP6f-pA plasmids into single-cell stage eggs. Injected fish were out-crossed with wild-type fish to screen for founders. Positive progenies were raised to

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Fig. 9 Mesoscale random-access imaging of mouse cortex in vivo. a, b Schematic of Thy1:GCaMP6f mouse brain in vivo (a) with cranial window over the somatosensory cortex (b). c, d 1024 × 1024 px nTC2 (c) and nTC1 (d) images as indicated. Red arrows indicate rapid transitions between scan regions, with the inset indicating the scan-profile. e–i 2 times 128 × 256 px (3.91 Hz) random access scan as indicated in (d) with mean-projection (e, f), activity-correlation (g, h, cf. Fig. 3c) and fluorescence traces (i), taken from the ROIs as indicated in (g, h). j–l nTC2 128 × 128 px xyz-tilted plane (7.82 Hz) traversing through cortical layers 1–4 at ~45° relative to vertical (such that the x-image dimension corresponds to the x-mirror, while the y-dimension in the image represents simultaneous and matched y- and z-movement, with mean image (k) and activity-correlation (l, cf. Fig. 3c). The scan was taken under nTC2 3.5 mm FOV configuration and zoomed in to the central ~0.6 mm.
establish transgenic lines. All plasmids were made using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids as follows: pTol2-isl2b-nlsTrpR-pA: pTol2pA58, p5E-isl2b59, pME-nlsTrpR60, p3E-pA; pBH-tUAS-memGaMP6f-pA: pBH61, p5E-tUAS60, pME-memGCaMP6f, p3E-pA. Plasmid pME-memGCaMP6f was generated by inserting a polymerase chain reaction (PCR)-amplified membrane targeting sequence from GAP-43 into pME plasmid and subsequently inserting a PCR amplified GCaMP6f at the 3′ end of the membrane targeting sequence.

**Acute brain slices.** One to two-month-old male Thy1-GCaMP6f-GP5.1764 mice were used. Acute transverse brain slices (300 µm) were prepared using a vibrisslicer (VT1200S, Leica Microsystems, Germany) in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 1.25 NaH2PO4, 2 NaHCO3, 1 MgCl2, 2 CaCl2 (bubbled with 95% O2 and 5% CO2, pH 7.3), and allowed to recover in the same buffer at 37 °C for 60 min. During imaging, slices were constantly perfused with 37 °C modified (epileptogenic) saline (37 °C) containing 125 NaCl, 2.5 KCl, 25 glucose, 1.25
Fig. 10 Multi-plane imaging and optogenetics for functional circuit mapping. a, b, DL (a) and nTC2 (b) 1024 x 1024 px scans of the ventral nerve cord of a 3rd instar (L3) VGlut:GCaMP6f Drosophila larva. c-f Scan-profiles taken in DL (c) and nTC2 (d) across 6 planes spaced ~15 µm apart. e, f Schematic of first instar (L1) elav:GCaMP6f; Ocro:CsChrimson Drosophila larva from top (e) and side (f), with CsChrimson (red) and GCaMP6f (green) expression pattern and scan-planes indicated. g-k Optogenetic circuit mapping of olfactory processing centres across the larval brain. Six scan planes (170 x 340 px each) were taken at 0.98 Hz/plane (i.e. volume rate) during the presentation of 587 nm light flashes (2 s) to activate CsChrimson in olfactory sensory neurons (OSNs). Brain anatomy (g) and false-colour coded fluorescence difference image (h–i) from each plane. To show the relative position of the two wavebands, the “green” and “red” channels each used a single-band bandpass filter (ET525/50 and ET 605/50, respectively, Chroma) and an aspheric condenser lens (GL1770000, Linos) to focus light on a PMT detector chip (HI0770PA-40, Hamamatsu).

Mouse surgical procedures for in vivo imaging of the barrel cortex. Head bar implantation surgery has been described elsewhere. Briefly, under aseptic conditions, a male mouse expressing a calcium indicator in pyramidal neurons (GCaMP6f; GPS17) was anaesthetised with isoflurane and implanted with a custom-made head bar. A circular 3.0 mm diameter craniotomy centred at 3.0 mm lateral and 1.0 mm posterior to bregma was made to expose the cranial surface. A cranial window, consisting of a 3.0 mm circular cover slip and a 5.0 mm circular cover slip (Harvard Apparatus), was placed over the craniotomy and secured in place with cyanoacrylate tissue sealant (Venbond, 3 M). Following 7 days of recovery, the mouse was handled daily and acclimated to a head fixation apparatus over a treadmill for a further 9 days. During 2P imaging, the head-fixed mouse could locomote freely on a custom-made treadmill. The mouse was awake and received fluid rewards between imaging batches. Cortical neurons were imaged at 960 nm and 100–150 mW.

Drosophila larval preparation and in vivo imaging. Flies were maintained at 25 °C in 12 h light:12 h dark conditions. Fly stocks were generated using standard procedures. The genotypes of the fly strains were: w; UAS-Ga4uk; LexAop-CsChrimson and w: UAS-Ga4uk; Ocro. These two strains were crossed to each other (collecting virgins from the pots had a grape juice agar plate with an added drop of yeast paste supplemented second one) and placed on laying-pots at 25 °C for larval collection. The laying-pots supplemented agar plates were changed every day and scanned for larval emergence. The larvae were transferred to DL 2P microscope (designed by W. Denk, MPI, Martinsried; purchased through Sutter Instruments) as described previously. Microscope (VISIR 1534SPR136, Leica) by removing the second lens (i.e. the one closer to the tube lens) from the compound mount which changed the focal length from 50 to 190 mm. Alternatively, the entire de-constructed scan lens could also be replaced by a similar power off-the-shelf plano-convex lens. Our 190 mm lens (L1) was placed exactly 190 mm in front of the tube lens (so shifted 60 mm forward from its original position). Next, we introduced an additional plano-convex 175 mm focal distance lens (L2) (LA1129, Thorlabs). L2 was held in place by custom 3D printed mount (cf. user manual) inside the MOM’s tube-lens holder and positioned anywhere between 0 and 100 mm in front of the tube lens. Depending on the exact position of L2 within this range, the effective FOV at the image plane could be adjusted between 1.2 mm (100 mm distance) to 1.8 mm (L2 and tube lens almost touching).

For nTC2 (FOV 2.5–3.5 mm), we replaced the original scan lens with a single, 200 mm focal length plano-convex lens L3 (LA1708, Thorlabs). Like L2 in nTC2, L3 was mounted on the same custom 3D printed holder and positioned anywhere within a distance of 0–100 mm from the tube lens. At the image plane could be adjusted between 2.5 mm (100 mm distance) to 3.5 mm (L3 and tube lens almost touching). For detailed instructions including photos of the optical path, consult the user manual.

We selected lens types and positions based on the available space within the Sutter MOM head such that for nTC2, and nTC2, the IJP was always located in front of or behind the TL, respectively. However, depending on the design of a given 2P setup’s excitation path, numerous alternative configurations are possible. Here, a straightforward means to rapidly estimate the nature and scale of a given configuration we use two multifunction I/O devices (PCIe-6363 and PCI-6110, National Instrument). Within ScanM, we defined custom scan-configurations: 1024 x 1024 and 512 x 512 pixel images with 2 ms per line were used for high-resolution morphology scans, while faster, 1 ms or 2 ms linespeed image sequences with 256 x 256 (3.91 Hz), 128 x 128 (7.81 Hz), 340 x 170 (5.88 Hz) or 128 x 64 (15.6 Hz) pixels were used for activity scans. All scans were unidirectional, and the laser was blanked via the Pockels cell during the turnarounds and retrace. This period was also used for light stimulation (zebrafish visual system and Drosophila optogenetics, see below).

Non-collimated 2P microscope modifications. We used two sets of modifications (nTC1 and nTC2) to de-collimate the excitation path to different degrees. For nTC1, we modified the original Sutter-MOM scan lens (VISIR 1534SPR136, Leica) by removing the second lens (i.e. the one closer to the tube lens) from the compound mount which changed the focal length from 50 to 190 mm. Alternatively, the entire de-constructed scan lens could also be replaced by a similar power off-the-shelf plano-convex lens. Our 190 mm lens (L1) was placed exactly 190 mm in front of the tube lens (so shifted 60 mm forward from its original position). Next, we introduced an additional plano-convex 175 mm focal distance lens (L2) (LA1129, Thorlabs). L2 was held in place by custom 3D printed mount (cf. user manual) inside the MOM’s tube-lens holder and positioned anywhere between 0 and 100 mm in front of the tube lens. Depending on the exact position of L2 within this range, the effective FOV at the image plane could be adjusted between 1.2 mm (100 mm distance) to 1.8 mm (L2 and tube lens almost touching).

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We selected lens types and positions based on the available space within the Sutter MOM head such that for nTC2, and nTC2, the IJP was always located in front of or behind the TL, respectively. However, depending on the design of a given 2P setup’s excitation path, numerous alternative configurations are possible. Here, a straightforward means to rapidly estimate the nature and scale of a given configuration we use to facilitate a fluorescence test-slide and observe the change in working distance and FOV as the scan path is modified.

Electrically tunable lens (ETL) for rapid axial focussing. For rapid z-focussing we added a horizontal ETL (EL-16-40-TC-20D, Optotune) into the vertical beam path after the silver mirror that reflected the excitation beam up to the MOM head, 200 mm in front of the scan-mirrors. To drive the ETL we used a custom

NaH2PO4, 26 NaHCO3, 2 CaCl2. Brain slices were imaged at 930 nm and 100–150 mW.

Drosophila larvae were dissected as described previously. In the Sutter-MOM head part of Sutter MOM stage (VISIR 1534SPR136, Leica) by removing the second lens (i.e. the one closer to the tube lens) from the compound mount which changed the focal length from 50 to 190 mm. Alternatively, the entire de-constructed scan lens could also be replaced by a similar power off-the-shelf plano-convex lens. Our 190 mm lens (L1) was placed exactly 190 mm in front of the tube lens (so shifted 60 mm forward from its original position). Next, we introduced an additional plano-convex 175 mm focal distance lens (L2) (LA1129, Thorlabs). L2 was held in place by custom 3D printed mount (cf. user manual) inside the MOM’s tube-lens holder and positioned anywhere between 0 and 100 mm in front of the tube lens. Depending on the exact position of L2 within this range, the effective FOV at the image plane could be adjusted between 1.2 mm (100 mm distance) to 1.8 mm (L2 and tube lens almost touching).

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current driver controlled by an Arduino Due microcontroller (see user manual), capable of generating positive currents between 0 and 300 mA. The Arduino Due receives the scan line command and in turn output commands to the current driver to effect line-synchronised changes in ETL. Prior to initiating a scan, the specific to-be-executed Arduino programme was uploaded to the Arduino via serial from a PC running a custom Matlab-script (Mathworks). This Matlab script launched a simple graphical user interface (GUI) that allowed the user to configure the scan path-during a custom scan (see user manual). Accordingly, ETL control remained flexible and fully independent of the scan software. In this way, our solution can be readily integrated with any 2P system without need to change the software or acquisition/driver hardware. Notably, this ETL implementation can also be used by itself, without need for implementing any of the other optical adjustments described in this work. However, depending on the system’s optics, the effective range of z-travel would likely be smaller. A detailed step-by-step guide to implement the ETL, including the control software and hardware is provided in the user manual.

Pockels cell. To control excitation laser intensity, we use a Pockels cell (Model 350-80, Conoptics; driver model 302, Conoptics). A line-synchronised blanking signal was sent from the DAQ to the drive to minimise laser power during the retrace. In addition, a custom circuit allowed controlling effective laser brightness during each scan line via a potentiometer (see user manual, designed by Ruediger Bernd, HIH, University of Tubingen). As required, this amplitude-modulated signal could be further modulated by a second Arduino Due controlled by a standalone Matlab GUI to automatically vary effective laser power as a function of scanline index. In this way, laser power could be arbitrarily modulated on a line by line basis, for example, to compensate for possible power loss when imaging at increased depth.

Light stimulation. For visual stimulation of zebrafish larvae (Figs. 3, 5–7, Figs. S2, 4) we used a full-field, broadband spot of light projected directly onto the eyes of the fish from the front via a liquid light guide (77555, Newport) connected to a custom collimated LED bank (Roithner LaserTechnik) with emission peak wavelengths between 650 and 390 nm to yield an approximately equal power spectrum over the zebrafish’s visual sensitivity range (described in detail in ref. 29). LEDs were line-synchronised to the scanner retrace by an Arduino Due. For CBrinnamon activation (Fig. 7) we used a custom 2 P line synchronised LED stimulator (https://github.com/BadenLab/Tetra-Chromatic-Stimulator) equipped with four 587 nm peak emission LEDs embedded in a custom 3D printed recording chamber.

Image brightness measurements. We imaged a uniform fluorescent sample consisting of two microscopy slides (S8902, Sigma-Aldrich) encapsulating a drop of low melting point agarose (Fisher Scientific, BP1360-100) mixed with low concentrated Acid Yellow 73 fluorescent solution (F6377 Sigma-Aldrich). Shown is the average brightness over the radius from the center to the edge of the FOV (Matlab, custom scripts).

PSF measurements. We used 0.175 ± 0.005 µm yellow-green (505/515) fluorescent beads (P2220, Invitrogen) embedded in a 1 mm depth block of 1% low melting point agarose (Fisher Scientific, BP1360-100). Image stacks were acquired across 30 × 30 µm lateral field of view with 256 × 256 pixels resolution (0.12 µm/pixel) and 0.5 µm axial steps/frame. Laser power at the sample was 15–20 mW. For x and z-dimensions, we calculated the full width at half maximum (FWHM) from Gaussian fits to the respective intensity profiles. Measurements were taken from set of the beads distributed across the entire FOV, and presented results are averages of at least 10 measurements of different beads, with error bars given in s.d.

To film the PSF and effective scan-plane(s) we focussed an air objective (Plan Apo 4x/0.20, Nikon) onto the excitation spot elicited in a plastic cuvette with fluorescein (F2456 Sigma-Aldrich) dissolved in water which was positioned beneath the excitation objective. The camera path was fitted with a single-band bandpass filter (ET525/50, Chroma) and a colour CCD camera (Manta G-031C, Allied Vision). The camera was controlled with its dedicated software (VIWBA, Allied Vision). Note that this technique is implemented here tended to overestimate PSF sizes (e.g. due to the low N.A. objective, and because the cuvette was slightly vertically tilted relative to the camera axis as implemented here tended to overestimate PSF sizes (e.g. due to the low N.A.)

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All systematic data generated in this study have been deposited on Zenodo (https://doi.org/10.5281/zenodo.5763750), and are further available in the Source data file. Source data are provided with this paper.

Code availability. Code used in this work is available on Zenodo/Github https://doi.org/10.5281/zenodo.5763750.

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References

1. Deng, W., Strickler, J. H. H., Webb, W. W. W., Series, N. & Apr, N. Two-photon laser scanning fluorescence microscopy. Science 248, 73–76 (1990).
2. Weisenburger, S. & Vaziri, A. A guide to emerging technologies for large-scale and whole brain optical imaging of neuronal activity. Annu. Rev. Neurosci. https://doi.org/10.1146/annurev-neuro-072116-051458 (2018).
3. Tra, K. et al. Fast, cell-resolution, contiguous-wide two-photon imaging to reveal functional network architectures across multi-modal cortical areas. Neuron https://doi.org/10.1016/j.neuron.2021.03.032 (2021).
4. Bumstead, J. R. Designing a large field-of-view two-photon microscope using optical invariant analysis. Neurophotonics 5, 1 (2018).
5. Tsai, P. S. et al. Ultra-large field-of-view two-photon microscopy. Opt. Express https://doi.org/10.1364/oe.23.021383 (2015).
6. Stirman, J. N., Smith, J. T., Kudrow, M. W. & Smith, S. L. Wide field-of-view, multi-region, two-photon imaging of neuronal activity in the mammalian brain. Nat. Biotechnol. 34, 857–862 (2016).
7. Yu, C. H. et al. Diesel2p microscope with dual independent scan engines for flexible capture of dynamics in distributed neural circuitry. Nat. Commun. https://doi.org/10.1038/s41467-021-26734-4 (2021).
8. Sofroniew, N. J., Flickeinger, D., King, J. & Svoboda, K. A large field of view two-photon microscope with subcellular resolution for in vivo imaging. Elife https://doi.org/10.7554/eLife.14472 (2016).
9. Han, S., Yang, W. & Yuste, R. Two-color volumetric imaging of neuronal activity of cortical columns. Cell Rep. https://doi.org/10.1016/j.celrep.2019.04.075 (2019).
10. Cheng, A., Gonçalves, J. T., Golshani, P., Arisaka, K. & Portera-Cailliau, C. Simultaneous two-photon calcium imaging at different depths with spatiotemporal multiplexing. Nat. Methods https://doi.org/10.1038/nmeth.1552 (2011).
11. Prevost, K. et al. Fast volumetric calcium imaging across multiple cortical layers using sculpted light. Nat. Methods https://doi.org/10.1038/nmeth.4040 (2016).
12. Weisenburger, S. et al. Volumetric Ca2+ Imaging in the mouse brain using hybrid multiplexed sculpted light microscopy. Cell https://doi.org/10.1016/j.cell.2019.03.011 (2019).
13. Lu, R. et al. Video-rate volumetric functional imaging of the brain at synaptic resolution. Nat. Neurosci. https://doi.org/10.1038/nn.4516 (2017).
14. Botcherby, E. J., Juskaitis, R. & Wilson, T. Scanning two photon fluorescence microscopy with extended depth of field. Opt. Commun. 268, 253–260 (2006).
15. Song, A. et al. Volumetric two-photon imaging of neurons using stereovision (VSTV). Nat. Methods https://doi.org/10.1038/nmeth.4226 (2017).
16. Grewe, B. F., Langer, D., Kasper, H., Kampa, B. M. & Helmchen, F. High-speed in vivo calcium imaging reveals neuronal network activity with millisecond precision. Nat. Methods. 7, 399–405 (2010).
17. Chong, E. Z., Pannilento, M., Barreiros, I., Kohl, M. M. & Booth, M. J. Quasi-simultaneous multiplane calcium imaging of neuronal circuits. Biomed. Opt. Express https://doi.org/10.1364/bio.10.000267 (2019).
18. Grewe, B. F., Voigt, F. E., van’t Hoff, M. & Helmechen, F. Fast two-layer two-photon imaging of neuronal cell populations using an electrically tunable lens. Biomed. Opt. Express https://doi.org/10.1117/1.oae.20.2.02035 (2011).
19. Yang, W., Carrillo-Reid, L., Bando, Y., Peterka, D. S. & Yuste, R. Simultaneous two-photon imaging and two-photon optogenetics of cortical circuits in three dimensions. Elife 7, e31673 (2018).
20. Sheffield, M. E. J. & Dombek, D. A. Calcium transient prevalence across the dendritic arbour predicts place field properties. Nature https://doi.org/10.1038/nature13871 (2015).
21. Zhao, Z. et al. The temporal structure of the inner retina at a single glance. Sci. Rep. 10, 1–17 (2020).
22. Denk, W. & Svoboda, K. Why multiphoton is more than a gimmick. Neuron https://doi.org/10.1016/j.neuron.2006.06.027 (2006).
23. Svoboda, K. & Yasuda, R. Principles of two-photon excitation microscopy and its applications to neuroscience. Neuron https://doi.org/10.1016/j.neuron.2006.05.019 (2006).
24. Born, M. & Wolf, E. Principles of Electromagnetic Theory of Propagation, Interference and Diffraction of Light. Princ. Opt. Electromagn. Theory Propagation, Interf. Diff. Light by Max Born, Emily Wolf Oxford, GB Pergamon Press. 1980 (1980).
28. Quirin, S. et al. Calcium imaging of neural circuits with extended depth-of-field light-sheet microscopy. Opt. Lett. https://doi.org/10.1364/OL.41.000855 (2016).

29. Fahrbach, F. O. et al. Rapid 3D light-sheet microscopy with a tunable lens. High Accuracy Quantum 3D Dot Tracking with Multifocal Plane Microscopy for the Study of Fast Intracellular Dynamics in Live Cells. Biophys. J https://doi.org/10.1016/0006-3495(07)7413-5 (2001).

30. Birge, R. R. Two-photon spectroscopy of protein-bound chromophores. Chem. Res 19, 138–146 (1986).

31. Patterson, G. H. & Pton, D. W. Photobleaching in two-photon excitation microscopy. Biophys. J https://doi.org/10.1016/S0006-3495(00)76762-2 (2000).

32. Sprangle, A. K. & Neher, E. Highly nonlinear photodamage in two-photon fluorescence microscopy. Biophys. J https://doi.org/10.1016/S0006-3495(01)74173-5 (2001).

33. Wu, Y., dal Maschio, M., Kubo, F. & Baier, H. An optical illusion pinpoints an aliasing in subsequent generations. Development 141, 1167–1174 (2014).

34. Sieracki, C. K., Levey, C. G. & Hansen, E. W. Simple binary optical elements for aberration correction in confocal microscopy. Opt. Lett. 20, 1213 (1995).

35. Zimmermann, M. J. Y. et al. Zebrafish differentially process color across visual space to match natural scenes. Curr. Biol. 28, 2018–2023.e5 (2018).

36. Kwan, K. M. et al. The Tollkit: a multistage gateway-based construction kit for two-photon transgenesis constructs. Dev. Dyn. 236, 3088–3099 (2007).

37. Pittman, A., J., Law, M.-Y. & Chien, C.-B. Pathfinding in a largeervebrate axon tract: isotropic interactions guide retinotaxal axons at multiple choice points. Development 129, 617–624 (2002).

38. Suii, A., Gulr, A. D., Rainhe, D. W. & Kimelma, D. A targeted gene expression system using the troy phosphopressor in zebrafish shows no silencing in subsequent generations. Development 141, 1167–1174 (2014).

39. Yoshimatsus, T. et al. Presynaptic partner selection during retinal cell reassembly varies with timing of neuronal regeneration in vivo. Nat. Commun. 7, 10590 (2016).

40. Ray, J. N. et al. Development. Development 128, 2497–2508 (2004).

41. Chen, T.-W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499, 295–300 (2013).

42. Deta, H. et al. Thy-1-GaMP6 transgenic mice for neuronal population imaging in vivo. PLoS ONE https://doi.org/10.1371/journal.pone.0108697 (2014).

43. Rey, S., Marra, V., Smith, C. & Staras, K. Nanoscale remodeling of functional synaptic vesicle pools in hebbian plasticity. Cell Rep. https://doi.org/10.1016/j.celrep.2020.01.051 (2020).

44. Bale, M. R. et al. Learning and recognition of tactile temporal sequences by mice and humans. Elife https://doi.org/10.7554/eLife.27337 (2017).

45. Prieto-Goñido, L. D., Diegelmann, S. & Bate, M. Embryonic origin of olfactory circuitry in Drosophila: contact and activity-mediated interactions pattern connectivity in the antennal lobe. PLoS Biol. 10, e1001400 (2012).

46. Euler, T. et al. Eyeecscope optical recordings of light stimulus-evoked fluorescence signals in the retina. Pflugers Arch. 457, 1393–1414 (2009).

47. Yoshimatsum, T., Bartel, P., Jianak, F. & Baden, T. Optimal rotation of colour space by zebrafish cones in vivo. FluoRes. 8, https://f1000research.com/posters/8-1730 (2019).

48. Franke, K. et al. Inhibition decorrelates visual feature representations in the inner retina. Nature 542, 439–444 (2017).

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Authors contributions

F.K.J. and T.B. designed the study, with inputs from T.E. and all authors; F.K.J. implemented and tested hardware and software modifications, with input from P.B., T.Y., T.B. and T.E. F.K.J. and T.B. analysed the data, with inputs from all authors. P.B. assisted with hardware and software testing and troubleshooting and built the visual stimulator. M.R.B. and M.M. provided mice for in vivo imaging and assisted with their handling and imaging. T.Y. and M.Z. generated Islet2b:mGCaMP line and assisted with zebrafish sample preparation and testing. E.K. and K.S. provided mouse brain acute slice samples and assisted with handling and imaging. L.L.P.G. provided Drosophila sample and assisted with handling and imaging. T.B. built the optogenetics stimulator. F.K.J. and T.B. wrote the manuscript with inputs from all authors.

Competing interests

The authors declare no competing interests.
