Multicolor two-photon tissue imaging by wavelength mixing

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We achieve simultaneous two-photon excitation of three chromophores with distinct absorption spectra using synchronized pulses from a femtosecond laser and an optical parametric oscillator. The two beams generate separate multiphoton processes, while their spatiotemporal overlap provides an additional two-photon excitation route, with sub micrometer overlay of the color channels. We report volume and live multicolor imaging of ‘Brainbow’-labeled tissues, and simultaneous three-color fluorescence and third-harmonic imaging of fly embryos.

Multicolor imaging approaches are increasingly used in biology for a variety of purposes: tracking cell anatomy, movement and lineage1, visualizing specific molecules, and recording activity and signaling events at the subcellular level2. A major challenge is to translate these approaches to the imaging of intact and live tissues. Two-photon–excited fluorescence (2PEF) microscopy allows subcellular-scale three-dimensional (3D) imaging of complex media over depths of several hundreds of micrometers, and it has become an indispensable tool for volume and live-tissue studies3. However, while any single particular fluorescent protein or dye can generally be observed using 2PEF microscopy3, current methods do not provide simultaneous, efficient and independent two-photon excitation of more than two spectrally distinct chromophores. To image three fluorophores with two-photon absorption spectra covering a large near-infrared spectrum (for example, 750–1,200 nm, as is needed to image the fluorescent proteins encoded by ‘Brainbow’ transgenes4), solutions have thus far included sequential imaging with a tunable laser, excitation with a single blue-shifted wavelength5,6, or the use of one laser to excite two chromophores with overlapping spectra and a second red-shifted laser7. However, the former approach is incompatible with multicolor imaging of fast events, and the two others do not allow independent optimization of each signal9.

Here we demonstrate a strategy for optimal and simultaneous two-photon imaging of three chromophores with distinct absorption spectra. We achieve three-color excitation by synchronizing the pulse trains from a femtosecond optical parametric oscillator (OPO) and its pump, which results in nonlinear processes produced by either one of the two sources and by their combination. Specifically, we use the combination as an additional ‘virtual’ excitation wavelength for two-photon–excited fluorescence. We show that this arrangement allows simultaneous two-photon excitation of blue, green-yellow and red fluorophores with independent control of their relative excitation efficiencies and automatic submicrometer overlay of the color channels. We report volume and live multicolor imaging of mouse brain and chicken spinal cord tissue labeled with ‘Brainbow’ transgenes, which enable stochastic expression of three fluorescent proteins in a Cre-dependent manner, and simultaneous three-channel fluorescence and third-harmonic generation (THG) imaging of developing Drosophila melanogaster embryos.

Red chromophores and fluorescent proteins4 can be efficiently excited at 1,000- to 1,300-nm wavelengths by an OPO pumped by a femtosecond laser such as a titanium:sapphire (Ti:S) oscillator (as in ref. 10). In such a setup, the OPO beam and a remaining fraction of the pump laser beam can be spatially overlapped to produce two independent nonlinear processes such as multiphoton excitation of two fluorophores. We also take advantage of the synchronous nature of the pump and OPO pulse trains and temporally overlap them in the microscope (Fig. 1a and Supplementary Fig. 1). This generates nonlinear processes resulting both from single-beam excitation (regular 2PEF, harmonic generation) and two-beam excitation (two-color 2PEF, sum-frequency generation (SFG), four-wave mixing and so on). We use this configuration to excite blue and red chromophores with the pump and OPO beams, respectively, and to simultaneously excite a third green-yellow chromophore not accessible by either beam alone using a two-beam two-photon process. In such a two-color two-photon mechanism11, the chromophore is excited by
the simultaneous absorption of one photon from each beam. Because the energy carried by a photon is $hc/\lambda$, where
$h$ is Planck’s constant, $c$ is the velocity of light and $\lambda$ is the wavelength associated with the photon, synchronizing pulses with center wavelengths $\lambda_1$ (750–860 nm) and $\lambda_2$ (1,000–1,300 nm) produces an effect equivalent to an additional two-photon excitation wavelength at $\lambda_3 = 2/(1/\lambda_1 + 1/\lambda_2)$ (850–1,000 nm). This ‘virtual’ wavelength is adjusted by tuning $\lambda_1$ and $\lambda_2$ (Supplementary Figs. 2 and 3), and it can be easily measured through an SFG process in nonlinear potassium dihydrogen phosphate (KDP) crystal powder (Fig. 1b). This strategy for multicolor excitation is illustrated in Figure 1c; we observed that only synchronized pulse trains provided an efficient simultaneous excitation of blue, green and red fluorescent proteins in human embryonic kidney (HEK) cells.

![Figure 1. Principles of multicolor two-photon imaging using synchronized pulses.](image)

Because of unequal fluorophore efficiencies and concentrations in samples, and because scattering-induced attenuation with depth is color dependent (Supplementary Fig. 4), it is crucial to have independent control over the strength of the three signals for quantitative multicolor imaging. Such an independent control is straightforward in our scheme: assuming Gaussian temporal profiles for the excitation pulses and no overlap of the excitation spectra, the intensities of the blue, red and green-yellow fluorescence respectively scale as $(P1)^2$, $(P2)^2$ and $2P1P2\exp(-\tau^2/2\sigma^2)$, where $P1$ and $P2$ are the Ti:S and OPO average powers, $\tau$ is the adjustable delay between the two pulse trains and $2\sigma$ is the 1/e2 width of the pulses’ temporal intensity intercorrelation (Fig. 1c,d). Therefore the efficiencies of the one-beam processes (blue and red fluorescence) are controlled by adjusting the Ti:S and OPO average power, and the efficiency of the two-beam process (green-yellow fluorescence) is controlled by adjusting the delay between the two pulse trains. A benefit of this scheme is that the detection of a ‘green’ image is a confirmation of the proper spatial overlap of the focused Ti:S and OPO beams (Fig. 1e and Supplementary Fig. 5), thus ensuring that the ‘red’,
We demonstrate volume multicolor two-photon microscopy using wavelength mixing by imaging mouse brains electroporated with Brainbow transgenes expressing mCerulean, mEYFP and tdTomato (and/or mCherry) (Fig. 2; Online Methods). Synchronized 850- and 1,100-nm pulses provide simultaneous and efficient excitation of these fluorescent proteins. Figure 2, Supplementary Figure 6 and Supplementary Videos 1–3 show neurons imaged with subcellular resolution in 900 × 720 × 370–μm³, 370 × 410 × 450–μm³ and 1,450 × 430 × 170–μm³ volumes of sparsely labeled mouse cortex. The method results in one-shot multicolor imaging of neurons with in-depth image quality equivalent to that obtained using sequential excitation (Supplementary Fig. 7). The respective powers of the two beams were automatically adjusted according to depth to compensate for the differential attenuation of the three signals, and color balance correction was performed across the field of view (Supplementary Figs. 8 and 9). This acquisition strategy maintains an unchanged balance among colors as a function of depth, which in turn allows comprehensive automated detection and quantitative colorimetric analysis of labeled cells (Fig. 2, Supplementary Fig. 10 and Supplementary Video 1) and color-based 3D segmentation in neurite tracing efforts (Supplementary Fig. 11 and Supplementary Videos 2 and 3).

Figure 2. Deep imaging and colorimetric analysis of Brainbow-labeled mouse cortical samples using multicolor two-photon imaging. (a) Region of mouse brain tissue imaged (dashed line and box). (b) Two-photon (2P) excitation spectra of the fluorescent proteins encoded by the Brainbow constructs mCerulean (CFP), mEYFP, tdTomato and/or mCherry. Arrows indicate the effective excitation wavelengths. (c) Multicolor images extracted from a 450-μm-thick z stack recorded on a Brainbow-electroporated mouse cortex (maximum-intensity projections of a resilience in the xz direction and of 100-μm-thick regions at different depths). (d) Three-dimensional view of a 900 × 720 × 370–μm³ volume of Brainbow-labeled mouse cortical tissue imaged with synchronized 850- and 1,100-nm pulses. (e,f) Automated detection of >3,000 cell bodies present in the imaged volume. Colorimetric analysis (e) of cells highlighted in red (f) confirms that the color balance is preserved over the imaged volume. (See also (See also Supplementary Videos 1-3 and Supplementary Fig. 10). Pixel dwell time, 5 μs. Scale bars, 100 μm.

A key advantage of the proposed method is that all signals are generated simultaneously. This results in an acquisition speed comparable to that of single-channel imaging and prevents motion-induced mismatch between color channels. We demonstrate this advantage through recordings of time-lapse 3D multichannel images of neural
development in live thick slices of embryonic chicken spinal cord electroporated with the Brainbow constructs and recorded with a multicolor pixel dwell time of 5 µs (Fig. 3 a,b and Supplementary Videos 4-6; Online Methods). The coloring allowed us to easily track the movements of individual cells in the developing neuroepithelium and the growth of motor and commissural neurons.

**Figure 3. Continuous three-color 2PEF and THG imaging of live embryonic tissue.** (a,b) 3D+t imaging of live Brainbow-labeled E3.5 (a) and E4 (b) chicken spinal cord tissue (see Online Methods). (a) 3D view of an electroporated slice, showing the section of a half-labeled neural tube. (b) Maximum intensity projections of 3D images of growing motor neurons extracted from continuous imaging with multicolor pixel dwell time 5µs. See also Supplementary Videos 4-6. (c,d) Multicolor 3D+t 2PEF / THG imaging of a developing Drosophila embryo. (c) Nonlinear processes used for simultaneous 4-channel imaging. (d) 2P excitation and emission spectra of endogenous fluorescence (NADH; ‘Endo’), GFP and RFP. Arrows indicate the effective excitation wavelengths and boxes indicate the detection range of the four channels. (e) Frames extracted from a simultaneous THG and three-channel fluorescence 3D+t data set. (f) 3D maximum intensity projections of the posterior pole part at different times. See also Supplementary Videos 7-10. Excitation wavelengths: 850/1,100 nm (a-c); 820/1,175 nm (f,g). Pixel dwell time: 5 µs. Scale bars, 50 µm.

We further illustrate the benefit of our approach for live imaging through multichannel images of gastrulating Drosophila embryos; these images combine three-color fluorescence (mRFP1, EGFP and blue endogenous fluorescence) and third-harmonic generation (Fig. 3 c-f, and Supplementary Video 7). Multicolor imaging of mesoderm invagination is performed with one 3D stack every 11 s (Supplementary Video 8). The rapid morphogenetic movement of germ-band extension in a *Drosophila* embryo is visualized with one four-channel image recorded every 1.5 s and one four-channel 3D stack recorded every 45 s (Fig. 3). Such a temporal resolution is necessary to catch the rapid cell movements at the posterior pole of the embryo (10–15 µm min−1) and cannot be obtained by sequential excitation of the various chromophores. Notably, simultaneous acquisition permits linear
unmixing of images despite rapid tissue evolution, providing an unbiased contrast on individual channels (Supplementary Fig. 12). These data also illustrate that our multicolor fluorescence imaging scheme is directly compatible with simultaneous imaging using coherent nonlinear signals such as THG, which is detected at one-third of the OPO wavelength. THG provides contrasted images of tissue morphology based on their intrinsic optical properties and has proven useful for imaging zebrafish embryos, mouse brain and other tissues. However, because of laser/OPO tunability limitations, THG had until now not been efficiently detected with, for instance, simultaneous GFP fluorescence. Its integration with diverse fluorescent protein imaging schemes as shown here should bring further additional applications.

Multicolor genetic labeling strategies are opening new avenues for reconstructing neuronal connectivity and tracking cell migrations or lineage during development. However, the lack of appropriate multicolor tissue imaging methods has hindered the use of these strategies in thick or live samples. In particular, rapid multicolor imaging with multiphoton microscopy has remained challenging. The simple and robust scheme proposed here addresses this issue with a number of unique features. First, it provides simultaneous and spatially colocalized excitation of the three fluorophores with internal control. This may prove critical for quantitative approaches such as multicolor fluorescence correlation spectroscopy, FRET and ratiometric imaging. Second, unlike linear microscopy-based setups such as confocal, this design has the practical advantages of multiphoton excitation: superior performance for deep-tissue imaging and increased detection efficiency due to the absence of overlap between excitation and emission spectra. Third, compared to other multiphoton strategies such as single-wavelength excitation of multiple chromophores or sequential illumination at three wavelengths, our scheme provides efficient and independent control over the strength of the three signals or reduced acquisition times, respectively. Besides the applications already discussed, this approach can also be used with a large range of fluorophore combinations such as Dendra2 Green, Dendra2 Red and mKate; DAPI, GFP and mCherry; and others (Supplementary Fig. 1). Finally, it can be adapted to other imaging geometries such as multiphoton light-sheet microscopy. Multicolor two-photon imaging with wavelength mixing should therefore find a broad range of applications in experimental systems biology.

Methods
Methods and any associated references are available in the online version of the paper.

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Competing financial interests
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References
ONLINE METHODS

Multicolor two-photon imaging setup. Imaging was performed on a custom-built laser scanning microscope incorporating a femtosecond Ti:S/OPO chain (Coherent and APE), orthogonal galvanometer mounted mirrors (GSI Lumonics) and a water-immersion objective (25× 1.05 numerical aperture (NA), Olympus). Ti:S and OPO beams were combined using a dichroic mirror (1000-DCXR, Chroma or DMSP-1000, ThorLabs). Pulses were synchronized with a motorized delay line (Supplementary Fig. 1), and the relative divergence of the two beams was controlled using telescopes. Beam polarizations were linear. Beam powers were independently controlled with wave plates and polarizers. Signals were detected in the epi- and trans-directions by photomultiplier modules (SensTech) counting electronics. Scanning and acquisition were synchronized using lab-written LabVIEW software and a multichannel I/O board (PCI-6115, National Instruments). Fluorescence was collected in the backward (epi) direction using a dichroic mirror (695dxr, Chroma) and directed toward three independent detectors with dichroics and filters, listed below. Coherent scattering (such as THG, SHG and SFG) was detected in the forward direction. We note that in addition to two-color 2PEF (2c-2PEF), other multiple-wave processes can happen in the sample. In particular, with femtosecond pulses, four-wave mixing (FWM) is efficiently produced at wavelength 1/(2/λ_{Ti:S}−1/λ_{OPO}) and, if present, should be separated from the red fluorescence. Since FWM is narrowband and mostly forward-scattered, fluorescence selection can generally be done using epidetection and appropriate filters. In addition, it is possible to red-shift the FWM signal away from the fluorescence wavelengths by choosing closer λ₁ and λ₂ wavelengths.

Laser chains and accessible two-photon excitation wavelengths. The wavelength combinations accessible using synchronized Ti:S/OPO pulses are illustrated in Supplementary Figure 2. We tested this excitation scheme with two different laser chains. The first chain (Ti:S1/OPO1) was the combination of a femtosecond oscillator (Chameleon-ultra2, Coherent) and a KTP-based optical parametric oscillator (OPO-Basic, APE). In this system, the OPO wavelength is controlled by the pump Ti:S wavelength and tunability is limited. OPO pulse duration was compressed to 80–100 fs at the objective focus using an external prism-based compressor, and Ti:S pulse duration at focus was approximately 250–280 fs. This configuration was used for simultaneous THG and three-channel 2PEF imaging of Drosophila embryos. The Ti:S1, OPO1 and two-color virtual excitation \( \lambda_3 = 2/(1/\lambda_1 + 1/\lambda_2) = 2/(1/\lambda_{Ti:S} + 1/\lambda_{OPO}) \) wavelengths were equal to 820 nm, 1,175 nm and 965 nm (Fig. 3 and Supplementary Videos 7, 9 and 10). The second chain (Ti:S2/OPO2) was the combination of a femtosecond oscillator (Chameleon-ultra2, Coherent) and an independently tunable periodically poled crystal-based OPO (OPO-automatic, APE). Pulse durations at the objective focus were approximately 160 fs and 400 fs, respectively. This configuration with tunable OPO wavelength was used for Brainbow-labeled mouse-brain and chick spinal cord samples as well as for Drosophila imaging. The Ti:S2, OPO2 and two-color virtual excitation wavelengths were respectively set to 850 nm, 1,100 nm and 959 nm for Brainbow-labeled tissue imaging, and to 820 nm, 1,100 nm and 930 nm for Drosophila imaging (Figs. 1–3 and Supplementary Videos 1–6 and 8).

Axial resolution. Beam magnifications were adjusted to the pupil size of the objective in order to obtain comparable resolutions for all channels. Resolutions were estimated from multiphoton imaging of fluorescent beads and KDP powder. Measured 2PEF FWHM z resolutions at the sample surface in the blue and red channels with the 25× 1.05NA objective were as follows: 2.5 μm and 3.0 μm for Drosophila experiments with Ti:S1 and OPO1 (820 nm/1,175 nm); 2.0 μm and 3.0 μm for Brainbow-labeled tissue experiments with Ti:S2 and OPO2 (850 nm/1,100 nm).

Experimental conditions for Brainbow imaging on fixed tissues (mCerulean, mEYFP and tdTomato/mCherry). Excitation: Ti:S2, OPO2 and two-color equivalent excitation \( \lambda_3 = 2/(1/\lambda_{Ti:S} + 1/\lambda_{OPO}) \) wavelengths were set to 850 nm, 1,100 nm and 959 nm, respectively (Supplementary Fig. 3a). Detection: we used FF520-Di02 and FF560-FD01 dichroics and FF01-475/64-25, FF01-538/40-25 and FF01-607/70-25 filters (Semrock) (Supplementary Fig. 3b). The pixel dwell time was 5 μs, and the voxel size was 0.60 × 0.60 × 1.0 μm³ (Fig. 1c), 0.48 × 0.48 × 1.0 μm³ (Fig. 2c,d,
Supplementary Figs. 6, 7 and 9c and Supplementary Videos 1–3) or $0.60 \times 0.60 \, \mu m^2$ (Supplementary Figs. 8 and 9a,b). Images were averaged two times for Figure 2c,d, Supplementary Figures 6, 7 and 9c and Supplementary Videos 1–3 and four times for Figure 1c. Laser power for both beams was automatically adjusted with depth.

Experimental conditions for dynamic Brainbow experiments (mCerulean, mEYFP and tdTomato/mCherry). Excitation and detection parameters were identical to those use for fixed Brainbow tissues imaging (Supplementary Fig. 3a,b). The pixel dwell time was 5 $\mu s$, and the voxel size was $0.80 \times 0.80 \times 2.0 \, \mu m^3$ (Fig. 3a and Supplementary Video 4) or $0.6 \times 0.6 \times 2.0 \, \mu m^3$ (Fig. 3b and Supplementary Videos 5 and 6). Laser power for both beams was automatically adjusted with depth, only for Figure 3a and Supplementary Video 4.

Experimental conditions for Drosophila embryo experiments (endogenous fluorescence, GFP, and RFP). Excitation: Ti:S1, OPO1 and two-color virtual excitation $\lambda_3 = 2/(1/\lambda_{TiS} + 1/\lambda_{OPO})$ wavelengths set to 820 nm, 1,175 nm, and 965 nm (Supplementary Fig. 3c) for Figure 3e,f, Supplementary Figure 12 and Supplementary Videos 7, 9 and 10, and to 820 nm, 1,100 nm and 930 nm (Supplementary Fig. 3e) for Supplementary Video 8. Detection: we used FF500-DI02 and FF560-DI01 dichroics, and FF01-447/55-25, FF01-525/50-25 and FF01-607/70-25 filters (Semrock, USA) (Supplementary Fig. 3d,f). The pixel dwell time was 5 $\mu s$, and the voxel size was $0.80 \times 0.80 \times 3.0 \, \mu m^3$ (Fig. 3e,f, Supplementary Fig. 12 and Supplementary Video 7), $0.80 \times 0.80 \times 1.5 \, \mu m^3$ (Supplementary Video 8) or $0.60 \times 0.60 \, \mu m^2$ (Supplementary Videos 9 and 10). Images were not averaged for Figure 3e,f, Supplementary Figure 12 or Supplementary Videos 7 and 8; images were averaged two times for Supplementary Videos 9 and 10.

Effect of spatial mismatch on two-color excitation. In the case of multiphoton microscopy, chromatic aberrations of the microscope and objective cause foci mismatches between the Ti:S and OPO beams, which must be avoided. The effect of wavelength-dependent aberrations can be described as follows: (i) at the center of the field of view, the two foci exhibit axial (z) mismatch; and (ii) toward the edge of the field of view, foci are mismatched both axially and laterally. The z mismatch at the center of the field was canceled by controlling the relative divergence of the beams using telescopes before injection inside the microscope. Then the extension of the field with overlapping foci was measured for different objectives by recording two-color 2PEF images of homogeneous fluorescent samples and SFG images of KDP powder. In practice, we found that two-thirds of the lateral field of view could be used with the 25× 1.05NA objectives for a second-order process with the pump and OPO wavelength set to 850 nm and 1,100 nm. Detection of the two-color process was a diagnostic of beam (and image) coalignment at the precision of the diffraction-limited foci (Supplementary Fig. 3).

Control of signal level and color balance with depth. Signal attenuation in thick tissues due to scattering of the excitation light (and fluorescence) is wavelength-dependent. This hinders quantitative colorimetric analysis (for example, for neuron tracing in 3D images). This differential signal attenuation was characterized and avoided as follows. First, blue, green-yellow and red 2PEF signal levels were adjusted at the tissue surface using the Ti:S and OPO powers and the delay between pulse trains. In the case of nonoverlapping excitation spectra, the three signals scale as: $I_{\text{blue}} \propto (P_{TiS})^2$, $I_{\text{red}} \propto (P_{OPO})^2$ and $I_{\text{green}} \propto 2P_{OPO} P_{TiS} \times g(t)$, where $P_{TiS}$ is the Ti:S power, $P_{OPO}$ is the OPO power, $t$ is the delay between the two pulse trains and $g(t)$ the temporal intensity intercorrelation of the pulse trains. In the case of Fourier transform–limited Gaussian temporal profiles, $g(t) \propto \exp(-t^2/2\sigma_3^2)$, where $2\sigma_3$ is related to the $1/e^2$ temporal width of the OPO and Ti:S pulses by the relation $\sigma = (\sigma_{OPO}^2 + \sigma_{TiS}^2)^{1/2}$. We note that if excitation spectra overlap so that two chromophores are excited by a single wavelength, the expressions above should be modified to take into account the excitation cross-talk. However, even in this case, the relative strength of the three signals can be adjusted independently using combinations of the experimental parameters $P_{TiS}$, $P_{OPO}$ and $t$. Signal attenuation with depth was characterized from low-resolution, large-scale tissue imaging (Supplementary Fig. 4a). Finally, this information was used in the microscope acquisition program to automatically adjust the powers of the two beams according to depth. This resulted in a constant balance between the different colors at all depths (Supplementary Figs. 4b and 10).

Image analysis. Image analysis was performed using ImageJ (US National Institutes of Health), Fiji (http://fiji.sc/), TrakEM2220 MATLAB (MathWorks) and Imaris (Bitplane).

Color balance correction across the field of view. Excitation and detection efficiency usually depends on the position in the field of view and may differ by channel. This must be corrected for in the case of colorimetric analysis. For Brainbow-labeled tissue-imaging applications, we normalized the images using images of homogeneous media (fluorescent plastic slides, Supplementary Fig. 8a,c). Supplementary Figure 8d,e shows an example of an image before and after correction.
**Multicolor mosaic stitching.** Mosaic stitching was performed using MATLAB or Fiji after color-balance correction. xy translation between images was evaluated using single z slices, or maximum-intensity projections when possible. **Supplementary Figure 9** and **Supplementary Videos 1** and **2** show examples of stitched images.

**Automated high-content cell detection and color analysis in large volumes of Brainbow tissue.** Analyses were carried out on a mosaic of Brainbow-labeled mouse cerebral cortex consisting of six z stacks encompassing a total volume of $900 \times 720 \times 370$ μm$^3$ (**Supplementary Video 1**). We ensured a constant color balance in the z and xy directions by adjusting laser powers with depth (**Supplementary Fig. 4**) and by lateral renormalization (**Supplementary Fig. 8**), respectively. We used Imaris to automatically detect >3,000 cells present in the imaged volume (**Fig. 2** and **Supplementary Fig. 10**) and extract the average cell-body signal intensity in each channel. Manual inspection indicated that there were 25% false negatives (labeled cells undetected by the algorithm), <5% false positives and that the detection efficiency did not depend on depth. These data were exported into MATLAB to measure the relative color intensities for each cell, as displayed in ternary graphs (**Fig. 2e** and **Supplementary Fig. 10**).

**Image segmentation.** A densely labeled volume (**Supplementary Fig. 11a**) was selected from the image stacks shown in **Supplementary Video 2**. After cropping and uniform level adjustment with Adobe Photoshop, the corresponding stack of 171 RGB TIFF images was loaded into TrakEM2 4 (available as a Fiji plug-in, http://www.ini.uzh.ch/~acardona/trakem2.html). Neurites were traced in each plane using the TrakEM2 Arealist mode. The area list of each segmented neuron was then exported as an image series so the neuron could be color-coded in Adobe Photoshop with a hue matching that in the parent image. In Fiji, traces of all segmented neurons were combined into a unique image series. The resulting segmented image stack was then rendered using the Fiji/ImageJ 3D viewer plug-in, and interpolation along the z axis was performed to allow isotropic rendering (**Supplementary Fig. 11b** and **Supplementary Video 3**).

**Linear spectral unmixing.** We perform linear spectral unmixing for the three-color *Drosophila* imaging experiments. Mixing was only observed between spectrally adjacent channels. To unmix the contributions of two chromophores A and B detected in channels C1 and C2, one solves the following linear equations:

$$\begin{pmatrix} C_1 \\ C_2 \end{pmatrix} = \begin{pmatrix} R_{A1} & R_{B1} \\ R_{A2} & R_{B2} \end{pmatrix} \begin{pmatrix} A \\ B \end{pmatrix}$$

where RA1 (and RA2) and RB1 (and RB2) are the normalized contributions of chromophores A and B in channel 1 (and 2), respectively. **Supplementary Figure 12** shows an example in the case of three-color imaging of a *Drosophila* embryo labeled with GFP and RFP. Mixing ratios between the green and red channels were estimated from images of GFP and GFP-RFP-labeled embryos. Mixing ratios between the blue and green channels were estimated from images of GFP-RFP-labeled embryos.

**Fluorescent HEK cells.** HEK 293 cells expressing a single color of fluorescent protein (mCerulean, EYFP or tdTomato) were separately generated by transient transfection with a CMV promoter-driven expression vector derived from pEGFP-N1 (Clontech) using Lipofectamine2000 reagent (Invitrogen). Two days after transfection, the 3 types of cells were harvested, mixed in equal proportions and plated on collagen-coated glass coverslips (50 μg ml$^{-1}$ in 10 mM HCl, Sigma C3867) at a density of 5.10$^4$ cells cm$^{-2}$. Cells were maintained for an additional day in culture prior to fixation with 4% Paraformaldehyde, and mounting in Vectashield medium (Vector Labs).

**Brainbow labeling.** Mouse brains and chicken spinal cords imaged in Figures 2 and 3 were produced by electroporating novel Brainbow transgenes expressing mCerulean$^{21}$, mEYFP$^{22}$ and tdTomato and/or mCherry$^{23}$ in the mouse embryonic cortical neuroepithelium or chicken spinal cord. The procedure and associated transgenes for creating semi-sparse trichromatic labeling of nerve cells will be described in a separate article. Mouse brains imaged in **Supplementary Figures 5** and **6** were produced as detailed in ref. 24 from Thy1-Brainbow-1.0 line L5, which expresses mCerulean$^{23}$, mEYFP and dTomato$^{23}$. Briefly, Brainbow mice were intercrossed with CAGGS-CreER animals$^{25}$, and newborn pups were injected with 20–50 μg Tamoxifen (Sigma T-5648). Mice were housed in a 14 h light/10 h dark cycle with free access to food, and all animal procedures were carried out in accordance with institutional guidelines (UPMC and Inserm).

**Tissue preparation.** For fixed brain imaging, 20-d-old mice were deeply anesthetized and perfused with 4% paraformaldehyde. Brains were harvested, postfixed overnight with the same fixative at 4 °C and washed in PBS. Sections 200–500 μm thick were cut using a vibrating microtome (Leica VT1000S) and mounted in Vectashield (Vector Labs) before imaging. For live imaging experiments, chicken embryos electroporated at E2 as described in
ref. 26 were harvested at E3 (Fig. 3a and Supplementary Video 4) or E4 (Fig. 3b,c and Supplementary Video 5 and 6) and dissected in PBS. The electroporated portions of the thoracic and lumbar regions were cut into 200- to 250-μm-thick slices with a McIlwain tissue chopper. Slices were mounted in 60–100 μL DMEM/F12 medium with 1% low melting point agarose in 35-mm glass-bottom culture dishes (MatTek) covered with 3 mL DMEM/F12 medium supplemented with 4% FCS, 1 mM sodium pyruvate, and 1× penicillin or streptomycin; they were maintained at 38 °C for 3–6 h before imaging.

**Drosophila embryos.** For fly imaging experiments, we used a transgenic line of *Drosophila melanogaster* exhibiting a ubiquitous expression of red (mRFP1) and green (EGFP) fluorescent proteins (gift from A. McMahon and A. Stathopoulos at the California Institute of Technology). In this line, the red labeling of nuclei (H2A‐RFP) is obtained with a fusion protein between histone 2A and the red fluorescent protein (such as that in Bloomington stock center strain #23650, http://flystocks.bio.indiana.edu/Reports/23650.html). In addition, a fusion protein between mouse lymphocyte marker mCD8 and the green fluorescent protein (mCD8-GFP) labels cell membranes27. Embryos from H2A-RFP/mCD8-GFP flies were collected, staged, dechorionated and glued on glass cover slips following standard procedure, as described in ref. 28. Gastrulating embryos were imaged in water at 21 °C.

**References:**