

Simultaneous whole-animal 3D imaging of neuronal activity using light-field microscopy

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High-speed, large-scale three-dimensional (3D) imaging of neuronal activity poses a major challenge in neuroscience. Here we demonstrate simultaneous functional imaging of neuronal activity at single-neuron resolution in an entire *Caenorhabditis elegans* and in larval zebrafish brain. Our technique captures the dynamics of spiking neurons in volumes of $\sim 700 \mu\text{m} \times 700 \mu\text{m} \times 200 \mu\text{m}$ at 20 Hz. Its simplicity makes it an attractive tool for high-speed volumetric calcium imaging.

Understanding how sensory inputs are dynamically mapped onto the functional activity of neuronal populations and how their processing leads to cognitive functions and behavior requires tools for non-invasive interrogation of neuronal circuits with high spatiotemporal resolution^{1,2}. A number of approaches for 3D neural activity imaging that take advantage of chemical and genetically encoded fluorescent reporters exist^{3,4}. Whereas some are based on scanning the excitation light in a volume, either sequentially⁵⁻⁷ or randomly^{8,9}, others try to capture 3D image data simultaneously by mapping axial information onto a single lateral plane using a range of approaches¹⁰⁻¹⁴.

Light-field microscopy (LFM)¹² is one such simultaneous 3D imaging method that has been applied to nonbiological and fixed biological samples^{12,13}. In contrast to conventional imaging schemes, a light-field microscope captures both the 2D location and 2D angle of the incident light. This is done by placing a microlens array in the native image plane such that sensor pixels capture the rays of the light field simultaneously. Such 4D light fields allow the synthesis of a focal stack computationally. In LFM, single sensor images are used to retrieve information for the entire

3D volume, a scheme that enables high-speed volumetric acquisition. However, despite its potentially superb temporal resolution, LFM has not to date been used for functional biological imaging. This is because capturing the 4D light-field information via a single sensor image comes at the cost of reduced spatial resolution and because of inherent trade-offs between axial imaging range and the spatial and axial resolution¹².

Here we report that neural tissues expressing calcium sensors can be imaged at volume rates of up to 50 Hz and at single-neuron resolution, using a 3D deconvolution algorithm^{15,16} applied to LFM. We achieved effective resolutions up to $\sim 1.4 \mu\text{m}$ and $2.6 \mu\text{m}$ in the lateral and axial dimensions, respectively, inside biological samples. To build our light-field deconvolution microscope (LFDM), we placed a microlens array at the image plane of an epifluorescence microscope (Fig. 1a and Online Methods), which captured the different perspectives of the sample (Fig. 1b) on the camera sensor. To overcome the trade-off between axial and lateral spatial resolution in LFM¹², we exploited aliasing of the recorded data and used computational reconstruction methods based on 3D deconvolution to effectively obtain improved lateral and axial resolution^{15,16} (Online Methods, **Supplementary Notes 1 and 2** and **Supplementary Software**).

To evaluate the spatial resolution of our LFDM, we imaged subdiffraction-sized beads and reconstructed the point spread function (PSF) of our system (Fig. 1b,c). Using a 40 \times objective, we found resolutions of $\sim 1.4 \mu\text{m}$ and $2.6 \mu\text{m}$ in the lateral and axial dimensions, respectively. To verify the suitability of the LFDM for capturing the activity of individual neurons, we imaged a sample consisting of 6- μm -diameter fluorescent beads randomly distributed in three dimensions in agarose and compared a conventional focal stack (taken without microlenses) (Fig. 1d,e) with the deconvolved light-field images (Fig. 1f,g).

Using the same objective with *C. elegans*, we were able to image the majority of a worm ($\sim 350 \mu\text{m} \times 350 \mu\text{m} \times 30 \mu\text{m}$) while maintaining single-neuron resolution (Fig. 2a-c, **Supplementary Figs. 1-4** and **Supplementary Videos 1-5**). We could record activity of neurons in the brain region surrounding the nerve ring and the ventral cord at a 5-Hz volume rate. We note that our LFDM allows for substantially higher volume rates than this, which we demonstrated by recording unrestrained worms at 50 Hz (**Supplementary Fig. 4** and **Supplementary Video 3**). Such volume rates would in principle be sufficient for performing whole-brain imaging in freely moving worms, especially if additional tracking is employed as previously shown for single neurons¹⁷. However, as Ca^{2+} signals in *C. elegans* typically occur at timescales of up to 1 Hz, we chose

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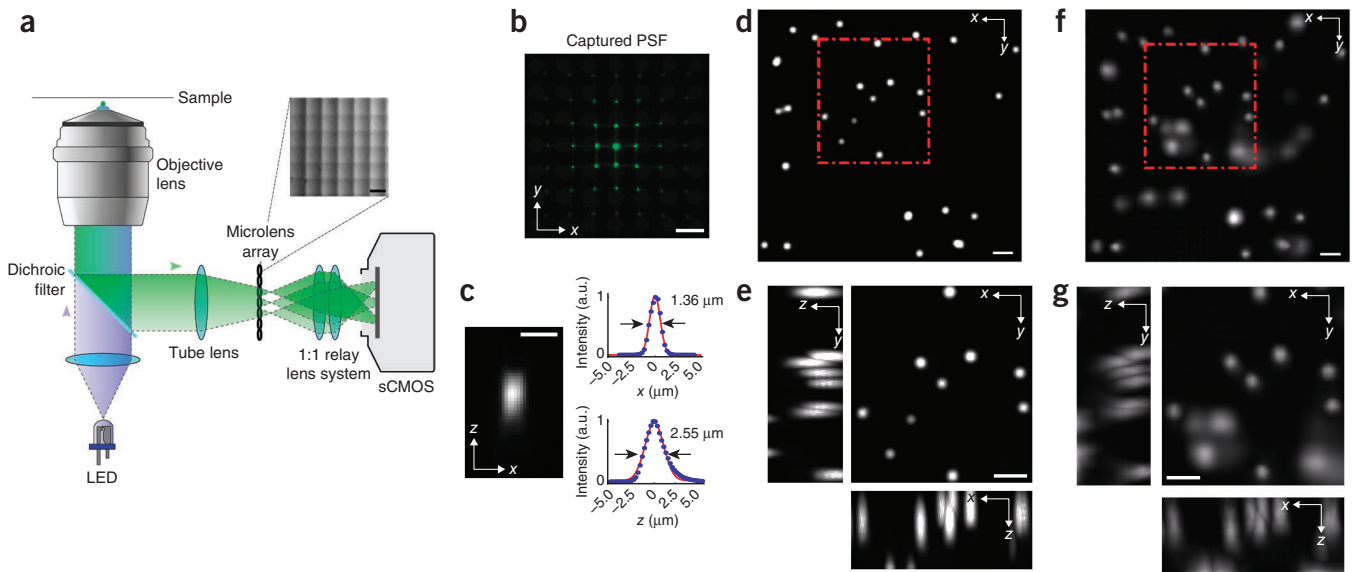


Figure 1 | Light-field deconvolution microscopy. **(a)** A microlens array was appended to the camera port of a wide-field microscope and placed in the primary image plane of the fluorescence microscope. The array itself was imaged with a 1:1 relay lens system onto the chip of a scientific complementary metal-oxide semiconductor (sCMOS) camera (Online Methods). Inset, close-up of the microlens array. **(b)** Point spread function (PSF) of a subdiffraction-sized bead located at $z = 7.5 \mu\text{m}$ off the focal plane, as seen through the microlens array. **(c)** Axial (xz) PSF at $z = 7.5 \mu\text{m}$, reconstructed using the LDFM, and corresponding x and z profiles showing lateral and axial resolution, respectively. a.u., arbitrary units. **(d)** Maximum-intensity projection (MIP) of a deconvolved wide-field focal stack taken without microlenses. The sample consists of $6\text{-}\mu\text{m}$ -sized fluorescent beads in agarose. **(e)** Red box in **d**; xz - and yz -section MIPs are shown. **(f,g)** Corresponding volume of the same beads in **d,e**, $4\text{--}28 \mu\text{m}$ off the focal plane, reconstructed via 15 iterations of the light-field deconvolution algorithm. Scale bars, $150 \mu\text{m}$ (**a,b**), $3 \mu\text{m}$ (**c**) and $10 \mu\text{m}$ (**d-g**).

slower volume rates (5 Hz) in order to maximize the signal-to-noise ratio and reduce potential photobleaching.

The wide field of view (FOV) of the LDFM and the intrinsic simultaneity of the acquisition allow one to study correlations in activity of neurons across the whole animal, which would not be feasible with other unbiased Ca^{2+} -imaging techniques. In our experiments, we observed correlated and anticorrelated activity patterns between the premotor interneurons in the head and motor neurons located along the ventral nerve cord, which connect to body-wall muscles according to the WormAtlas (Fig. 2a–c).

We used the location, morphology and activity patterns of some of these neurons to identify specific premotor interneuron classes such as AVA, AVE, RIM, AIB and AVB, and A- and B-class motor neurons that have been associated with motor-program selection¹⁸ (Supplementary Fig. 3). AVA neurons have been associated with a switch from forward to backward directed crawling, which depends on A-class motor neurons¹⁹ and is associated with a change in the relative activities of A- and B-class motor neurons¹⁸. What we observed was consistent with these findings: a high correlation of AVA and A-class motor neuron activity and an anticorrelation of AVA and B-class motor neuron activity. Further, we used the LDFM and sensory stimulation to identify neuron classes (Supplementary Fig. 3 and Supplementary Video 5). Applying consecutive 30-s intervals of high and low oxygen levels, we observed two neuron classes with increasing Ca^{2+} transients upon oxygen up- and downshift, respectively. Morphology, location and activity patterns of these neuron classes matched those of the oxygen chemosensory neurons BAG and URX⁵.

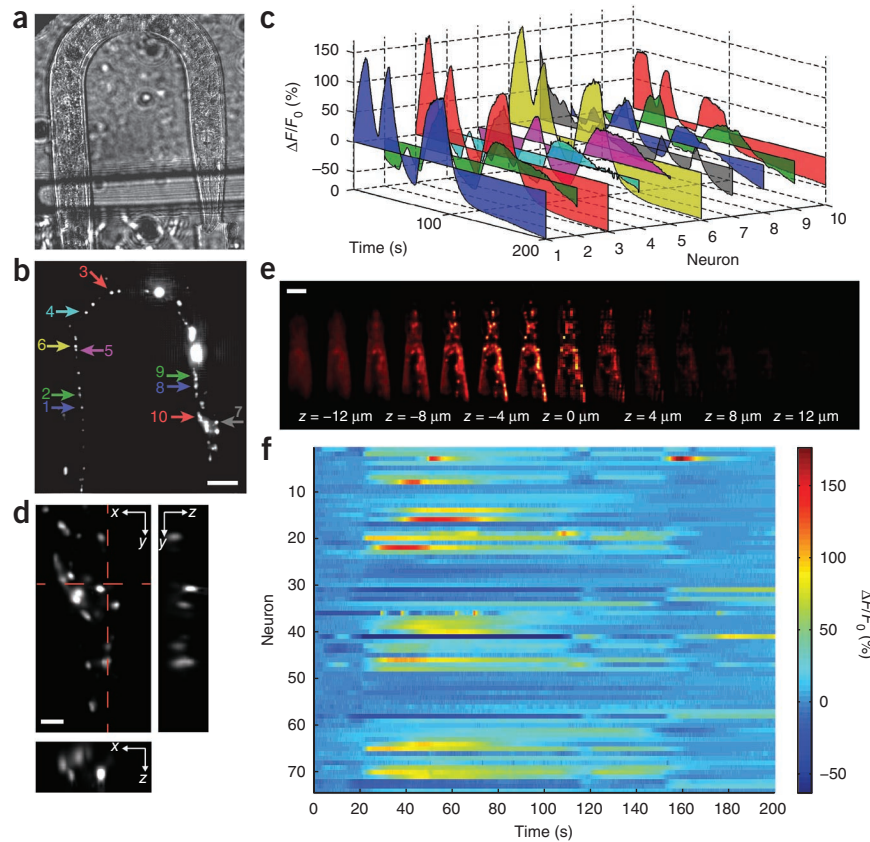
We also recorded exclusively from brain regions surrounding the nerve ring (Fig. 2d–f and Supplementary Fig. 2). Imaging

smaller FOVs ($\sim 200 \mu\text{m} \times 70 \mu\text{m} \times 30 \mu\text{m}$) led to faster volume reconstructions and better image quality owing to the lack of undesired fluorescence from coelomocytes, which were partially labeled in our transgenes. Similarly to previous findings⁵, we were able to resolve up to 74 individual neurons in a typical recording, around 30 of which showed pronounced activity over the recording time of 200 s (Fig. 2d–f and Supplementary Fig. 2).

In order to highlight the temporal resolution and the broader applicability of our technique for capturing dynamics of large populations of spiking neurons, we performed Ca^{2+} imaging in live zebrafish larvae brains expressing the Ca^{2+} indicator GCaMP5 pan-neuronally. Employing a $20\times$ objective, we demonstrated whole-brain Ca^{2+} imaging for volumes spanning $\sim 700 \mu\text{m} \times 700 \mu\text{m} \times 200 \mu\text{m}$ at a 20-Hz volume rate. Although in this case optical single-cell resolution had to be compromised in favor of larger FOVs, we could still recover spatially resolved cellular signals over the entire time series using standard signal extraction and unmixing techniques²⁰. Implementing this approach, we extracted neuronal activity for $\sim 5,000$ cells across the brain and followed their fast Ca^{2+} transients on a millisecond timescale (Fig. 3 and Supplementary Video 6).

By applying an aversive odor to the fish (Online Methods), we evoked neuronal activity and inferred dynamics of Ca^{2+} signals across the olfactory system, the midbrain and parts of the hind-brain, results consistent with previous demonstrations of the neuronal dynamics in these regions^{6,7,21–23}. The high temporal resolution of the LDFM revealed subtle differences in the exact timing of the onset of the response for different groups of neurons located close to each other (Fig. 3c). Whereas the neurons in each group exhibited a nearly synchronous onset of their activity,

Figure 2 | Whole-animal Ca^{2+} imaging of *C. elegans* using LFDM. (a) Wide-field image of the worm inside a microfluidic poly(dimethylsiloxane) (PDMS) device used for immobilization. The head is at the bottom right. (b) Maximum-intensity projection (MIP) of a light-field deconvolved image (15 iterations) containing 14 distinct z planes. Arrows and numbers indicate individual neurons in the head ganglia and ventral cord. (c) Ca^{2+} intensity traces ($\Delta F/F_0$) of NLS-GCaMP5K fluorescence of selected neurons as marked in b and imaged volumetrically at 5 Hz for 200 s (Supplementary Video 1). (d) Close-up of the brain region, with the MIP of the xy plane as well as xz and yz cross-sections indicated by the dashed lines (Supplementary Video 2). (e) Individual z planes of a typical recording of the worm's brain, reconstructed from a single camera exposure (see Supplementary Fig. 2 for neuron IDs). In this recording, the worm's center along the lateral (left-right) (z) axis was placed at the focal plane of the objective. (f) Activity of all 74 neurons identified in e (Supplementary Video 4). Each row shows a time-series heat map of an individual neuron. Color indicates percent fluorescence changes ($\Delta F/F_0$); scaling is indicated by the color bar on the right. Scale bars, 50 μm (b,e), 10 μm (d).



the collective response of each group was delayed with respect to those of the other groups. Overall, our imaging speed, which was more than an order of magnitude faster than in previous whole-brain functional imaging^{6,7}, was thus able to reliably capture the dynamic activity of a large number of cells with high spatial and temporal resolution.

In summary, we have implemented an LFDM and demonstrated its ability to capture the neuronal activity of the entire nervous system of *C. elegans* simultaneously at single-cell resolution as well as record dynamics of spiking neurons by performing whole-brain Ca^{2+} imaging in larval zebrafish at 20 Hz. The increase in spatial resolution compared to that of LFM was achieved by performing deconvolution during postprocessing. The simultaneity of acquisition of volumes in LFDM imaging eliminates spatiotemporal ambiguity associated with sequentially recorded approaches and decouples temporal resolution from volume size. Resolutions in all three dimensions are set by the objective and microlens properties, and FOV and acquisition rate are determined by the camera chip size, frame rates and signal intensity. The LFDM is easy to set up and is cost effective and compatible with standard microscopes. Both the temporal resolution and the obtainable FOVs make light-field deconvolution microscopy an attractive technique for future combination with behavioral studies. Future work will focus on obtaining higher spatial resolutions and larger FOVs as well as faster and more efficient computational reconstruction techniques, both of which are expected to improve with technological advancements in camera sensors and processors. Finally, the use of red-shifted Ca^{2+} sensors²⁴ and the combination of the LFDM with techniques for imaging at depth in biological tissue²⁵ bears further potential for widespread use of this method.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.P. designed microlenses, built the imaging system and performed experiments together with M.H. Y.-G.Y. designed and wrote 3D-deconvolution software with contributions from G.W. under the guidance of R.R. R.P. and M.H. refined and rebuilt the imaging system and analyzed data together with Y.-G.Y. N.P. implemented and tested the LFDM prototype. T.S. generated transgenic animals, provided microfluidic devices and performed cell identifications under the guidance of M.Z. S.K. wrote analysis software.

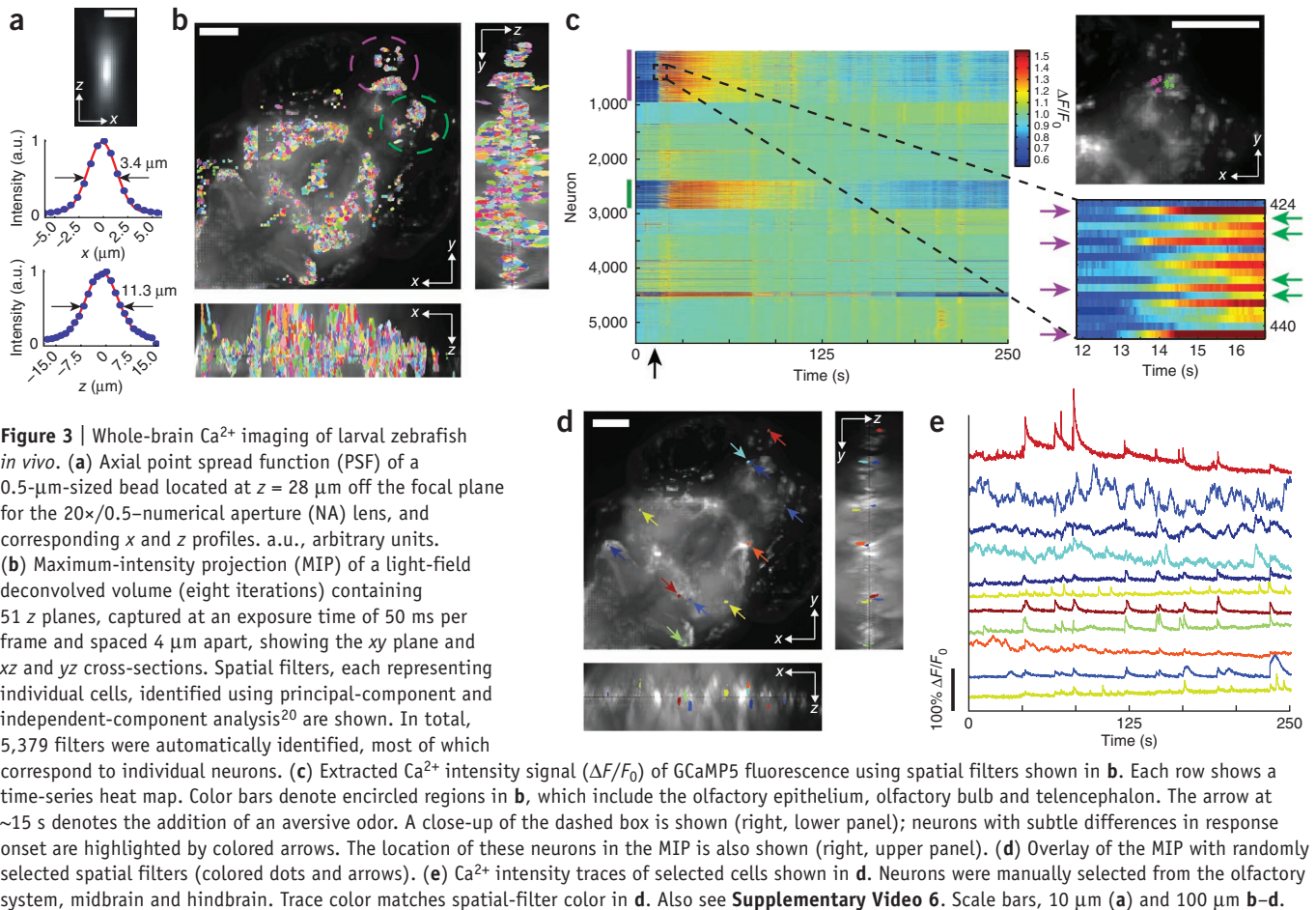


Figure 3 | Whole-brain Ca^{2+} imaging of larval zebrafish *in vivo*. (a) Axial point spread function (PSF) of a $0.5\text{-}\mu\text{m}$ -sized bead located at $z = 28\text{ }\mu\text{m}$ off the focal plane for the $20\times/0.5$ -numerical aperture (NA) lens, and corresponding x and z profiles. a.u., arbitrary units. (b) Maximum-intensity projection (MIP) of a light-field deconvolved volume (eight iterations) containing 51 z planes, captured at an exposure time of 50 ms per frame and spaced $4\text{ }\mu\text{m}$ apart, showing the xy plane and xz and yz cross-sections. Spatial filters, each representing individual cells, identified using principal-component and independent-component analysis²⁰ are shown. In total, 5,379 filters were automatically identified, most of which correspond to individual neurons. (c) Extracted Ca^{2+} intensity signal ($\Delta F/F_0$) of GCaMP5 fluorescence using spatial filters shown in b. Each row shows a time-series heat map. Color bars denote encircled regions in b, which include the olfactory epithelium, olfactory bulb and telencephalon. The arrow at $\sim 15\text{ s}$ denotes the addition of an aversive odor. A close-up of the dashed box is shown (right, lower panel); neurons with subtle differences in response onset are highlighted by colored arrows. The location of these neurons in the MIP is also shown (right, upper panel). (d) Overlay of the MIP with randomly selected spatial filters (colored dots and arrows). (e) Ca^{2+} intensity traces of selected cells shown in d. Neurons were manually selected from the olfactory system, midbrain and hindbrain. Trace color matches spatial-filter color in d. Also see **Supplementary Video 6**. Scale bars, $10\text{ }\mu\text{m}$ (a) and $100\text{ }\mu\text{m}$ b–d.

E.S.B. and A.V. conceived of and led project. R.P., Y.-G.Y. and A.V. wrote the manuscript, with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Setup. The LFM system is appended to an epifluorescence microscope (Zeiss, Axiovert 200) equipped with an LED excitation light source ($\lambda = 465$ nm, 300 mW, CoolLED) and a standard GFP filter set (Zeiss). In all *C. elegans* imaging experiments, we used a 40 \times /0.95-NA dry objective (Zeiss Aplanachromat), whereas zebrafish imaging was performed with a 20 \times /0.5-NA dry objective (Zeiss Plan-Neofluar). The microlens array is mounted inside a five-axis kinematic mount (Thorlabs) to allow fine adjustment of the array orthogonal to the optics axis, which we found crucial for high-quality results. The array is further imaged onto a 5.5-megapixel (2,560 \times 2,160 pixels) sCMOS camera (Andor Zyla) using a 1:1 relay macro lens objective (Nikon AF-S 105mm 2.8 G VR IF-ED Micro) (**Fig. 1a**). Details on optical design choices and their effect on resolution are discussed in **Supplementary Note 1**.

***C. elegans* experiments.** To record neuronal activity from *C. elegans*, we loaded adult worms (1- to 4-egg stage) expressing NLS-GCaMP5K under the *unc-31* promoter (strains ZIM294 and ZIM617) into a microfluidic channel that was connected to a reservoir containing S-basal buffer with 5 mM tetramisole, an acetylcholine receptor-specific agonist that mildly paralyzes the animal's muscles to reduce motion⁵. The worm was placed off the native focal plane and toward the objective using a piezo stepper motor (PI-721, Physik Instrumente) such that the entire worm was ideally contained in the region spanning ~ 30 μ m to 0 μ m. By doing so, we exploited the highest resolution of LFDI while avoiding artifacts near the focal plane. When we recorded from the head region only, the worm's head ganglia were placed at the center of the FOV, and excitation was limited to this area by the use of an iris in the excitation pathway. For the experiments involving chemosensory stimulation, we followed the procedure described in ref. 5. Neurons were identified by classification according to size, shape and relative positions of cell nuclei using the WormAtlas²⁶; previously described characteristic activity patterns⁵ were used as further confirmations. AVA neurons are located in the anterior-ventral part of the lateral ganglia and exhibit an elongated nucleus. AVE neurons are situated posteriorly-medially to AVA and have a similar activity pattern¹⁸. RIM neurons are located in the posterior ventral part of the lateral ganglia; their position is often ambiguous with that of RIB and AIB neurons, which also exhibit activity patterns similar to RIM. VB01 is located in the anterior-to-middle part of the retrovesicular ganglion; its position is ambiguous with other motor neurons in this region such as DB02. DA01 is located at the posterior end of the retrovesicular ganglion. AVB neurons are located central to the lateral ganglia and typically show anticorrelated activity with that of AVA. Ambiguities are posed by the nearby neurons AIN, AVD, AVH and AVJ. BAG neurons are located at the posterior end of the anterior ganglion and exhibit the largest cell nucleus in this region; they reliably respond to oxygen downshift. URX neurons are located at the anterior dorsal end of the lateral ganglia directly ventrally to the unambiguously identifiable nucleus of ALA. URX neurons reliably respond to oxygen upshift.

Zebrafish larvae experiments. For zebrafish experiments, *mitfa*^{-/-} larvae with pan-neuronal GCaMP5 expression have been imaged 5–8 d.p.f. (days post fertilization) using stable lines HuC:GCaMP5G and HuC:Gal4/UAS:GCaMP5G. We immobilized fish

by embedding them in 2% agarose with the mouth and tail cleared of agarose to allow for odor stimulation and tail movement. Odor stimulation was performed during imaging by manually supplying decomposed fish water (an intrinsically aversive odor) into the recording chamber.

Light-field deconvolution. The volume reconstruction itself can be formulated as a tomographic inverse problem²⁷, wherein multiple different perspectives of a 3D volume are observed and linear reconstruction methods—implemented via deconvolution—are employed for computational 3D volume reconstruction. The image formation in light-field microscopes involves diffraction from both the objective and microlenses. PSFs for the deconvolution can be computed from scalar diffraction theory²⁸. More details are given in **Supplementary Note 2**.

After we recorded the raw light-field images, the digital images were cropped to regions of interest (ROIs) and resampled to contain 11 \times 11 or 15 \times 15 angular light-field samples under each lenslet. Two calibration images, one showing the microlenses with collimated rear illumination and one showing a uniform fluorescent slide, were used for digital image rectification, in which camera pixels are assigned to individual microlenses. Reconstruction of each frame of an image sequence took between 2 and 30 min, depending on the size of the image, number of iterations of the deconvolution algorithm, reconstruction method and workstation used. Computational resources are further discussed in **Supplementary Note 2**. A software package for 3D volume reconstruction from light-field images is included as **Supplementary Software**.

Ca²⁺ imaging data analysis. To extract a fluorescence time series for individual neurons from the 4D data, we employed different strategies for *C. elegans* and zebrafish. For *C. elegans*, we first applied rigid-body motion correction to each individual *z*-plane movie. We then computed a median-intensity projection through time for each motion-corrected *z* plane movie and used a maxima-finding algorithm to identify areas of peaked intensity in each projection. A circular ROI was created surrounding each intensity peak, and overlapping ROI areas within *z* planes were eliminated. ROIs in adjacent *z* planes within an *xy* distance of 7 pixels were considered to be a component of the same neuron, up to a maximum of five planes; and for each neuron at each time point, the brightest 100 pixels of the aggregate of all pixels within the neuron's component ROIs were averaged to produce a single fluorescence value and de-trended with an exponential decay function to account for photobleaching. For zebrafish, the data were first de-trended on the basis of the overall intensity of each frame. Then, to reduce time-series data, first we discarded inactive voxels on the basis of their time-domain variance. Splitting the volume into smaller subvolumes further reduced data size. We followed the strategy proposed in ref. 20 to extract cellular signals from the Ca²⁺ imaging data. Each subvolume datum underwent PCA/ICA for automated spatial-filter extraction where ideally each spatial filter corresponds to the location of a neuron²⁰. After automatically rejecting spatial filters on the basis of size and dispersion, we applied the spatial filters to the 4D data to extract their fluorescence intensity. Time points during which the fish seemed to contract were discarded and replaced with nearest-neighbor fluorescence intensities. These contractions typically lasted between 200 ms and 1 s only and were temporally very

sparse. Therefore, we regarded them negligible compared to the overall recording time. Fish that moved substantially during image acquisition were discarded from analysis. To extract $\Delta F/F_0$, we calculated $\Delta F/F_0 = 100 \times (F(t) - F_0)/F_0$, with F_0 being the mean fluorescence intensity of each corrected trace.

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