Universal autofocus for quantitative volumetric microscopy of whole mouse brains

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Abstract

Unbiased quantitative analysis of macroscopic biological samples demands fast imaging systems capable of maintaining high resolution across large volumes. Here, we introduce RAPID (Rapid Autofocusing via Pupil-split Image phase Detection), a real time autofocus applicable in every wide-field-based microscope. RAPID-enabled light-sheet microscopy reliably reconstructs entire mouse brains with subcellular resolution, and allowed us to characterize the 3D spatial clustering of somatostatin-positive neurons in the whole encephalon, including densely labelled areas. It further enabled 3D morphological analysis of microglia cells across the entire brain. Beyond light-sheet microscopy, we demonstrate that RAPID maintains high image quality in various settings, from in vivo fluorescence imaging to 3D tracking of fast-moving organisms. RAPID thus provide a flexible autofocus solution, suitable for traditional automated microscopy tasks as well as for unprecedented quantitative analysis of large biological specimens.

Introduction

Light-sheet microscopy (LSM) is widely used for fast imaging of large, clarified specimens, such as entire mouse brains[1] Fluorescent staining of these samples is typically achieved with whole-mount immunohistochemistry[2] (IHC), injection of viruses or tracers[3], or with transgenic strategies[4]. Despite the wide variety of labeling methods, quantitative whole-brain analysis with LSM has been achieved only using either sparse viral labeling[5], or whole-mount staining restricted to the cell body[2,6] which facilitates cell detection even at low resolution (a few microns per pixel). The quantitative application of LSM to dense staining, filling also small structures like those present in many transgenic models and in IHC against several important proteins (e.g. parvalbumin), has not been reported on a brain-wide scale because of the inability to maintain sub-cellular resolution consistently across the entire sample. Although the microscope *per se* affords sub-cellular resolution, the presence of a macroscopic sample introduces optical aberrations, mainly defocus[7]. In low-resolution settings, the detection depth of field is large enough to tolerate even tens of microns of defocus. However, in high-resolution LSM implementations with sub-micron sampling, necessary to disentangle cell bodies in crowded environments and resolve fine neuronal processes, defocus can compromise the coincidence of the light sheet and the focal plane of the detection objective, frustrating the very principle of LSM, and introducing severe blur in the collected images.

Defocus in LSM has been previously tackled with optimization methods, which search for the best focus while suspending data collection, significantly reducing imaging throughput[7–10] (Supplementary Note 1). Online optimization approaches have been recently reported, but their effectiveness has been demonstrated only in samples stained against cell nuclei[11]. Autofocusing methods compatible with real-time correction are well established in the general field of microscopy[12], however they require reflective surfaces such as coverslips and are therefore not suitable for LSM (Supplementary Fig. 1, Supplementary Note 2). Here, we introduce RAPID (Rapid Autofocus via Pupil-split Image phase Detection), a method for real-time image-based focus stabilization that, unlike contrast optimization methods, is agnostic with respect to image content.

Results

RAPID operating principle

RAPID is based on phase detection[13], an optical principle which exploits the fact that rays passing through distinct portions of the objective pupil intersect the image plane at different lateral positions when the object is defocused (Fig. 1a). In RAPID, the two ray bundles are physically separated using a wedge plate in a conjugated Fourier plane, and the resulting images are collected with an auxiliary camera (Fig. 1b). When the focus varies, the two images are not only blurred but also laterally displaced (Fig. 1c, Supplementary Video 1). This mutual displacement (the 'phase') is directly proportional to the focal state of the microscope (Fig. 1d, Extended Data Figs. 1-3) and thus provides a direct feedback for focus stabilization. The quantitative relation between the lateral motion of RAPID images and defocus can be obtained by considering the two ray bundles originated from the two halves of the pupil (Supplementary Fig. 2) separately. Depending on the amount of defocus Δf, the lateral displacement of the center of mass of ray bundles (with respect to the central ray) is given by:

$$\Delta x_{\pm} = \pm\, G \cdot \mathrm{NA} \cdot M_{RAPID} \cdot \Delta f \qquad (1)$$

where ± refers to the two different halves, NA is the numerical aperture of the microscope objective used, M_{RAPID} is the effective magnification in the image space of the RAPID system, and *G* is a geometric factor that is dependent on the shape of the pupil portion used and on the light distribution in the pupil. In the case of two perfect halves of a uniformly filled circular pupil, *G* is given by $4/3\pi$ (the center of mass of half a unity circle); in general, *G* is of the order of unity. It follows from Eq. (1), that the mutual distance *d* between the centers of mass of the two ray bundles is linearly dependent on defocus:

$$d = d_0 + a \cdot \Delta f \qquad (2)$$

d_0 being the in-focus mutual distance and with $a = 2 \cdot G \cdot \mathrm{NA} \cdot M_{RAPID}$.

The above analysis has been derived assuming a single point source. However, since modern microscopes are usually telecentric, such analysis is valid for all the points in the field of view (and in particular *G* is constant throughout the field of view). Therefore, Eq. (2) also provides the mutual displacement of the two bidimensional images produced onto the RAPID auxiliary camera. As far as the system is perfectly

telecentric and spatially homogeneous (i.e. neglecting vignetting and distortion), the mutual displacement between the two images is perfectly rigid (Fig. 1c and Supplementary Video 1).

Closed-loop operation of RAPID is achieved in parallel to image acquisition by inserting a beam splitter and a motion actuator controlling the detection objective (Fig. 1b). The feedback loop can be closed in a few hundreds of milliseconds, depending on the size of the auxiliary images (Supplementary Fig. 3). For sample-induced focus changes that vary more slowly than this refresh rate, RAPID effectively provides real-time focus stabilization (Supplementary Note 3). An additional guide on the choice of components for the implementation of RAPID in a given microscope can be found in Supplementary Note 4.

We experimentally verified the relation between d and Δf, obtaining d by means of cross-correlation (see Supplementary Methods, Supplementary Fig. 5). Lateral shift between pupil-split images was found to be linearly dependent on defocus in a variety of illumination conditions, objective magnifications and NAs (Fig. 1d, Extended Data Figs. 1-3). Exploiting this linear behavior, it is possible to infer the focal state of the system from d by inverting eq. (2) and use this information to keep the system focused using a simple feedback loop. We observed reliable focus discrimination over a range 70 times larger than the objective depth of focus (Extended Data Fig. 3, Supplementary Note 5). The measured focus discrimination accuracy was approximately 70% of the depth of focus (104% in the case of LSM) and can theoretically be reduced further (Supplementary Note 5 and Supplementary Table 1).

RAPID enables effective focus stabilization in light-sheet microscopy of cleared mouse brains

We demonstrate the capabilities of RAPID in a high-resolution light-sheet microscope designed for clarified mouse brains, finding that a feedback rate of about 1 Hz is sufficient to achieve proper autofocusing (Supplementary Note 3). RAPID can effectively correct defocus across different tiles (Fig. 1e, Extended Data Fig. 4), allowing the detection of small neuronal processes that are not visible without autofocus (Fig. 1f). In general, RAPID leads to a substantial increase in image contrast (18.5% ± 0.2%, 18000 images, $p < 0.001$, Student one-sample t-test) which exceeds 50% in 4% of the images (Fig. 1g). Further, RAPID stabilizes focus also along stack depth and with different staining and image content, including vasculature (Fig. 1h-i, Supplementary Video 2) and nuclei (Fig. 1j). In the latter, due to the globular shape of the labeled structures,

we can also quantify the resolution enhancement through the support radius of the optical transfer function[8], finding a significant increase in resolution in out-of-focus areas (26% ± 4%, 100 images, $p < 0.001$, Student one-sample t-test).

RAPID is fully compatible with a variety of clearing methods, including CLARITY (Fig. 1e), uDISCO (Extended Data Fig. 5) and PEGASOS (Extended Data Fig. 6), in line with the fact that the phase-detection principle is independent of image content or sample details. Further, as long as the microscope itself is achromatic, autofocus performances are independent of the wavelength used, making RAPID suited also for multi-color imaging (Extended Data Fig. 7, Supplementary Note 6).

RAPID-enabled LSM allows brain-wide quantitative analysis of 3D cell distribution

The superior contrast and resolution provided by RAPID enable unprecedented quantitative studies in large clarified samples. Here, we report for the first time a complete analysis of the 3D spatial clustering of somatostatin-positive (SST+) neurons, a geometrical feature with profound functional implications[14,15]. As a first step, we analyzed the spatial distribution of transgenically labeled SST+ neurons across the entire mouse brain (Fig. 2a, Supplementary Video 3). The sharp, high-resolution images afforded by RAPID-enabled LSM allows reliable automatic localization of neurons (Fig. 2b,c), guaranteeing in all brain regions an accuracy comparable or superior to the concordance between different human annotators (Fig. 2d). The same annotators failed to label neurons manually in crowded areas if the resolution was artificially lowered (Supplementary Figs. 6,7). Remarkably, previous LSM implementations failed to provide high-quality images amenable of automated detection of densely labeled cells across all brain regions[16]. Further, localization of neurons in absence of defocus correction fails to detect a large number of cells (Extended Data Fig. 8). In contrast, in the datasets collected with RAPID, we were able to produce a point-cloud representation of all the SST+ neurons in a mouse brain (Fig. 2e). This analysis, besides providing regional counts of this kind of cells (Fig. 2h), allowed computing the local 3D density of cells at the position of each single neuron, being able to appreciate large variations not only between different areas but also within single regions (Fig. 2f,i, Extended Data Fig. 9). Finally, we exploited local density to estimate a clustering index based on the 3D Ripley's *K*-function[17] (see Methods). This analysis shows large variability in local 3D clustering tendency across the entire brain

(Fig. 2g,j), without any apparent correlation between local density and clustering (Fig. 2k, Supplementary Fig. 8). The patchwork-like distribution of cells with higher 3D clustering index indicates that spatial clusters of SST+ neurons are present across the whole encephalon, with large clusters clearly visible in the cerebellum and in the olfactory bulbs (Fig. 2g, Supplementary Fig. 9). Interestingly, clustering tendency is found to be higher in deep brain areas rather than in the isocortex, suggesting potential correlations between the level of spatial clustering of SST+ interneurons and brain functions. Since RAPID-enabled LSM allows reliable cell localization even in densely labeled samples, this kind of analysis can be scaled to other neuronal populations and developmental stages, providing a comprehensive yet detailed view of mouse brain cytoarchitecture, and complementing cell counting information provided by serial sectioning methods[18].

RAPID-enabled LSM allows morphological analysis of microglia across multiple brain areas

In addition to studies on the spatial distribution of neuronal populations, the subcellular resolution enabled by RAPID allows quantitative analysis of cell morphologies on a brain-wide scale. As an example, we studied the morphology of microglia cells across the entire mouse brain (Fig. 3, Supplementary Video 4). This is an important task in neurobiology as microglial shape is known to change according to cellular state and function[19]. Thanks to the whole-brain sub-cellular resolution afforded by RAPID-enabled LSM, we were able to segment cell bodies and processes in various brain areas (Fig. 3b,c, Extended Data Fig. 10, Supplementary Fig. 10). Notably, resolution and image quality of RAPID-enabled LSM were sufficiently good to perform this analysis by simple feature-based machine learning in ilastik, without the development of specialized deep learning pipelines and extensive manual labeling. Quantitative analysis of the shape of the soma of microglia cells highlights different distributions of cell body volume and ellipticity in different brain areas (Fig. 3d-g). For instance, we observed larger and more elliptical cells in the hippocampus than in the cortex, consistent with previous observations[20]. Together, our brain-wide morphological analysis supports the hypothesis that selected brain areas are characterized by region-specific microglia phenotypes[19].

RAPID applications beyond LSM

The real-time image-based focus stabilization of RAPID is by no means limited to LSM but is instead universally suited to all wide-field microscopy methods and applications (Supplementary Note 7). We demonstrated RAPID operation in whole-slide histological imaging of atherosclerotic human carotid human keloid under bright-field illumination (Fig. 4a-c). Long-term stability of focus stabilization was assessed for over 12 h by imaging living yeast cells under either bright-field or epi-fluorescence illumination (Fig. 4d,e, Supplementary Videos 7,8). Finally, high-speed defocus correction was demonstrated by imaging living *C. elegans* moving in 3D at speed as high as 400 µm/s (Fig. 4f-h, Supplementary Fig. 11, Supplementary Video 9). Notably, in this last experiment no autofocus method based on triangulation or on contrast maximization could have been employed (Supplementary Note 7).

Discussion

Here we presented RAPID, an image-based autofocus method that runs concurrent to data acquisition. Being agnostic to image content, this method affords robust and effective focus stabilization regardless of the labeling strategy. We exploited RAPID in LSM to image entire murine brains treated with various clearing methods, based on either tissue transformation or organic solvents, and with multiple staining methods including nuclear and vascular staining and dense labeling of neurons or microglia. Beyond LSM, we successfully applied RAPID to bright-field and epi-fluorescence microscopy. We successfully tested a wide range of NAs (from 0.3 to 1.4, Supplementary Table 2), and imaging scenarios with signal-to-noise and signal-to-background ratios as low as 4.53 ± 0.07 and 1.66 ± 0.05, respectively (mean ± s.e.m., N = 10 images, n = 5 measurements per image, Supplementary Note 7). Overall, our results show the universal applicability of RAPID to stabilize focus in microscopy techniques based on wide-field detection most prominently amongst those LSM.

In our experiments, the RAPID refresh rate was always capable of following defocus changes in the analyzed specimens, providing effective real-time autofocusing (Supplementary Note 3). In more demanding situations, faster operation can be achieved by reducing the size of the phase comparison images, or by

exploiting faster devices to change focus, like electrically tunable lenses[9] or remote refocusing with voice coil devices[21].

The pupil-split approach we propose holds the promise for fast correction of higher-order aberrations. Indeed, by dividing the pupil in more than two sectors, it would be possible to implement pupil segmentation adaptive optics[22] in a parallel rather than serial fashion, significantly speeding up wavefront detection if sufficient computational power is available to calculate cross-correlations between all the images obtained from the different pupil segments.

Since defocus is responsible for most of image degradation[23], its correction is critical but importantly also sufficient to enable quantitative and comprehensive analysis of entire murine brains at sub-cellular resolution using LSM. RAPID unlocks the full potential of this microscopy technique – especially of novel implementations providing improved axial resolution[24] –, leaving the user complete choice of the labeling strategy according to experimental or financial demands. RAPID uses only off-the-shelf optical components and well-established image registration algorithms, and can be easily implemented even by non-experts in optics (Supplementary Methods). Given the simplicity of the method and its universal applicability, we anticipate that RAPID will have a significant impact in the neurosciences and in the entire microscopy community.

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Author contributions statement

L. Sil. devised RAPID; L. Sil., L. Sac. and F.S.P. designed the experiments; L. Sil. and M.C.M. implemented RAPID; I.C. prepared the yeast and nematode samples; A.P.D.G. and I.C. prepared the cleared mouse brains; L. Sil., I.C. and A.P.D.G. performed the experiments; L. Sil. and M.C.M. analyzed data; L. Sil., L. O. T., A. F. and I. C. manually annotated ground truth data for cell localization; C. C., L. O. T., G. M., and P. F. analyzed whole-brain SST+ datasets, performed stitching and cell localization; D. K. and A. K. analyzed microglia morphology data; L. Sil. and M.C.M. wrote the paper with contributions from all the Authors.

Competing interests statement

L. Sil., M. C. M., L. Sac., F. S. P. are inventors of patent IT201600132604A1, which is related to RAPID.

Figure Legends

Figure 1. RAPID autofocusing in high-resolution light-sheet microscopy. (a) Lateral motion of the center of mass of rays passing through different pupil portions, depicted in red and green. (b) Implementation of RAPID in a standard light-sheet microscope. (c) Lateral shift of pupil-split images. (d) Experimental shift plotted as a function of defocus, together with a linear fit. (e) A virtual slab (500 μm thick) from the brain of a thy1-GFP-M transgenic mouse, with RAPID defocus correction across different tiles (insets). (f) Intensity profiles along the dashed lines. Gray regions highlight fine sample details lost without autofocus. (g) Histogram of contrast enhancement for all the images forming the slab in (e). Red arrowheads highlight positive outliers, whereas the inset shows the cumulative density function (CDF). (h) 3D rendering of an image stack from a vasculature-stained mouse brain showing insets at different depths. (i) RAPID contrast enhancement as a function of depth for the stack in (h). (j) 3D rendering of an image stack from a mouse brain with nuclear staining. The constant shape of the nuclei allows evaluation of resolution enhancement with RAPID looking at the radius of the Fourier transforms (insets, middle line). Scale bars: 1 mm (e), 20 μm (insets).

Figure 2. Whole-brain quantitative analysis of cell distribution. (a) Sagittal maximum intensity projection of a mouse brain where SST+ neurons express tdTomato. (b,c) zoom-in renderings from superior colliculus and olfactory bulb, respectively. Colored dots representing the position of localized neurons are superimposed to the grayscale image. Scale bar, 100 μm. (d) Performances obtained by the cell detection algorithm in different brain areas, compared to inter-human variability in manual annotations. (e-g) Sagittal views of the point cloud of SST+ neurons across the entire mouse brains, colored according to brain region (e), local cell density (f) or 3D clustering (g). Total cell counts across the different areas are reported, together with their uncertainty (see Methods) in (h). The distributions of local cell density and of 3D clustering index are reported in (i) and (j), respectively (n = 1'567'553 cells). Data are presented as box plots with minima at 5 percentile, maxima at 95 percentile, centre at 50 percentile, and bounds of box at 25 and 75 percentile, respectively. Scatter plot of the average cell density vs the average 3D clustering index for different regions is reported in (k), highlighting significant differences between various brain areas. CB is cerebellum, CN cerebral nuclei, CS cortical subplate, HB hindbrain, HF hippocampal formation, HT hypothalamus, IC isocortex, MB midbrain, OA olfactory areas, TH thalamus. Scale bars: 1 mm (a), 20 μm (b,c).

Figure 3. Microglia shape analysis in multiple brain areas. (a) Transversal maximum intensity projection of a mouse brain where microglia cells express GFP. (b) left: 3D rendering of a substack extracted from the somatosensory cortex; right: zoom-ins from a single cell taken at the position of the yellow arrowhead, showing raw imaging data (top) and results of segmentation (bottom). (c) same as (b), but for a substack extracted from CA1. (d) Cell densities in different brain regions (mean +/- s.d. n = 8 subvolumes; data points report densities measured in single subvolumes). Distribution of cell volume and ellipticity (see Methods) in the same areas are reported in (e) and (f), respectively (n = 3'685 cells). Data are presented as box plots with minima at 5 percentile, maxima at 95 percentile, centre at 50 percentile, and bounds of box at 25 and 75 percentile, respectively. (g) Scatter plot of the average cell volume vs the average ellipticity for the same regions, highlighting significant differences between various brain areas. In all the box plots: rectangles enclose 25 to 75 percentiles, with the median indicated by the central line; external lines indicate 5 to 95 percentiles. Scale bars: 1 mm (a), 50 μm for the renderings in (b,c), 20 μm in the insets.

Figure 4. **RAPID applications beyond light-sheet microscopy.** (a) RAPID application in whole-slide histological imaging of atherosclerotic human carotid. (b) Insets corresponding to the yellow and blue arrows depicted in (a). (c) Defocus map showing large defocus variability across the slide. (d,e) Images of cultured yeast cells taken at different time points show long-term focus stabilization with RAPID in bright-field and epifluorescence microscopy, respectively. (e) RAPID autofocusing in imaging of fast-moving nematodes; the displayed images were acquired at different XY (planar) positions (in mm). (g) High-frequency (HF) content of the images as a function of time. (h) RAPID-enabled 3D tracking of the worm. All experiments shown in the figure were successfully replicated on three different samples, only a representative one per type is reported here. Scale bars: 1 mm (a), 10 µm (b,c), 1 µm (d,e), 100 µm (f).

Methods

RAPID implementation

In our setup, a 90:10 (transmission : reflection) beam splitter was placed in the infinity-corrected space behind the microscope objective in all experiments except the live imaging of fluorescent yeasts, where a 50:50 beam splitter was used. Light reflected from the beam splitter was sent to a 4f system to create an image of the objective back aperture. The magnification of this 4f system was 200:150 for the bright-field (BF) and epi-fluorescence (EF) experiments and 75:200 for the light-sheet experiments. In the secondary pupil plane created by the 4f system, a wedge plate (BSF2550-SIDES-A-SP, Thorlabs, Newton, NJ) was used to spatially separate the two portions of the pupil. A third lens (f = 100 mm for all experiments) was used to create two images of the microscope field of view onto an auxiliary camera, which was Retiga SRV (QImaging, Surrey, BC, Canada) for BF and EF, and Cascade II:512 (Photometrics, Tucson, AZ) for light-sheet experiments. A field stop was placed in the intermediate plane of the 4f system, an image plane of the microscope, to avoid

superposition of the two pupil-split images. Field stop size for each experiment are reported in Supplementary Table 2.

The images formed onto two pre-defined portions of the auxiliary camera were mutually aligned by determining the cross-correlation peak. Quality checks of the images and alignment, as well as several image pre-processing strategies were employed to maximize the accuracy and reliability of the system (see Supplementary Methods). The mutual displacement between the pupil-split images was fed to a proportional-integrative feedback loop executed in LabVIEW 2012 (National Instruments, Austin, TX) to correct the objective position. The RAPID software is freely available from https://github.com/lens-biophotonics/RAPID-AF. The hardware and software parameters used in the various experiments presented in this paper are summarized in Supplementary Table 2.

Animals

For this study, we used male mice of 8-12 weeks age from Jackson Laboratory, Bar Harbor, ME. Staining of nuclei and of blood vessels was performed on C57B6J mice. Transgenic lines used were thy1-GFP-M (JAX stock #007788) where a random subset of neurons express GFP, B6N.Cg-*Sst*$^{tm2.1(cre)Zjh}$/J × B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (JAX stocks #018973 and #007909, respectively) where SST+ cells express tdTomato, B6.129(Cg)-Fos$^{tm1.1(cre/ERT2)Luo}$/J × B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (JAX stocks #021882 and #007909, respectively) where neurons expressing c-fos in a given time window are labeled with tdTomato. To achieve expression of tdTomato in the latter line, 50 mg/Kg of 4-hydroxytamoxifene dissolved in corn oil were injected at least 7 days before euthanasia, following the protocol by Guenthner et al.[25].

Brains of B6.129P2(Cg)-*Cx3cr1*^{tm1Litt}/J mice (JAX stock #005582), expressing GFP in microglia, were kindly provided by Dr. Federico Del Gallo, University of Verona, Italy. All the experimental protocols were designed in accordance with Italian laws and were approved by the Italian Ministry of Health (authorization n. 790/2016-PR). Mice were housed at 22 ± 2 °C, with 55% ± 5% relative humidity, under a 12 h light/dark cycle and were given ad libitum access to water and food.

Mouse brain clearing and staining

CLARITY[26] was used as the clearing procedure. Briefly, animals were deeply anesthetized isoflurane (1.5%–2%) and then transcardially perfused with 50 mL of ice-cold 0.01 M phosphate buffered saline (PBS) solution (pH 7.6), followed by 75 mL of freshly prepared paraformaldehyde (PFA) 4% (wt/vol, pH 7.6). Brains were subsequently extracted and post-fixed in PFA 4% overnight at 4 °C. The day after, samples were incubated in the hydrogel solution (4% w/v acrylamide, 0.05% w/v bis-acrylamide, and 0.25% w/v VA044 in PBS) at 4 °C for three days. The brains were then degassed in nitrogen atmosphere and incubated at 37 °C to initiate polymerization. The embedded samples were extracted from the gel and incubated in clearing solution (sodium borate buffer 200 mM, sodium dodecyl sulfate 4% w/v, and pH 8.5) at 37 °C with gentle shaking for one month. Before imaging, CLARITY-treated samples were optically cleared using successive incubations in 50 ml of 2,2'-thiodiethanol (30% and 63% v/v) in 0.01 M PBS (TDE/PBS)[27], each for one day, at 37 °C while gently shaking.

For whole-brain nuclei staining, the CLARITY-processed murine samples were incubated at 37 °C for two days with a 1:50 propidium iodide (P3566, LifeTechnologies, Carlsbad, CA) solution in $PBST_{0.1}$, followed by washing in a $PBST_{0.1}$ solution at 37 °C for one day. Subsequently, they were optically cleared with 63% TDE/PBS before imaging with a light-sheet microscope.

Blood vessels were stained by perfusion with a fluorescent gel as described previously[28]. Mice were euthanized by overdoses of anesthetic (isoflurane) and then transcardially perfused first with 30 ml of a 0.01 M PBS solution (pH 7.6) and then with 60 ml of 4% w/v paraformaldehyde (PFA) in PBS. This was followed by perfusion with 10 ml of a fluorescent gel perfusate containing 0.05% tetramethylrhodamine-conjugated albumin (A23016, Thermo Fisher Scientific, Waltham, MA) as a fluorescent marker. Mice bodies were submerged in ice water, with the heart clamped, to rapidly cool and solidify the gel. Brains were extracted after 30 min of cooling and were incubated overnight in a solution of 4% w/v PFA in PBS at 4 °C. On the next day, brains were rinsed three times with PBS. The fixed brains were incubated in a hydrogel solution for 5 days, followed by degassing and hydrogel polymerization at 37 °C. Subsequently, they were incubated in a clearing solution at 37 °C with gentle shaking for one month. Finally, brains were cleared in 63% TDE for imaging.

Clearing with the uDISCO and PEGASOS was performed following the original protocols[29,30]. Briefly, for uDISCO the PFA-fixed brains were dehydrated by incubation in *tert*-butanol (Sigma, 360538) / water mixtures at rising concentration: 30%, 50%, 70%, 80%, 90%, 96%, 100%, 12 hours each at 35 °C. Samples were than delipidated by incubation in Dichloromethane (Sigma, 270997) for 1 hour, and finally cleared by incubation in 10:1 mixture of BABB (Benzyl Alcohol + Benzyl Benzoate 1:2, Sigma, 24122 and Sigma, W213802, respectively) and Dyphenyl Ether (Alfa Aesar, A15791), with the addition of 0.4% vitamin E (Alfa Aesar, A17039). For PEGASOS, PFA-fixed brains were decolorized in 25% Quadrol (Sigma, 122262) / water mixture for 2 days, then incubated in 30%, 50% and 70% *tert*-butanol / water mixtures with the addition of 3% Quadrol (4 hours, 6 hours, 1 day respectively). Then, samples were additionally dehydrated by 2-days incubation in 70% *tert*-butanol, 27% PEG methacrylate Mn 500 (PEGMMA500) (Sigma, 409529) and 3% Quadrol. Finally, clearing was achieved by immersion in 75% Benzyl Benzoate and 25% PEGMMA500, with the addition of 3% Quadrol. All steps were performed at 37 °C.

Light-sheet microscopy

The custom-made light-sheet microscope used in the experiments has been described in detail by Müllenbroich and colleagues[31]. Briefly, the sample was illuminated from the side using a virtual light sheet created with a galvo scanner (6220H, Cambridge Technology, Bedford, MA), which was coupled via a 4f system to an air objective (Plan Fluor EPI 10X NA 0.3, Nikon) covered with a protective coverslip. Light emitted from the specimen was detected orthogonally to the illumination plane using an immersion objective corrected for clearing solutions (XLPLN10XSVMP 10X NA 0.6, Olympus, Tokyo, Japan). Then, it was bandpass-filtered to isolate fluorescence light and projected by a tube lens onto the chip of a sCMOS camera (Orca Flash 2.0, Hamamatsu) operating in rolling-shutter mode to guarantee confocal line detection. During imaging, the sample was fixed in a refractive-index-matched quartz cuvette (3/Q/15/TW, Starna Scientific, Hainault, United Kingdom) and moved using a set of high-accuracy linear translators (M-122.2DD, Physik Instrumente, Karlsruhe, Germany). Defocus correction was implemented by moving the objective with an additional identical linear translation stage. The entire system was controlled by custom software written in

LabVIEW 2012 using the Murmex library (Distrio, Amsterdam, The Netherlands). The software can be freely downloaded from https://github.com/ludovicosilvestri/RAPID_CLSM.

Image analysis

Tiles from whole-slide imaging were stitched together using the FIJI Grid/Collection stitching plugin[32] (https://fiji.sc). FIJI was also used to produce the images and videos. Three-dimensional rendering was performed with Amira 5.0 (FEI Visualization Sciences Group, Bordeaux, France). The high-frequency content of the nematode time-lapse images was evaluated using a MATLAB R2016 script (MathWorks, Natick, MA). Tiled images acquired with LSM were stitched together using ZetaStitcher (https://github.com/lens-biophotonics/ZetaStitcher). Besides generating a low-resolution view of the entire imaging volume, this software includes an API (VirtualFusedVolume) to programmatically access the high-resolution volume. The image contrast of the original images was evaluated using the discrete cosine transform entropy method[8] and was implemented using MATLAB R2016. Image resolution increase was evaluated by calculating the support radius in Fourier space of the Optical Transfer Function (OTF) [8]. To this aim, the radial average of the OTF was calculated for each image, and the support radius was defined as the one where this average went below a threshold, chosen as 1 log unit above the minimum plateau.

Whole-brain cell detection

Fluorescently labeled neurons were localized in the whole-brain images using a modified version of BrainCell Finder[33]. Briefly, patches of the original dataset (accessed via VirtualFusedVolume), were fed into a UNet with 4 contraction layers of 3D convolutions with exponentially increasing number of filters and 4 expansion layers of transposed 3D convolutions with decreasing number of filters. UNet training was carried out with binary cross-entropy loss and Adam optimizer. The goal of this network is to perform ‘semantic deconvolution’, i.e. to transform the original image into an ideal one where cell bodies are clearly visible, whereas other structures like dendrites and axons are removed. The network was previously trained on a ground-truth dataset where a human expert has localized the center of neuronal somata. The training dataset

was composed of 162 image stacks for a total volume of about 5 mm^3 and 15'355 manually labeled cells. The stacks were randomly selected from different areas of the brain, in order to train the network to recognize the large variability in cell shape that can be found across the sample. The images deconvolved by the network are then processed with a standard blob detection algorithm (Difference of Gaussians, DoG), providing center of bright structures, which in this case are the neurons. The overall performance of the method is evaluated by comparing the list of neuronal centers found by the software with human-annotated ground truth on a test set of 165 image stacks for a total volume of about 5.1 mm^3 and 12'909 manually labeled cells. Again, these stacks were randomly selected from different areas of the brain, to test network performance in different brain regions. If two neuronal centers from the two annotations (automatic and manual) are closer than 10 μm (roughly half of the average diameter of a neuron), they are considered to be the same cell, i.e. a true positive (TP). If a center is present only in the manual annotation, it is considered a false negative (FN), whereas if it is present only in the results of the algorithm, it is considered a false positive (FP). Count of TP, FP and FN was carried out with Maximum Bipartite Matching algorithm[34]. We evaluated localization performances using precision = TP/(TP+FP), recall = TP/(TP+FN) and F1-score (defined as the harmonic mean of precision and recall). On our test set, we found precision 0.83, recall 0.90 and F1-score 0.86.

Spatial registration to atlas

A downsampled version of the whole-brain dataset (voxel size 25 μm) was spatially registered to the Allen reference atlas using Advanced Normalization Tools[35], with a sequence of rigid, affine and diffeomorphic (SyN) transformations. The same transformations were applied to the point cloud produced by the BrainCell Finder, representing the position of SST+ neurons. Each cell was then assigned to a selected brain area based on its position. To evaluate errors in cell counting introduced by wrong alignment to atlas, for each brain region we counted the number of cells lying on the region border, weighted with a 3D gaussian kernel with a sigma of 75 μm.

493 **Whole-brain spatial clustering analysis**

494 To assess spatial clustering of neurons, we evaluated for each neuron the 3D Ripley's *K*-function[17].
495 Given the spatial density of cells λ, the *K*-function is defined as:

496 $$K(r) = \frac{\#\ of\ cells\ within\ a\ ball\ of\ radius\ r}{\lambda}$$

497 In three dimensions, under the hypothesis of complete spatial randomness (CSR), the expected value
498 of $K(r)$ is just the volume of the sphere:

499 $$E[K(r)]_{CSR} = \frac{4\pi r^3}{3}$$

500 As discussed by Jafari-Mamaghani and co-authors[36], deviations from the CSR hypothesis are best
501 described by the deviations from this expected value: $K(r) - E[K(r)]_{CSR}$. We define as 3D clustering index
502 *I* the defined integral of this function on a specific range of *r*:

503 $$I = \int_{r_{min}}^{r_{max}} K(r) - E[K(r)]_{CSR}\ dr$$

504 We chose this definition given the linear nature of the integration operator.

505 To estimate the *K*-function from our point cloud, we first evaluated for each neuron the local density
506 of cells in a ball of radius 300 μm surrounding the neuron itself. This sphere is significantly larger than cell
507 size but still smaller than the main anatomical subdivision of the mouse brain. After estimating the local density
508 at the position of each single neuron, we computed the 3D clustering index using the above formula, with r_{min}
509 = 10 μm (comparable to cell size) and r_{max} = 100 μm (smaller than the radius used for density estimation), with
510 an integration step of 10 μm. The local density as well as the 3D clustering index were associated with each
511 cell, and used for subsequent analysis. Images of the point clouds were produced using CloudCompare
512 (https://www.cloudcompare.org). Graphs of the distribution of densities and of 3D clustering index were
513 produced using OriginLab (https://www.originlab.com).

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Microglia morphological analysis

Substacks from selected brain regions were manually extracted from the whole-brain dataset. Microglia somata were segmented using ilastik[37]. In more detail, we used the Autocontext workflow[38] and semantic classes for somata, processes and background. We have trained a single project with sparse annotations on five regions (CA1, granular layer of cerebellum, molecular layer of cerebellum, hypothalamus, thalamus) with four label classes in the first round of Autocontext: soma, background, and two classes for processes. In the second Autocontext round, we have labeled with three different labels, for soma, background, and process. Probability images were generated for all 16 image volumes with ilastik in headless mode using the trained Autocontext project.

We have relied on the feature extraction pipeline of the ilastik Object Classification workflow to extract morphological features. Before processing in ilastik, we have rescaled the probability images to an isotropic resolution of 0.65 µm. Furthermore, the images were threshold at 0.5 in the soma probability channel, followed by connected component analysis. We quantified ellipticity by comparing the largest to the smallest eigenvalue of the principal component analysis (PCA) of the object voxels.

Histological sample preparation

Samples of atherosclerotic human carotid and human keloid (courtesy of Dr. Cicchi, National Institute of Optics, Italy) were fixed with paraformaldehyde, cut into 5-µm slices with a microtome, stained with standard hematoxylin/eosin, and mounted in glycerol.

Yeast cultures

The strains used in this study were wild-type *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, MO) and *Schizosaccharomyces pombe* (that express GFP-tubulin under the nmt promoter, courtesy of Prof. Tolić, Ruđer Bošković Institute, Croatia). The yeasts were grown in a standard liquid yeast culture medium (Yeast Peptone D-Glucose) and imaged at 37 °C using a warmed plate. To enhance the expression of GFP, 2 µM of thiamine were added to the growing medium of *Schizosaccharomyces pombe*.

C. elegans motion assay

Wild-type *Caenorhabditis elegans* (C. elegans Behavior Kit, Bio-Rad Laboratories, Hercules, CA) were grown according to the protocol recommended by the supplier. To perform the motion assay, a few *C. elegans* worms were transferred with a spatula onto a fresh agar plate and placed under the microscope. Custom software written in LabVIEW 2012 (available from https://github.com/ludovicosilvestri/RAPID_CLSM) was used to keep the worm in the camera field of view. The same software also recorded the XY positions of the stage and worm in the field of view, providing the absolute XY position of the worm. The Z position was tracked using the position of the Z stage, which was continuously corrected by the RAPID module.

Bright-field and epifluorescence microscopy

An Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with an XYZ stage (L-STEP 13, LANG, Hüttenberg, Germany) was integrated with RAPID for the BF and EF experiments. In the BF modality, a mercury lamp coupled with a red bandpass filter (630/10, Thorlabs) was used to illuminate the sample. In the EF modality, light from a blue LED (M470L3, Thorlabs) was bandpass-filtered (469/35, Semrock, Rochester, NY) to avoid contamination in the fluorescence channel and then reflected to a long-pass dichroic mirror (496 nm edge, Semrock) to illuminate the sample. Light emitted from the sample and transmitted by the dichroic was further bandpass-filtered (520/35, Semrock) to isolate the fluorescence contribution. Images were collected using a sCMOS camera (Orca Flash 2.0, Hamamatsu, Japan). Defocus was corrected by moving the objective either with a piezo scanner (PIFOC P-721.LLQ, Physik Instrumente, Karlsruhe, Germany – for the yeast cultures) or with the Z axis of the sample translation stage (for the *c. elegans* tracking). The imaging parameters for the different experiments are summarized in Supplementary Table 2.

Data availability statement

The datasets acquired for this study are available at LINK.

Code availability statement

RAPID stand-alone code is publicly available at https://github.com/lens-biophotonics/RAPID-AF. Code used to control RAPID-enabled LSM is publicly available at https://github.com/ludovicosilvestri/RAPID_CLSM. ZetaStitcher and ilastik are available at https://github.com/lens-biophotonics/ZetaStitcher and https://www.ilastik.org, respectively. BCFind software is available at https://github.com/lens-biophotonics/BCFind2.1.

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