We present cleared-tissue axially swept light-sheet microscopy (ctASLM), which enables isotropic, subcellular resolution imaging with high optical sectioning capability and a large field of view over a broad range of immersion media. ctASLM can image live, expanded, and both aqueous and nonaqueous chemically cleared tissue preparations. Depending on the optical configuration, ctASLM provides up to 260 nm of axial resolution, a three to tenfold improvement over confocal and other reported cleared-tissue light-sheet microscopes.

We imaged millimeter-scale cleared tissues with subcellular three-dimensional resolution, which enabled automated detection of multicellular tissue architectures, individual cells, synaptic spines and rare cell-cell interactions.

Human tissues are composed of multiple polarized cell types organized in well-defined three-dimensional (3D) architectures. Interestingly, it has been shown that rare subsets of cells play a crucial role in disease progression and interdisciplinary efforts now aim to generate comprehensive atlases of human cells in diverse tissue types. To date, this has largely relied on massively parallel sequencing and machine-learning-based analyses to identify unique subpopulations of cells. Combined with advanced imaging, such efforts could not only shed light on the diversity of cell types, but also on the biological context in which each population operates. However, imaging large tissues with subcellular resolution remains challenging owing to the heterogeneous refractive index and composition of tissues, which results in complex aberrations and an increased scattering coefficient, both of which decrease spatial resolution and limit the depth of imaging.

To overcome this challenge, a large variety of clearing techniques have been developed that aim to homogenize the optical properties of the tissue using aqueous or organic solvents. These clearing techniques routinely render biological specimens sufficiently transparent, such that, if the imaging technology was to exist, entire organisms could be imaged with subcellular resolution and with molecular specificity. Because the specimens are 3D, an ideal imaging system should possess isotropic resolution to accurately measure 3D cellular morphology, biomolecular localization and signaling activity. Nevertheless, imaging chemically cleared specimens with diffraction-limited or super resolution presents technical challenges. For example, each clearing mechanism provides advantages and disadvantages and requires unique immersion media with refractive indices that range between 1.33 and 1.559. Thus, the imaging system must optimally operate throughout this refractive index range without suffering from deleterious aberrations that decrease resolution and sensitivity.

Light-sheet fluorescence microscopy (LSFM), because of its parallelized image acquisition, inherent optical sectioning and ability to image large biological structures quickly and with high optical resolution, serves as an ideal candidate for cleared tissue imaging (Supplementary Table 1). Nevertheless, to the best of our knowledge, there is not yet a light-sheet microscope for cleared tissue specimens that possesses submicron, isotropic resolution and is also compatible with the full range of clearing methods. Although lattice light-sheet microscopy readily achieves high spatial resolution, it relies on specialized high numerical aperture lenses that are only compatible with aqueous solvents (refractive index = 1.333) and its field of view is relatively small. Here, we address these limitations and present a scalable imaging platform that provides subcellular anatomical detail in any spatial dimension across millimeter-wide cubes of tissue. The system that we refer to as ctASLM, builds on previous work where the sample was mechanically scanned through a thin light-sheet. Instead of scanning samples slowly, ctASLM scans the illumination in its propagation direction using fast aberration-free remote focusing (Supplementary Note 1 and Supplementary Fig. 1), refractive index-independent illumination and detection optics, residual-spherical-aberration correction in...
the remote focusing arm and a synchronous camera readout to deliver isotropic high-resolution, a large field of view and high-contrast imaging (Fig. 1a)12,13. Compared to other light-sheet variants that lack aberration-free refocusing (such as mesoSPIM) and camera synchronization (such as tiling light-sheet microscopy), ctASLM is better suited for using thin light-sheets over large fields of view (Supplementary Note 1) 14,15. Consequently, we present two implementations of ctASLM using matched multi-immersion objectives of numerical aperture (NA) 0.4 or 0.7, which provide isotropic subcellular imaging at two resolution levels (~600 nm and ~300 nm, respectively; Supplementary Fig. 2 and Supplementary Note 2). By tiling multiple volumes16, ctASLM permits routine visualization of subcellular features throughout millimeters of tissue, regardless of the clearing method (Supplementary Fig. 3 and Supplementary Video 1).

To evaluate optical performance, we imaged subdiffraction beads with ctASLM, conventional light-sheet microscopy and an Airyscan confocal microscope (Fig. 1b), each equipped with long working distance multi-immersion objectives (detailed full-width half-maximum values are given in Table 1). Importantly, ctASLM improves axial resolution sixfold over conventional LSFM and three to sevenfold over Airyscan confocal microscopy. Depending on the refractive index of the immersion media, ctASLM achieves an isotropic, raw resolution of 800–900 nm with the NA 0.4 objectives and 400–500 nm with the NA 0.7 objectives, over the same field of view encompassed by conventional light-sheet microscopy (Fig. 1b, Supplementary Figs. 4 and 5, Supplementary Tables 2 and 3 and Supplementary Note 3)17,18. Iterative deconvolution improves the resolution of ctASLM by ~30%, leading to 300 nm resolution with the NA 0.7 objective (Table 1 and Supplementary Figs. 6 and 7), which we further confirmed inside a biological sample (Supplementary Fig. 8). To the best of our knowledge, the axial resolving power of ctASLM of 260–290 nm in solvent-based media is the highest reported z-resolution in diffraction-limited light-sheet microscopy (without requiring super-resolution mechanisms such as stimulated emission depletion, nonlinear structured illumination or localization approaches). Owing to the excellent optical sectioning of ctASLM and high-quality raw data, deconvolution...
is not always needed (Supplementary Fig. 9). In comparison to a conventional light-sheet microscope (Fig. 1c), individual synaptic spines could be clearly resolved in any spatial dimension (Fig. 1d) and neurons could be traced in 3D using unprocessed data.

Because of its large field of view and long working distance, we explored the ability of ctASLM equipped with the NA 0.4 objectives to image large tissues. Figure 1e shows a PEGASOS-cleared Thy1-GFP brain (Supplementary Video 2) and Fig. 1f shows a volume rendering of a neuron at a depth of 2.5 mm. Even at this depth, we were able to resolve fine neuronal structures and individual spines (Supplementary Videos 3 and 4 and Supplementary Figs. 10 and 11). Using benzyl alcohol and benzyl benzoate (BABB) clearing, we imaged a neonatal kidney labeled with the vasculature marker Flk1-GFP and maintained sufficiently high spatial resolution to distinguish individual cells throughout the 3.4 × 2.6 × 2.5 mm³ volume (Fig. 1g and Supplementary Video 5). We also imaged the hematopoietic stem cell (HSC) niche in a PEGASOS-cleared bone marrow plug (Fig. 1h–k). HSCs are responsible for the continued production of blood and immune cells throughout life and HSCs reside within a perisinusoidal niche in the bone marrow, where leptin-receptor-positive (lepR⁺) stromal cells and endothelial cells synthesize the factors required for HSC maintenance⁹. However, careful analysis of the interactions between these cells has not been performed because HSCs are rare (0.003% of all bone marrow cells) and imaging large specimens has been prohibitively time-consuming⁴. ctASLM imaging allowed us to identify α-catulin⁺-c-Kit⁺ HSCs, markers that identify all HSCs in young adult bone marrow and that give very high levels of purity (Fig. 1). The isotropic resolution revealed extensive interactions of lepR⁺ cell projections and HSCs (Fig. 1j,k), which were distorted when imaged with a confocal microscope (Supplementary Video 6). Further, we imaged the entire PEGASOS cleared murine olfactory bulb (Supplementary Video 7), which included ossified structures that would otherwise be incompatible with aqueous clearing methods and demonstrated the importance of being able to image in solvents that range in refractive index from 1.333 to 1.559.

Next, we tested whether the quality of ctASLM data was sufficient for the application of automated and unbiased approaches to image quantification. Using the open source software u-shape3D, we could readily detect dendritic spines and cluster them on the basis of their 3D morphology (Fig. 2a and and Supplementary Videos 8 and 9)⁵⁶. These clusters spanned a wide range of morphologies (Fig. 2b), formed a structured space following principal-component analysis (Fig. 2c) and were separable via interpretable measures, such as the ratio of spine neck area to spine surface area

| Microscope | NA | Field of view (µm³) | Lateral resolution (µm) | Axial resolution (µm) | Field of view (µm³) | Lateral resolution (µm) | Axial resolution (µm) |
|------------|----|-------------------|------------------------|----------------------|-------------------|------------------------|----------------------|
| ctASLM1    | 0.4 | 870 × 870         | 0.95 ± 0.30 (raw, n = 10) | 0.95 ± 0.115 (raw, n = 10) | 737 × 737       | 0.83 ± 0.035 (raw, n = 10) | 0.94 ± 0.075 (raw, n = 10) |
| ctASLM2    | 0.7 | 327 × 327         | 0.48 ± 0.012 (raw, n = 10) | 0.48 ± 0.029 (raw, n = 10) | 310 × 310       | 0.45 ± 0.016 (raw, n = 6)  | 0.38 ± 0.032 (raw, n = 6)  |
| LSFM1      | 0.4 | 870 × 870         | 1.04 ± 0.04 (raw, n = 5)  | 6.7 ± 0.4 (raw, n = 5)   | 737 × 737       | —                       | —                    |
| LSFM2      | 0.7 | 327 × 327         | 0.5 ± 0.02 (raw, n = 5)   | 2.66 ± 0.04 (raw, n = 5) | 310 × 310       | —                       | —                    |
| Airyscan   | 0.8 | —                 | —                       | —                     | —                | 0.32 ± 0.03 (deconv., n = 3) | 3.1 ± 0.4 (deconv., n = 3) |

Note that the magnification and resolving power slightly change for the multi-immersion lenses used for ctASLM for refractive indices (η) between 1.333 and 1.56, respectively. All numbers presented are the mean ± the s.d. of the full-width half-maximum and the sample size n was 3–10 for each measurement. deconv., deconvolution; —, not measured.

![Fig. 2](image-url)
but were readily resolved by ctASLM, owing to its improved resolution. Importantly, detailed features of the HSCs could not migrate through these fenestrations to enter and exit circulation, consistent with observations that HSCs are associated with fenestrations in the walls of the sinusoids. ctASLM allows visualization of subcellular interactions among cells in large tissues. (Fig. 3e–h)19. An x–y cross-section of c-Kit+ cells residing in the sinusoids. c-Kit+ cells are positioned along fenestrations in the walls of the sinusoids. A 3D rendering of a cell highlighted in close-up view (segmented and rendered) to c-Kit+ cells in a BABB-cleared mouse bone marrow specimen. Close-up view of a possible interaction. y–z view along a plane indicated by white triangles in showing an interaction between a nerve fiber and a c-Kit+ cell. Volume rendering of Data were acquired with NA 0.7 objectives. Scale bars, 500 μm (a), 10 μm (b,c), 2.5 μm (d), 5 μm (e,f) 10 μm (f) and 2.5 μm (j,k).

( Supplementary Fig. 12). For the kidney, damage to glomeruli is associated with a spectrum of clinical outcomes, including proteinuria and reduced filtration, which are the hallmarks of chronic kidney diseases. To evaluate tissue composition in an automated fashion, we developed a multiscale watershed algorithm to identify glomeruli (Fig. 2d). Further, owing to the high axial resolution, we could detect endothelial cells within an individual glomerulus (Fig. 2e, Supplementary Fig. 13 and Supplementary Video 10). Formerly, such an analysis was performed manually on hundreds of serial-sectioned tissue preparations, because identification of individual cells in confocal datasets was particularly challenging owing to its diminished z-resolution.

Lastly, we imaged PEGASOS-cleared HSCs, nerve fibers and vasculature with a mouse bone marrow with the NA 0.7 objectives and 300-nm-scale resolution (Fig. 3a–d and Supplementary Fig. 14). Many c-Kit+ HSCs were associated with fenestrations in the sinusoidal blood vessels, consistent with observations that HSCs migrate through these fenestrations to enter and exit circulation. Intriguingly, in BABB-cleared bone marrow plugs we observed punctate staining along nerve fibers that resembled synapses, raising the possibility that nerve fibers directly interact with a subset of c-Kit+ cells (Fig. 3i–l and Supplementary Video 11).

In summary, ctASLM simultaneously provides subcellular detail and tissue-scale anatomy. Because tissues are inherently 3D, the isotropic resolution of ctASLM is an important attribute to describe 3D morphologies and molecular concentrations in an unbiased way. Indeed, even higher resolutions can be achieved by combining ctASLM with expansion microscopy (Supplementary Figs. 16–18 and Supplementary Videos 12 and 13), albeit with one order of magnitude larger field of view than lattice light-sheet microscopy. Of note, ctASLM imposes a higher out-of-focus excitation load on the sample than a conventional light-sheet system and illuminates a smaller portion at a time, both of which can increase photobleaching. However, even at the highest resolution level (NA 0.7), we were not limited by photobleaching and were able to image a sample multiple times with a high signal-to-noise ratio. Some steering of the light-sheet was observed deep in the PEGASOS brain and likely higher-order aberrations were present in some of our CLARITY-cleared samples (Supplementary Fig. 19). Automated alignment routines, as well as adaptive optics, could help to achieve diffraction-limited performance in less than optimally cleared samples. With the given resolution level, we readily show that we can combine ctASLM with computer vision to detect and classify biological features throughout millimeters of tissue. Consequently, we believe that ctASLM will expedite human cell atlas efforts, providing much-needed insight into how tissue function manifests in health and disease, from the heterogenous cellular populations that compose it.

Online content
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Methods

Animal specimens. All animal protocols were approved by local Institutional Animal Care and Use Committees (IACUC) as directed by the National Institutes of Health, and strictly followed. These protocols include AC-AAAR04157 (to R.T., Columbia University, 2017-102370 to J.M., UT Southwestern Medical Center), 101917 (to D.M., UT Southwestern Medical Center), 101715 (to L.B., UT Southwestern Medical Center) and 102632 (to S.M., UT Southwestern Medical Center).

Microscope control. The data acquisition computer for ctASLM v.1 was a Dell Precision T350 tower equipped with an Intel Xeon E3-1270 v3 processor operating at 3.5 GHz with six cores and 12 threads, 128 GB of 2.133 MHz DDR4 RAM and an integrated Intel AHCI chipset controlling 4×512 GB SSDs in a RAID0 configuration. The data acquisition computer for ctASLM v.2 was a Colfax ProEdge SXT980 workstation equipped with two Intel Xeon Silver 4112 processors operating at 2.6 GHz with eight cores and 16 threads, 96 GB of 2.667 GHz DDR4 RAM, a Intel DC P3100 1024 GB M.2 NVMe drive and a Micron 5200 ECO 7,680 GB hard drive for file storage. All software was developed using a 64-bit version of LabView 2016 equipped with the LabView Run-Time Engine, Vision Development Module, Vision Run-Time Module and all appropriate device drivers, including NI-RIO drivers (National Instruments). Software communicated with the camera (Flash 4.0, Hamamatsu) via the DCAM-API for the Active Silicon Firebird frame-grabber and delivered a series of deterministic transistor–transistor logic (TTL) triggers with a field programmable gate array (PCIE 7852R, National Instruments). These triggers included control of the resonant mirror galvanometers, voice coils, stage positioning, laser modulation and blanking, camera triggering and other functions. The microscope control software are licensed under a Material Transfer Agreement from Howard Hughes Medical Institute, Janelia Research Campus. The control software for ctASLM can be requested from the corresponding authors and will be distributed under a material transfer agreement with Howard Hughes Medical Institute, Janelia Research Campus.

tcASLM v.1 microscope layout. A schematic layout of the ctASLM v.1 microscope is shown in Supplementary Fig. 20 and a parts list is provided in Supplementary Table 4. The illumination train consisted of four Coherent Obis lasers (LX 405-100C, LX 488-50C, LS 561-60 and LX 637-140C) that were combined with dichroic beam splitters (LM04-427-25, LM04-503-25 and LM04-613-25, Semrock), focused through a 30-µm pinhole (P30D, Thorlabs), recollimated with a 200-µm achromatic doublet (AC254-200-A, Thorlabs), and directed through a 3x Galilean beam expander (GB03-9). Thus, the initial beams were expanded by a factor of ×4 Galilean focused through a 30-µm pinhole (P30D, Thorlabs), with a 50-mm achromatic doublet (AC254-200-A-M1, Thorlabs), and directed through a 3x Galilean beam expander (GB03-9). The mirror was mounted on a voice coil with 10 mm of travel, sub-30-µm positioning repeatability and a 2.5-µm response time (LFA-2010, Equipment Solutions). The reflected light was recollected by the same x4 objective, the polarization state was rotated by the second passage through the quarter waveplate, reflected with the cube polarizing beam splitter (10FC16PB.7, Newport) and imaged onto a mirror with a x40 microscope objective (CFI Plan Fluor ELWD ×40, NA 0.6, Nikon Instruments). The mirror was mounted on a voice coil with 10 mm of travel, sub-30-µm positioning repeatability and a 2.5-µm response time (LFA-2010, Equipment Solutions). The reflected light was recollected by the same x4 objective, the polarization state was rotated by the second passage through the quarter waveplate, reflected with the cube polarizing beam splitter and directed to the illumination objective (Cleared Tissue Objective, NA 0.7 Advanced Scientific Imaging) with 80-µm and 200-µm achromatic doubles (AC308-200-A and AC308-75-A, Thorlabs).

Sample mounting. Samples were glued with cyanacrylate (PT909, Pacer Technology) or with silicone (GE Silicone + onto a 1×2.5 cm glass slide cut from a 2×5×7.5 cm microscope slide (catalog no. 300, Thermo Scientific). The glass slides were mounted with a custom holder to a sample stage on a computer-aided design drawings of the sample holder assembly are shown in Supplementary Fig. 23.

Conventional light-sheet microscopy. For conventional low-NA light-sheet imaging, the same optical train was used in ctASLM v.1, but with the presence of a variable width slit aperture conjugate to the back pupil of the cylindrical lens and without axial scanning of the illumination beam. The illumination NA was selected so that the entire field of view (870×870 µm) resided within two Rayleigh lengths of the illumination beam. This resulted in a Gaussian beam with a 10−3 effective waist of ~7 µm, which is in agreement with resolution measurements obtained using subdiffraction fluorescence beads. With the ctASLM v.2 microscope (NA 0.7 objectives), an axial resolution of 2.5 µm resulted, which we believe is in large part owing to the close to diffraction-limited detection PSF.

Confocal microscopy. Imaging of the bone plug was performed on a Zeiss LSM880 confocal microscope equipped with a long working distance multi-immersion objective (LD LCI Plan-Apo ×25×0.8 NA) as previously described22,23. PEGASOS-cleared mouse brain was also imaged using a Leica SP8 confocal laser scanning microscope equipped with an HCX AP0 ×20/0.95 immersion objective. For the best performance a 56-µm pinhole size with a pixel dwell time of 1.845 µs was used. A comparison of ctASLM to the data acquired with the Leica SP8 is shown in Supplementary Fig. 24.

Sample preparation. Zebrafish. The zebrafish embryo was a transgenic line (krdlmCherry) expressing mCherry on the vascular endothelium. A 3-d post-fertilization zebrafish embryo was mounted in 2% low-melting agarose (AH0945-25G, Sigma-Aldrich) in a fluorinated ethylene propylene (FEP) tube (FEP HS 0.029/0.018 RC, Zeus). The working solvent of the agarose was E3 medium. The FEP tube was sonicated in 70% ethanol and then washed with clean distilled water before mounting the zebrafish embryo. We first selected and left a zebrafish embryo in ~30% of E3 medium on a Petri dish. Next, 5 µl of 0.1% tricaine was added to anesthetize the embryo. Approximately 50 µl of the mixture of that tricaine and E3 medium was removed and liquid low-melting agarose was added to the embryo. The embryo in the liquid agarose was aspirated directly into the clean FEP tube with a pipette. Within a few minutes the agarose was solidified and the zebrafish embryo was mounted in the FEP tube. For the last step, we used a needle to make two holes in the FEP tube above and below the embryo to increase gaseous

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**Isolated hippocampus.** The Thy1-GFP mouse was transcardially perfused with cold PBS, then 4% PFA in PBS for fixation. The brain was taken out and put into 4% PFA in PBS overnight at 4 °C, then 2-mm thick hippocampal slices were cut on a cryotome with a vibratome. The hippocampal slices were then washed with PEGASOS-clearing protocol, previously described by Jing et al. Briefly, the slices were decolorized in 25% Quadrol for 1 d, then delipidated in series of 30%, 50% 70% tert-butanol solutions, dehydrated in tB-PEG and finally cleared in BB-PEG.

**Expanded hippocampus.** For imaging, expanded hippocampal samples from either Thy1-GFP mice (Supplementary Figs. 16–18) or wild-type mice injected with Synaptogat AAV (Supplementary Fig. 17) were used. The mice were transcardially perfused with cold PBS, then 4% PFA in PBS for fixation. The brain was taken out and put into 4% PFA in PBS overnight at 4 °C, then 0.1-mm thick coronal hippocampal slices were cut with a vibratome. Samples were processed according to the original protocol for expansion microscopy procedure. Briefly, the slices were permeabilized using 5% BSA, 0.5% Triton X-100 in PBS for 1 h at room temperature and stained with rat anti-mCherry (Thermo Scientific M11217, 1:500; Supplementary Fig. 17) or chicken anti-GFP (Abcam ab13970, 1:500; Supplementary Fig. 18) and rabbit anti-Homer1 (Synaptic systems, 160003, 1:200) antibodies (Supplementary Fig. 18) in permeabilization buffer for 2 d at 4 °C. For Homer1 staining, antigen was retrieved by incubation in 1 mM EDTA at 60 °C for 45 mins. Slices were washed three times in PBS and stained with donkey anti-rat-Alexa594 (Invitrogen, 1:200; Supplementary Fig. 17) or goat anti-chicken-Alexa 488 (Invitrogen, 1:200) and goat anti-rabbit-Atto647N (Sigma-Aldrich, 1:200; Supplementary Fig. 18) conjugates. Following antibody incubation, after thorough washing steps, samples were incubated overnight with a coupling reagent 0.1% acryloyl-X succinimidyl ester in PBS. For results shown in Supplementary Fig. 16, the samples were directly crosslinked with acryloyl-X succinimidyl ester reagent without further antibody staining against GFP. Slices were washed three times with PBS for 5 min and incubated in polymerization solution (8.6% sodium acrylate, 0.1% 1,4-butanediol, 15% N,N'-methylenebisacrylamide, 2 M NaCl, 0.2% APS, 0.2% TEMED and 0.01% TEMPO) for 1 h at 4 °C polymerization at 37 °C for 2 h. Embedded tissue was transferred to isotonic protease K buffer (50 mM Tris-HCl (pH 8.0), 2 M NaCl, 1 mM EDTA, 0.5% Triton X-100 and 8 μl/mL protease K) and incubated for 2 h at room temperature. Complete proteolysis overnight at room temperature in protease K buffer (20 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.5% Triton X-100 and 8 μl/mL protease K) the next day, samples were expanded for 1 h in double-distilled H2O with water three changes. Glass holders were covered with 0.1% poly-L-lysine for 1 h, washed three times with double-distilled H2O and allowed to air dry for 1 h. The hippocampus regions were excised from coronal slices and mounted on poly-L-lysine-coated glass holders using wax as additional support on the sides. For long-term storage and image acquisition, samples were transferred to 1 mM NaOH buffered solution.

**Neonatal kidney preparation.** On postnatal day 3, kidneys from an Flk1-GFP (Jackson Laboratories, 017006) mouse were harvested and fixed for 2 h at room temperature in 4% paraformaldehyde in PBS. Procedures were performed according to University of Texas Southwestern Medical Center IACUC-approved guidelines. Fixed kidneys were incubated with tert-butanol/water mixtures of increasing alcohol concentrations (30%, 50%, 70%, 80%, 96% and twice 100%) with 2% trehalolamine. The kidneys were left in BABB in a 1:2 volume ratio with 16% trehalolamine for 3–4 weeks. After polymerization medium (2–4 d), samples were stored at 4 °C. Given that the tissue was harvested from neonatal mice, and no genotyping was performed, the sex of the mouse was unknown.

**Stitching and 3D visualization.** Image processing was performed on a Windows 10-based workstation equipped with two Intel Xeon Gold 5120 CPUs, 1 TB of RAM and an NVIDIA Quadro P6000 GPU. To stitch the subvolumes, the Fiji-based plugin BigSticher was used. Image analysis was performed with Fiji and MATLAB (Mathworks) and 3D renderings were produced with ChimeraX or Avizo.

**Deconvolution.** We tested the Richardson–Lucy deconvolution and blind deconvolution (Supplementary Table 6 lists details for each dataset shown in this manuscript). When using the Richardson–Lucy algorithm to deconvolve the images, the PSF was extracted from the image of a single bead, centered and background-subtracted and 3D deconvolution was performed with 20 iterations of the Richardson–Lucy algorithm (Microvision). For the blind deconvolution, we used the deconvblind function in MATLAB (Mathworks) and the following procedure. Before the deconvolution, we rotationally averaged, along the optical axis, the experimental image of a single 200-nm bead. We measured the mean value of the background in the rotationally averaged PSF and subtracted it from the experimental PSF: we then extracted a single image with the rotationally averaged and background-subtracted PSF for 5 iterations. This yielded a restored PSF from the deconvblnde routine (the resulting PSF more closely approximated the real PSF in the measurement). In a second step, we used this restored PSF to perform blind deconvolution on the raw image data, which then resulted in the final deconvolved image. For biological data we
used 5–10 iterations, whereas for the bead analysis, we limited the number of iterations to five. The raw images and the experimental PSF were resampled twice with the bicubic algorithm with ImageJ before the deconvolution, which was particularly necessary for the ctaSLM v1 data (NA 0.4), which did not have much Nyquist oversampling in its data acquisition. ctaSLM v2 was designed with more oversampling (a larger optical magnification), hence computational resampling before deconvolution was not necessary.

Data analysis. HSC detection. HSCs were detected as previously described. Briefly, individual α-catenin-GFP and c-Kit+ HSCs were identified manually using the ortholocator function of Bitplane Imaris v9.2.1 software. HSC coordinates and size were interactively annotated using the Imaris spots function in the manual mode. c-Kit and leptin tdTomato surfaces were generated in Imaris using the isosurface function on the basis of an empirically chosen intensity threshold.

Glomeruli detection. In an effort to automatically detect the abundance of glomeruli throughout the entire neonatal kidney tissue, subvolumes (1,024 × 1,024 × 470 pixels) were retrieved from the stitched volume (3,042 × 4,018 × 2,757 pixels) and loaded into MATLAB. Subvolumes were subjected to a 3D Gaussian blur of 20 pixels before individual glomeruli were detected using an autoadaptive watershed algorithm implemented in MATLAB. This algorithm uses a difference of Gaussians to suppress features that are either too large or small, here defined as 100 and 10 pixels, respectively. Segmented objects touching the border of the subvolume were removed. After identification of glomeruli, the encompassing region was again loaded into MATLAB and the full resolution was subjected to the same watershed algorithm, but with a difference of Gaussians of 10 and 2 pixels, respectively.

Spine detection and clustering. We detected spines using previously described software. Images were first deconvolved for ten iterations using the MATLAB function deconvblind(), which uses a maximum likelihood algorithm to infer the fluorescence distribution in the sample. Following a gamma transform of 0.4 and 3D hole filling, a triangle mesh representing the cell surface was created as an isosurface at the Otsu threshold value of the image. The mesh was then decomposed into approximately convex patches using the parameters previously described, except that merging via the triangle criterion was disabled and merging via the line-of-sight criterion was performed more conservatively with a parameter of 0.4. Training data for patch merging and patch classification machine-learning models were then generated from nine non-overlapping images of size 200 pixels in the lateral dimensions and approximately 150 pixels in the axial dimension. These nine images were cropped from eight larger images. Using leave-one-out cross validation on patches labeled as certainly spines or certainly not spines, we calculated that our spine detection workflow had a precision of 0.86 and a recall of 0.88.

We performed an unsupervised clustering of detected spines into 15 clusters using agglomerative hierarchical clustering. For each cluster, we calculated the median of each of the morphological feature vectors used for clustering and plotted the spine closest to that median (Fig. 2b). Only the clusters with a median spine attached to the neuron body were plotted. Principal-component analysis was then performed on these same features. We defined the spine neck surface area as the patch closure surface area. We previously defined the closure surface area as the additional surface area needed to minimally generate a closed polyhedron from the open polyhedron comprising the dendritic spine. We also defined the variation from a sphere by fitting each spine to a sphere and measuring the ratio of the standard deviation of distances from the sphere at each mesh face to the sphere radius.

Segmentation of neurons. The mouse brain volume was tiled in subvolumes for segmentation of neurons. The mouse brain volume was tiled in subvolumes for segmentation of neurons. The mouse brain volume was tiled in subvolumes for segmentation of neurons. The mouse brain volume was tiled in subvolumes for segmentation of neurons.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a  Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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- A description of any covariates tested

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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

- Give P values as exact values whenever suitable.

- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection: Labview control software that was developed by Coleman technologies.

- Data analysis: ImageJ, Matlab

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

We have no restrictions on data availability. Data can be requested from the corresponding author.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf
# Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Resolution measurements of our mASLM microscopes were conducted by averaging over 5 green fluorescent microspheres. Kidneys, olfactory nodes, expanded hippocampus slices, and bone marrow niches have been imaged multiple times (e.g., >5) for ongoing research efforts. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded intentionally. |
| Replication | Replicate experiments were successful |
| Randomization | not applicable |
| Blinding | not applicable |

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology         |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChiP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**
- chicken anti-GFP: GFP-1020, Avex Labs
- goat anti-c-kit: BAF1356, R&D Systems
- Alexa Fluor 488-AffiniPure F(ab')2 fragment donkey anti-chicken IgG', 703-546-155, JacksonImmuno
- Alexa Fluor 647-AffiniPure F(ab')2 fragment donkey anti-goat IgG, 703-546-147, JacksonImmuno

**Validation**
- Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer’s website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.

### Animals and other organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research

#### Laboratory animals
- *Danio rerio* (Zebrafish), Strain: wib with transgenic krd:imCherry, sex: male and female, age: 3dpf.
- Olfactory bulv: Age - 3.32 month old male transgenic mouse derived from strain C57Bl/6.
- Expanded hippocampus: 3 month old female wild-type 86 mouse.
- Kidney: 3 day old neonatal mouse, C57BL/6
- Bone Marrow: 8-8 week old female mouse [Jackson Laboratory 008320, LepR-Cre (Leprtm2[Cre]Rck/J)]
- Rs26-tetTomato [B6 Cp-Gt(ROSA)265sortm9(CAG-tetTomato)Hze/J, JAX:007909]
- a-catenin-GFP [Ctnn1tm1.1Jsm/J, JAX:028342]

#### Wild animals
- n/a

#### Field-collected samples
- n/a

#### Ethics oversight
- Mice and Zebrafish maintenance and experiments were conducted in strict accordance with UTSW and Columbia university IACUC guidelines. UTSW approved the study protocol and have complied with all relevant ethical regulations

Note that full information on the approval of the study protocol must also be provided in the manuscript.