Optical super-resolution microscopy allows nanoscale imaging of protein molecules in intact biological tissues. However, it is still challenging to perform large volume super-resolution imaging for entire animal organs. Here we develop a single-wavelength Bessel lightsheet method, optimized for refractive-index matching with clarified specimens to overcome the aberrations encountered in imaging thick tissues. Using spontaneous blinking fluorophores to label proteins of interest, we resolve the morphology of most, if not all, dopaminergic neurons in the whole adult brain ($3.64 \times 10^7 \mu m^3$) of *Drosophila melanogaster* at the nanometer scale with high imaging speed (436 $\mu m^3$ per second) for localization. Quantitative single-molecule localization reveals the subcellular distribution of a monoamine transporter protein in the axons of a single, identified serotonergic Dorsal Paired Medial (DPM) neuron. Large datasets are obtained from imaging one brain per day to provide a robust statistical analysis of these imaging data.
Exceeding the Abbe diffraction limit of optical microscopy has been one of the most exciting technological developments in life science. The unprecedented nanometric resolution achieved by several super-resolution techniques, including stimulated emission depletion microscopy\(^1\), structured illumination microscopy\(^2\), photo-activated localization microscopy\(^3\) and stochastic optical reconstruction microscopy\(^4\), has led to numerous breakthroughs in biology. Despite their successes, these techniques require the use of a high numerical aperture (NA) objective with short working distance which limited the observation depth to the vicinity of a coverslip surface\(^5,6\), yielding relatively small imaging volumes compared to the size of most tissue specimens. Several research groups have attempted to modify the designs of localization microscopy techniques to accommodate larger imaging volumes\(^7–10\). Among these studies, a combination of ultra-thin Bessel\(^11\) or lattice lightsheet\(^12\) and point accumulation for imaging in nanoscale topography microscopy has achieved sub-100 nm resolution in samples with over 20-μm thickness\(^10\). Premature photo-bleaching was effectively suppressed by utilizing the inherent optical sectioning of lightsheets\(^7–11\), whereby an oversampling localizations density was attained\(^10,11\). Moreover, lightsheet illumination extends the imaging area away from the coverslip, thereby enabling the use of thicker specimens, such as neuromast organs and zebrafish embryos\(^10\). By choosing appropriate imaging parameters, the combination of lightsheet and localization microscopy can be used with living cell for tracking protein complex\(^11\).

A more ambitious goal is to apply this technology and to perform three-dimensional (3D) imaging of subcellular features in large biological tissues. Due to photon scattering in these tissues, however, the quality of the image significantly degrades with observation depth, making super-resolution imaging much more difficult. To reduce the out-of-focus signal, genetic tools can be used to limit the expression of proteins of interest with spontaneously blinking fluorophores\(^16\). With mechanical sectioning, super-resolution imaging can, therefore, be carried out on 8-μm cryosections to reveal the subcellular features of neurons\(^14\). Because the structural integrity of a tissue is easily disrupted by mechanical sectioning\(^14\), the challenge has remained in implementing such techniques to larger specimens or to map protein distributions within the whole brain. Two recent studies have used a mounting medium with a high refractive-index (RI) to capture super-resolution images of neurons and mRNA molecules from the whole fly brain using Airyscan\(^15\) or Bessel Beam Structured Illumination Microscopy\(^16\), respectively. The optical resolution in these studies nonetheless was still limited to greater than 100 nm. Further, the dehydration caused by organic solvents in the mounting medium likely altered the structural integrity of the samples and the chemical properties of the labeled fluorophores\(^16\).

To address these issues, we use a water-based optical clearing agent to eradicate the RI mismatch arising from different cell morphologies and constituents within the fly brain, thereby minimizing light scattering and ensuring minimal perturbation of the sample structure. To break the depth of field (DOF) limit of detection objective with long working distance, the profile of the ultra-thin Bessel lightsheet convolves with the detection point spread function (PSF) to create a thin optical sectioning plane. Refined by post-processing based on the defocusing model, the signal-to-background ratio is improved\(^11\), thus we are able to confined single-molecule localization within each optical section, and also to achieve super-resolved sub-100 nm lateral resolution throughout the entire sample. In addition, we use spontaneously blinking fluorophores HMSiR\(^17\), which have been synthesized specially for super-resolution localization microscopy. By densely labeling the proteins of interest with spontaneously blinking fluorophores, we are able to map complex nanoscale structures within a considerable imaging volume using our lightsheet localization microscopy. Our success in establishing a strategy for 3D super-resolution microscopy at nanometric resolution opens up exciting applications, including the quantification of synaptic protein densities through large brain volumes.

Results

Lightsheet localization microscopy for clarified tissue. Our lightsheet localization microscopy for clarified tissue (LLM-CT) shares a similar optical path with lattice lightsheet microscopy (LLSM)\(^13\), Fig. 1a–d and Methods). Instead of using a spatial light modulator to generate lightsheet in LLSM, the lightsheet was generated from a scanning Bessel beam created by a lens-axicon doublet, illuminated by a Gaussian laser beam\(^18\) (Fig. 1b). Illuminated by varied Gaussian beam sizes, different ring-shaped focal patterns were formed, which were tuned to modulate the lightsheet length (Supplementary Fig. 1). The resulting ring pattern was further filtered by an annular ring mask placed on the Fourier plane conjugated to the back aperture of the customized excitation objective (Fig. 1a, c; N.A. = 0.5, working distance = 12.8 mm; Supplementary Fig. 2b)\(^19,20\). The principle of scanning Bessel beam lightsheet microscopy has been discussed in previous papers\(^19,20\), and a detailed description of our system can be found in the methods section. The Bessel beam has been described as a self-reconstruction light beam that is particularly effective for penetrating a thick tissue\(^19,20\). In optically clarified tissues (Fig. 1d), the axicon-generated Bessel beam of over 200 μm (Fig. 1e, Supplementary Fig. 3i, o) is scanned through the imaging plane to form a time-averaged virtual lightsheet. The axial energy distribution of the illumination is mostly confined within a 0.55 μm thick layer which reduces premature bleaching of the fluorophore outside the imaging plane (Supplementary Fig. 3m, n). Volumetric imaging is conducted by scanning simultaneously the detection objective and the excitation lightsheet. Because the imaging volume created by scanning the lightsheet is relatively large in relation to the volume of a fly brain, whole-brain super-resolution can be reconstructed by stitching together only 4 sub-volumes (Fig. 1f).

Localization precision in optically cleared fly brains. Traditional localization microscopy relies on photon-triggered quantum state switching of fluorophores either with exposure to a short wavelength or with high-power illumination leading to ground state depletion\(^3,4,22\). Use of an additional activation laser prolongs image acquisition time and potentially introduces more photo-bleaching. The high intensity illumination, on the other hand, will exert exceeding laser fluence to the surroundings near the imaging plane, which causes premature photo-bleaching and loss of localization density. To produce a localization microscope system with a moderate excitation power, we employed a spontaneously blinking fluorophore, HMSiR\(^17\), which can be excited efficiently by a red-wavelength laser (λ = 637 nm) at a relatively low power density. This low excitation power requirement allows the imaging area in our setup from a single exposure to be as large as 2 × 10^4 μm^2 (Fig. 2a).

To demonstrate our localization microscopy technique, a tissue sample maintained in phosphate-buffered saline (PBS) first was imaged with a water-based lightsheet system\(^11\) (Supplementary Figs. 2a, 4). As depicted in Supplementary Fig. 4, the positions of fluorophores were identified unambiguously by using ThunderSTORM\(^22\), and the reconstructed images demonstrated improved resolution. Due to tissue-induced aberrations, we observed an increase in lateral localization uncertainty in deeply located structures (Supplementary Fig. 4a). This increase in localization uncertainty resulted in a deterioration of the resolution of the reconstructed image and the increasing observed line width of dendritic structures, compared to images derived from shallower regions (Supplementary Fig. 4b). To improve this technique for
imaging deep tissues, we integrated lightsheet with optical tissue clearing technology\textsuperscript{23,24}. When the fly brain was cleared with neutral ScaleView-A2 solution (Methods)\textsuperscript{25}, the blinking signals observed in MZ19-Gal4-labeled olfactory neurons, located around 100 µm deep, became more distinguishable and had a better signal-to-noise ratio than those observed with PBS-based images (Fig. 2b–d, Supplementary Fig. 5). As we imaged through all HMSiR-labeled MZ19-Gal4 projection neurons, whose dendrites extend from the antennal lobe (AL) at the frontal surface of the brain to the axon terminals innervating the calyx of mushroom body (MB) at the posterior brain surface, we determined that our system was able to localize all blinking signals through an entire brain with minimum change in localization uncertainty and without mechanical sectioning (Fig. 2e, f).

**Whole-brain imaging using LLM-CT for HMSiR-labeled neurons.** LLM-CT allowed 3D nanoscopic imaging of MZ19-Gal4-labeled neurons. The superior quality of these images represents a substantial improvement over conventional lightsheet images acquired using the same system configuration (lightsheet microscopy for clarified tissue, LM-CT), including excitation wavelength and optics (Supplementary Fig. 6, Supplementary Movie 1). Due to the dense labeling of HMSiR (Fig. 3a), over 50 million molecules were localized in just 3 glomeruli on one side of antennal lobes with a lateral precision of ~20 nm over 60 µm depth in 1200 repeated imaging volumes (Fig. 3b; enlarged in Fig. 3c, Supplementary Movie 2). To determine a realistic acquisition time with a reliable statistical basis, we plotted the theoretical resolution with respect to time based on the structural features of our samples (Fig. 3d–g).
Two-dimensional (2D) localization density (number of localization events/area of the structure) increases exponentially with the number of frames. As the number of sampling frames increased, growth of localization density eventually slowed because of photobleaching and a depletion of dye molecules (Fig. 3d). The theoretical resolution limit of localization density was estimated by localization precision and the Nyquist resolution26. Resolution power increased rapidly in the first 400 frames (Fig. 3e) but then slowed down and converged at about 60 nm. This represents a lower bound at which the finest structures can be resolved with the current imaging parameters (Fig. 3e). Similarly, the Fourier ring correlation (FRC) value, which reflects the finest structure can be distinguished in the image, decreases with the frame (Fig. 3f)27,28. If an infinite photon budget existed and no photo-bleaching occurred, the localization density would increase monotonically with the number of sampling frames. Such modeling indicates that the theoretical resolution may reach 20 nm when 15,000 frames per layer are used for image reconstruction, which represents a 25-fold increase in the acquisition time used for this study (Fig. 3g).

With LLM-CT, the super-resolved image quality of olfactory projection neurons was maintained throughout the entire adult brain (Fig. 4a, b, Supplementary Movie 3). Notably, the photo-electric sensors in conventional fluorescence imaging have a limited dynamic range for detection sensitivity. Thus, simultaneously capturing structures with both strong and weak fluorescence signals in a single image is difficult (Fig. 4c). In localization microscopy, the individual molecules are reconstructed separately based on the histogram of localization events, and consequently the imaging is less sensitive to intensity differences in a single image. When imaging densely bundled neural fibers connecting the brain with the body, for instance, LLM-CT captured significantly more fibers than LM-CT (Fig. 4d), and individual brain-ascending/descending fibers could be manually segmented (Fig. 4e). LLM-CT also enabled the 3D visualization of individual synaptic proteins. For example, we were able to image the Down syndrome cell adhesion molecules (Dscam) within a single spine-like protrusion on a dendrite of the giant fiber neuron (Fig. 4f, g).

Visualizing fine neurites in the whole adult brain. To apply our LLM-CT system to intact adult brain, HMSiR was immunostained to the green fluorescent protein (GFP) labeled dopaminergic neurons with a TH-Gal429 using anti-GFP antibody (Methods), and mapped the distribution and wiring patterns of all TH-Gal4 neurons throughout the adult brain (Fig. 5a). Typically, more than 100 million HMSiR blinking signal were detected within each of the four sub-volumes used to reconstruct super-resolution images of the entire brain during a <1-day acquisition time (Methods). To analyze the resulting large dataset of raw blinking images for whole-brain reconstruction, we created a parallel computing pipeline process based on ThunderSTORM22 on a Lustre-backed Torque cluster (Supplementary Fig. 7). This large-volume super-resolution protein mapping enabled simultaneous visualization of the putative dopaminergic neurons labeled with strong GFP signal in the central brain, as well as the fine neurites labeled with weak GFP signal in the optic lobe (Fig. 5b, c, e). A majority of MB-innervated dopamine neurons direct their axons into the vertical lobe30. We calculated the innervation density of TH-Gal4-labeled MB neurons in each sector of the vertical lobe and determined that the middle part of the
vertical lobe (sectors α2, α′2) had the highest innervation density (Fig. 5d).

By applying the defocusing PSF model during post-processing of the data (Supplementary Note 1; Supplementary Figs. 8 and 9), digital segmentation of individual neurons, with nominal axial overlap, was achieved in a whole-brain 3D image (Fig. 5e, f). Serial optical slices and 3D navigation demonstrated extensive yet separable neurites (Supplementary Movie 4).

Visualizing protein spatial distribution within a single neuron. Expression level and spatial distribution of proteins are essential to the function of a cell. Scientists historically quantify such endogenous proteins with immunostaining and visualize the label intensity at the cell body with confocal or wide-field microscopy to represent the expression level (Fig. 6a). Like many proteins, vesicular monoamine transporter (VMAT) needs to be transported and/or locally synthesized at neuronal terminals to be functional and might be regulated locally by modulatory signals, resulting in variable expression within a given neuron.

We explored this idea by visualizing VMAT molecules in a specific MB local neuron—the dorsal paired medial (DPM) neuron, located in the central brain, and calculated the density of VMAT protein along the different sub-regions in the MB. We labeled VMAT with HMSiR and used our LLM-CT system to reconstruct VAMT locations. VMAT molecules along the MB lobes were classified into two groups: DPM positive (DPM+) and DPM negative (DPM−), based on the 3D digital intersection between VMAT and DPM (Fig. 6b, e, h; Supplementary Movie 5). Importantly, this classification could be accomplished only by using our LLM-CT method (Fig. 6b) and not by traditional Airyscan.
**Fig. 4** Super-resolution images in *D. melanogaster* brain with LLM-CT. Comparison between LLM-CT and LM-CT. a, b Images of MZ19-Gal4-labeled olfactory projection neurons in the antennal lobe (0–66 μm), lateral horn (90–144 μm), and calyx (129–189 μm) taken with LM-CT (a) or LLM-CT (b). c–e Images of fru-Gal4-labeled fly neck neurons taken with LM-CT (c) or LLM-CT (d). f More than 30 neurons were segmented manually across a 15-μm imaging depth. g The dendritic arbors of a giant-fibre neuron expressing UAS-Dscam::GFP imaged with LM-CT (f) or LLM-CT (g). 3D images were color-depth coded in each figure. Scale bars = 10 μm, unless otherwise indicated.

**Fig. 5** LLM-CT imaging of all dopaminergic neurons in *D. melanogaster* brain. a A whole-brain super-resolution mapping of all dopaminergic neurons labeled by HMSiR via anti-green fluorescent protein immunostaining in TH-Gal4 > UAS-GCaMP6f flies. b Maximum intensity projection of several optical slices showing dopaminergic innervations in the ellipsoid body (EB) and fan-shaped body (FB). c Dopaminergic innervations in the mushroom body (MB). Inset: magnified view of the optical slices at the α2α′2 sector of the MB vertical lobe. The colors indicate different depths beneath the surface. Scale bars = 20 μm, unless otherwise indicated. d Super-resolved dopamine neuron fiber density distribution in each sector of the MB. e Volume rendering of whole-brain dopaminergic neurons. Zoomed-in images show distinguishable interweaved neurites. f Digital segmentation of local neurons in the medulla. Inset: enlarged green neuron. The experimental flies carried TH-Gal4; UAS-GCaMP6f transgenes.
super-resolution confocal microscopy (Fig. 6c, f). To determine the imaging number of frames needed to map VMAT proteins (Supplementary Fig. 10; Supplementary Note 2), we determined theoretical resolution and localization density as discussed in Fig. 3. The reliability of DPM+ VMAT localization was validated by targeting VMATRNAi expression to DPM neurons, which reduced the total number of VMAT molecules in DPM+ neurites but not in DPM- regions of MB lobes (Fig. 6d, g, i; Methods).

DPM neurites completely engulf both the vertical and horizontal lobes (axonal outputs) of the MBs33 but activity in the vertical lobes appears to subserve behavioral responses after a few sessions of olfactory classical conditioning34. Furthermore, MB output neurons exhibit learning-gene dependent tuning plasticity35 and synthesize new proteins during long-term memory formation36,37. We hypothesized that VMAT in a DPM neuron might be distributed differently to modulate functional differences among MB sectors. Accordingly, we manually segmented DPM neurites into 15 MB sectors: α1, α2, α3, α’1, α’2, α’3, β1, β2, β’1, β’2, and γ1-γ5, based on the pattern of input innervations from dopaminergic neurons (Supplementary Fig. 11; Methods). With quantitative single-molecule localization by LLM-CT, we found that VMAT proteins distribute unevenly within a DPM neuron and their density in each MB sector is highly variable among different flies (Fig. 6j, k). The plasticity of VMAT distribution we observe here may account for the translation of transient MB activity into long-term memory coding at the MB outputs neurons.

**Fig. 6** Co-localization between VMAT molecules and DPM neurites in the whole MB. a VMAT signal in the soma taken by Zeiss 880 confocal microscopy. b LLM-CT showed distinguished signal of anti-VMAT immuno-positive molecules (magenta) were within or outside the DPM neurites (green). c As a comparison, Airyscan super-resolution confocal microscopy provides relatively poorer resolution. The ambiguous overlapping signals prevent the high accuracy co-localization analysis. d Levels of anti-VMAT immuno-positive molecules decreased within but not outside the DPM neuron in the flies carrying UAS-mCD8::GFP;VT64246-Gal4/UAS-VMAT-RNAi transgenes. e-g Magnified views of the images in the white boxes of (b-d). VMAT was immunolabeled with Alexa Fluor 635 (a, c, f) or HMs8iR (b, d, e, g). h Visualization of VMAT molecules. Left, VMAT proteins (gray) distributed within (red) or outside (gray) the DPM neurons (green). Right, an enlarged view. i VMATRNAi-mediated changes in VMAT density in the DPM+ neurites (left) but not in DPM-regions (right) within the MB. Each bar represents mean ± S.E.M. (n = 3). *P < 0.05. j A representative volume image showing the 3D distribution of VMAT in the DPM neurites innervating MB lobes: α/β (α1, α2, α3, β1, β2), α’/β’ (α’1, α’2, α’3, β’1, β’2) and γ (γ1 – γ5). Color code indicates VMATDPM+ signals in different MB sectors. k Comparison of VMAT distribution in the different MB sectors within the DPM neurites across 5 flies. Scale bar = 5 μm in (b-d), 1 μm in (e-g) and 20 μm in (j) unless otherwise indicated. Source data are provided as a Source Data file.
Recently, a combination of expansion microscopy and lattice light-sheet microscopy (ExLLSM) has achieved also nanometric resolution in the adult fly brain. For direct comparison between LL-CT and ExLLSM, we replicated the optical setup in ExLLSM and imaged VMAT expression in the DPM neuron within a 4x expanded fly brain with lattice light-sheet microscopy and compare the result with our data in Fig. 6 (Supplementary Fig. 12). While LL-CT and ExLLSM both produced high quality images, each has its advantage and limitation (Supplementary Table 2). Though acquisition time only a few more hours than LL-CT, ExLLSM required to image ~25,000 sub-volumes to cover the expanded fly brain. To record thousands of sub-volumes and process multiterabyte data, customized algorithms to minimize discontinuities between boundaries are required in the lattice light-sheet system. In contrast, LL-CT imaged the entire brain with only 4 sub-volumes and post-processing using ThunderSTROM could reconstruct the entire brain within a day. For quantitative analysis of protein distribution, LL-CT using standard immunostaining protocol is preferable (Fig. 6f, h). In ExLLSM, at equivalent high resolution (see Supplementary Table 2), the quality of fluorescent signal in expanded hydrogel is too capricious for quantitative analysis of protein molecules (Supplementary Fig. 12h, i). Finally, LL-CT exhibits better single-molecule sensitivity than ExLLSM since the latter method suffers from poor detection of signals with a wide intensity distribution (also demonstrated in Fig. 4c).

Discussion

By combining lightsheet, tissue clearing and spontaneously blinking fluorophores, our LL-CT method offers a powerful imaging tool for resolving nanoscale 3D structures in an intact adult fly brain. With high-speed localization of single molecules and high-dynamic-range detection of fluorescence, LL-CT enables high quality images for quantitative investigation of most, if not all, biological processes within or between subjects in details (Methods and Supplementary Tables 1 and 3).

A caveat of using Bessel beam to cover a large sample is the unwanted background induced by the concentric sidelobe. To minimize the deterioration of signal contrast, we used a weak astigmatism and excluded signals with asymmetric PSF during post-acquisition data processing (Figs. 5 and 6; Supplementary Note 1; Supplementary Figures 8 and 9). Alternatively, we can use two-photon Bessel beam to directly reduce the background in the future (see also Supplementary Figs. 13 and 14).

Another way to improve resolution is using a clearing reagent with high refractive index. With such, the overlap of signals should be reduced and an increase in localization density is expected (Supplementary Figs. 9 and 10). Several clearing reagents with high refractive index can effectively improve tissue transparency, such as FocusClear24 used in CLARITY40, uDISCO41, SeeDB25, CUBIC42 and the recently developed FlyClea43. Notably, the properties of HMSiR, such as photons per blink and the population of open-forms, are largely affected by the tissue-clearing environment. For this study, we identified ScaleView-A2 (pH 7.4) as the only commercially ready clearing reagent compatible with HMSiR. Going forward, we will need new blinking fluorophores with properties matching diverse tissue clearing reagents and exhibited different emission wavelengths for improved resolution and multi-color imaging, respectively. We need also new algorithms and processing pipelines to improve further the throughput for ever-larger datasets (Supplementary Table 3).

In conclusion, our LL-CT method provides neurobiologists with a new tool to study nano-scale structures in an intact adult fly brain with less sample perturbation. The high throughput of this process allows for more efficient and reliable statistical analyses over a large number of samples. Our method can be applied to biological studies investigating 3D cellular morphologies and protein densities in mm-scale samples. Applying this imaging technique to larger tissue, such as whole mouse brains, may become possible in the future.

Methods

Microscope optics and image acquisition. The schematic of the optical system (also demonstrated in Fig. 4c).

The laser beam passes through an axial ring mask (outer NA = 0.12 kW/cm²). Although this setup is flexible to vary the beam length using different annular masks, the maximum power measured at the back aperture of the excitation objective is merely 3 mW due to losses within the system and requiring a power density of 0.12 kW/cm² to cover a subarea along the projection neuron (250 μm²). Although this power loss caused by the mask hinders its applicability to a larger field of view. With the axicon lens combined with an achromatic lens to concentrate the laser energy into a ring pattern, we were able to focus the laser energy on the annular ring mask (outer NA = 0.26, inner NA = 0.185 formed in the excitation objective), a Bessel beam with a full-width-at-half-maximum (FWHM) width comparable to the thickness of the specimen. In the inset of Supplementary Fig. 2a, a water dipping objective lens (Nikon, CFI Apo LWD 23X, 0.8 NA, 2 mm WD for imaging in PBS, Olympus, XPLN25XMP2, 25X, 1.05 NA for imaging in ScaleView-A2), orthogonal to the illumination plane and mounted on a piezo scanner (Physik Instrumente, P-725.4CD PIFOC), used to collect the fluorescence signal, which then passes an emission filter (Semrock Filter: LP02–750RU) onto a scientiﬁc complementary metal-oxide-semiconductor (sCMOS) camera (Hamamatsu, Orca Flash4.0v3 (COMS) by a 500 μm tube lens ( Edmund 49-290, 500 mm FL/50 mm diameter; Tube Lens). A weak astigmatism, used for subdiffraction imaging in the axial direction, was introduced by inserting a pair of cylindrical lenses (Thorlabs, KL1002 RM-A, L11516RM-A) between the tube lens and camera. Although it is reported that HMSiR probe can be excited with very low power density (40 W/cm²), we found it reasonable to increase the power density to 0.1 kW/cm² to increase the photon count per localization event to reduce the localization uncertainty. The power density is estimated by dividing the power measured at the back aperture of the excitation objective to the area scanned by the Bessel beam of specified length. In the optical setup using an annular mask (outer NA = 0.26, inner NA = 0.185 formed in the excitation objective), a Bessel beam with an axial FWHM of 50 μm is generated (Supplementary Figures 2a, 3g, h). The maximum power measured at the back aperture of the excitation objective is merely 3 mW due to losses within the system and requiring a power density of 0.12 kW/cm² for imaging on the annular ring mask. The unwanted components were sequentially filtered using an annular ring mask (outer NA = 0.187, inner NA = 0.174 formed in the excitation objective) (Fig. 1, Supplementary Figs. 1 and 3). The length of the light sheet generated by the axicon lens can be extended over 200 μm to cover the entire cross-section of the fly fruit body. The power measured at the back aperture is increased to 20 mW, and the power density can thus be maintained at about 0.10 kW/cm² while extending the image area to 20,000 μm² (Fig. 2a).

Simulation of lens-axicon combination for Bessel beam generation. The field distribution of the Gaussian beam E(x, z) at the input side of the lens-axicon...
conjugated IgG were detected using Alexa Fluor 635-streptavidin (1:500 dilution; Thermo Fisher Scientific, Waltham, MA). For goat anti-rabbit labeling of HMSiR, we incubated 250 μl PBS at pH 7.2 with 0.8 μl of 10 mM HMSiR-NHS in DMSO, after which we utilized the same protocol as that used for antibody labeling. Concentrations were then measured at an absorbance of 280 nm, after which the HMSiR conjugates were stored at 4 °C until use.

**Localization image reconstruction.** Localization images were reconstructed using ThunderSTORM, an ImageJ plugin with an in-built macro for batch processing of large data. As schematically depicted in Supplementary Fig. 7, the data were initially transposed to a time-lapse series on a local workstation, after which they were transferred to Lustre storage and distributed to a three-node Torque cluster (Intel Xeon X5660 with 48 GB memory each, connected to Lustre storage). After a particle list was generated for each layer, the list was deposited into one of the clusters, after which a single list containing all localization events within the imaging volume was generated. The list was then used to render the reconstructed image for presentation and analysis. ThunderSTORM was used to perform drift correction using fiducial marker tracking or the cross-correlation method. For 3D volume rendering, the image stack was resampled to minimize discontinuities in structural integrity.

**Image rendering, segmentation, and quantification.** Color-coded depth projections of 3D images were created via ImageJ. Blinking signal in Fig. 2d was rendered in Imaris (Bitplane). We further used the Volume Rendering tool in Avizo 9.4 (Thermo Fisher Scientific Inc) for 3D visualization of whole brain dopamine neurons (Fig. 5). For single neuron segmentation, as depicted in Fig. 5, we used the Brush tool in the segmentation mode of Avizo 9.4 (Thermo Fisher Scientific Inc) to manually select the boundary of each single neuron to generate a single neuron boundary mask. We further used the arithmetic tool to intersect the original data with the manually segmented single neuron mask to generate single neuron images with the original gray scale. In Figs. 5d and 6, we used the Material Statistics module in Avizo 9.4 (Thermo Fisher Scientific Inc) to quantify the volume of the MB sectors, DPM neurons, and VMAT protein expression. This module calculates the voxel numbers inside a labeled area, which can be transformed into volumes by multiplying with a known voxel size in each image. We manually segmented the boundaries of the MB sectors using the Lasso tool in segmentation mode. We used the Magic Wand tool to select one seed within the DPM neuron and to determine a reasonable threshold for selecting the connecting voxels. We directly used the Threshold tool for whole-volume VMAT images, setting the lower bound to 78 (8-bit; 0-255).

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

**Data availability**

The materials and the data that support the findings of this study are available from the corresponding author upon reasonable request. The source data underlying Figs. 2f, 3d, 4 are provided as a Source Data file.

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**Immunohistochemistry.** Fly brains were dissected in PBS (pH 7.2) and immediately transferred to a microwave-safe 24-well plate containing 4% paraformaldehyde prepared in PBS. The plate was placed on a shaker for 25 min. The fixed tissues were then permeabilized and blocked in PBS containing 2% Triton X-100 and 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) at 4 °C overnight. Immunostaining was sequentially performed in PBS containing 1% Triton X-100 and 0.25% NGS using the following primary antibodies: mouse anti-Discs large antibody (1:250 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-GFP antibodies (1:250 dilution; Thermo Fisher Scientific Inc, A11122), rabbit anti-VMAT (1:250 dilution), and secondary antibodies including either biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:250 dilutions; Thermo Fisher Scientific Inc). The biotin-conjugated IgG were detected using Alexa Fluor 635-streptavidin (1:500 dilution; Thermo Fisher Scientific Inc) or HMStreptavidin (1:1000 dilution). Each step was carried out over the course of 2 days, with extensive washes between the steps at room temperature (25 °C). The samples were then transferred to ScaleView-A2 for 2 h before imaging, or to FocusClear/DeepClear (CellExplorer. Labs Co.) immediately before imaging (Supplementary Fig. 15).

**HMSiR conjugates.** For goat anti-rabbit labeling of HMSiR, we incubated 250 μg of goat anti-rabbit Fab(ab)2 fragment (Jackson Immunoresearch, West Grove, PA) in 0.1 M sodium borate buffer at pH 8.5 with 0.8 μl of 10 mM HMSiR-N-hydroxysuccinimide (NHS) (GORY Chemical, Bunkyo-ku, Tokyo) in dimethyl sulfoxide (DMSO) at 37 °C for 30 min, after which the sample was incubated at 4 °C overnight. The conjugated antibody was then passed through a Desal Z-25 column (empBiotech GmbH, Berlin) to remove the unconjugated HMSiR-NHS and the medium was replaced with PBS (pH 7.2). To label HMSiR with streptavidin, we incubated 125 μg of streptavidin (Sigma-Aldrich, St. Louis, MO) in 250 μl PBS at pH 7.2 with 0.8 μl of 10 mM HMBiR-NHS in DMSO, after which we utilized the same protocol as that used for antibody labeling. Concentrations were then measured at an absorbance of 280 nm, after which the HMSiR conjugates were stored at 4 °C until use.

**References**

1. Hell, S. W. & Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* 19, 780–782 (1994).
2. Gustafsson, M. G. L., Agard, D. A. & Sedat, J. W. Doubling the lateral resolution of wide-field fluorescence microscopy using structured illumination. *SPIE* https://doi.org/10.1117/12.3814890 (2006).
3. Betzig, E. et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313, 1642–1645 (2006).
4. Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* 3, 793–795 (2006).
5. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science* 319, 810–813 (2008).
6. Pavan, S. R. et al. Three-dimensional, single-molecule fluorescence imaging beyond the diffraction limit by using a double-helix point spread function. *Proc. Natl. Acad. Sci. U. S. A.* 106, 2995–2999 (2009).
7. Vaziri, A., Tang, I., Shroff, H. & Shank, C. V. Multilayer three-dimensional super-resolution imaging of thick biological samples. *Proc. Natl Acad. Sci. USA* 105, 20221–20226 (2008).
8. Abrahamsson, S. et al. Fast multicolor 3D imaging using aberration-corrected multifocus microscopy. *Nat. Methods* 10, 60–63 (2013).

**Fly brain mounting.** The samples were loaded on a 5-mm round, glass coverslips (Warner Instruments) in 100 nl of Cell-Tak™ (Corning®). During the image acquisition, the seesaw objectives were immersed in a drop of water to maintain optical clarity. All experiments were conducted at room temperature.

**Fly stocks.** Fly stocks were raised on cornmeal food at a temperature of 25 °C and a relative humidity of 70% under a 12 h light/dark cycle. The following fly lines were used in the current study: Fruitless-Gal4 (66696, Bloomington Drosophila Stock Center) was used to label the neck neurons, MZ19-Gal4 (34497, Bloomington Drosophila Stock Center) for the factory projection neurons, 12862-Gal4 (111501, DGLRC) for the giant fiber neurons, TH-Gal4 (8848, Bloomington Drosophila Stock Center) for the dopaminergic neurons, VT6426-Gal4 (204311, VDRC) for the DPM neurons, UAS-mCD8:GFP (5137 and 5310, Bloomington Drosophila Stock Center) and UAS-GaMP8 (42747, Bloomington Drosophila Stock Center) were used as reporters for expression. UAS-Flp (F1) (Dr. Michael Bellen, Howard Hughes Medical Institute, Ashburn, VA) was used to label Dscam in the Gal4-labeled neurons, and UAS-VMAT RNAi (v104072 and v4856, VDRC) was used to assess the downregulation of VMAT protein expression in the DPM neurons.
9. Cella Zanacchi, F. et al. Live-cell 3D super-resolution imaging in thick biological samples. Nat. Methods 8, 1047–1049 (2011).
10. Legant, W. R. et al. High-density three-dimensional localization microscopy across large volumes. Nat. Methods 13, 359–365 (2016).
11. Lu, C.-H. et al. Lightsheet localization microscopy enables fast, large-scale, and three-dimensional super-resolution imaging. Commun. Biol. 2, 177 (2019).
12. Planchon, T. A. et al. Rapid three-dimensional isotropic imaging of living cells using intramolecular spirocyclization. Nat. Methods 8, 417–423 (2011).
13. Chen, B.-C. et al. Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. Science 346, 1257998 (2014).
14. Spuhler, I. A., Conley, G. M., Scheffold, F. & Sprecher, S. G. Super resolution imaging of genetically labeled synapses in Drosophila brain tissue. Front. Cell. Neurosci. 10, 142 (2016).
15. Ke, M.-T. et al. Super-resolution mapping of neuronal circuitry with an index-optimized clearing agent. Cell Rep. 14, 2718–2732 (2016).
16. Long, X., Colonell, J., Wong, A. M., Singer, R. H. & Lionnet, T. Quantitative mRNA imaging throughout the entire Drosophila brain. Nat. Methods 14, 703–706 (2017).
17. Uno, S. N. et al. A spontaneously blinking fluorophore based on intramolecular spirocyclization for live-cell super-resolution imaging. Nat. Chem. 6, 681–689 (2014).
18. Rioux, M., Tremblay, R. & Belanger, P. A. Linear, annular, and radial focusing with axicons and applications to laser machining. Appl. Opt. 17, 1532–1536 (1978).
19. Fairenbach, F. O. & Rohrbach, A. Propagation stability of self-reconstructing Bessel beams enables contrast-enhanced imaging in thick media. Nat. Commun. 3, 632 (2012).
20. Gao, L., Shao, L., Chen, B. C. & Betzig, E. 3D live fluorescence imaging of cellular dynamics using Bessel beam plane illumination microscopy. Nat. Protoc. 9, 1083–1101 (2014).
21. Heilemann, M. et al. Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. Angew. Chem. Int. Ed. Engl. 47, 6172–6176 (2008).
22. Ovesny, M., Krizek, P., Borkovec, J., Svindrych, Z. & Hagen, G. M. ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinformatics 30, 2389–2390 (2014).
23. Richardson, D. S. & Lichtman, J. W. Clarifying tissue clearing. Nat. Methods 9, 1049 (2011).
24. Chiang, A. S. et al. Three-dimensional mapping of brain neuropils in the Drosophila brain. Nat. Methods 10, 618–627 (2013).
25. Nieuwenhuizen, R. P. et al. Measuring image resolution in optical nanoscopy. Appl. Opt. 44, 6172–6176 (2005).
26. Shim, S. H. et al. Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. Proc. Natl. Acad. Sci. U. S. A. 109, 13978–13983 (2012).
27. Nieuwenhuizen, R. P. et al. Measuring image resolution in optical nanoscopy. Nat. Methods 10, 557–562 (2013).
28. Banterle, N., Bui, K. H., Lemke, E. A. & Beck, M. Fourier ring correlation as a simulation tool. Cell 162, 246–257 (2015).
29. Chiang, A. S. et al. Three-dimensional mapping of brain neuropils in the cockroach, Diploptera punctata. J. Comp. Neurol. 440, 1–11 (2001).
30. Hama, H. et al. Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat. Methods 10, 557–562 (2013).
31. Aso, Y., Rubin G. M. Dopaminergic neurons write and update memories with axonal plasticity. Elife. 5, e16135 (2016).
32. Tauber, J. M., Vanlandingham, P. A. & Zhang, B. Elevated levels of the vesicular monoamine transporter and a novel repetitive behavior in the Drosophila model of fragile X syndrome. PLoS. One. 6, e27110 (2011).
33. Yu, D., Keene, A. C., Srivatsan, A., Waddell, S. & Davis, R. L. Drosophila DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning. Cell 123, 945–957 (2005).
34. Waddell, S., Armstrong, J. D., Kitamoto, T., Kaiser, K. & Quinn, W. G. The amnesiac gene product is expressed in two neurons in the Drosophila brain that are critical for memory. Cell 103, 805–813 (2000).
35. Keene, A. C., Korsching, S. I. & Waddell, S. Drosophila dorsal paired medial neurons provide a general mechanism for memory consolidation. Curr. Biol. 16, 1524–1530 (2006).
36. Hige, T., Aso, Y., Rubin, G. M. & Turner, G. C. Plasticity-driven individualization of olfactory coding in mushroom body output neurons. Nature 526, 258–262 (2015).
37. Pai, T. P. et al. Drosophila ORB protein in two mushroom body output neurons is necessary for long-term memory formation. Proc. Natl. Acad. Sci. USA. 110, 7789–7793 (2013).
38. Gao, R. et al. Cortical column and whole-brain imaging with molecular contrast and nanoscale resolution. Science 363, pii eaau3302 (2019).
39. Truong, T. V., Supatto, W., Koos, D. S., Choi, J. M. & Fraser, S. E. Deep and fast live imaging with two-photon scanned light-sheet microscopy. Nat. Methods 8, 757–760 (2011).
40. Chung, K. et al. Structural and molecular interrogation of intact biological systems. Nature 497, 332–337 (2013).
41. Pan, C. et al. Shrinkage-mediated imaging of entire organs and organisms using UIDSCO. Nat. Methods 13, 859–867 (2016).
42. Susaki, E. A. et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157, 726–739 (2014).
43. Pende, M. et al. High-resolution ultramicroscopy of the developing and adult nervous system in optically cleared Drosophila melanogaster. Nat. Commun. 9, 4731 (2018).

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Author contributions
L.-A.C. and C.-H.L. planned and performed the imaging experiments and image processing. L.-A.C., C.-H.L., Y.-T.L., and B.C.C. designed and constructed the LLM-CT. S.-M.Y. produced the HSMIR conjugates. Y.-T.L. designed the automated blinking imaging processing protocol and software. K.-L.F. and L.-A.C. performed the VMAT experiments. L.-A.C., C.-H.L., and Y.-C.T. performed the image analysis. W.-K.C. performed the graphic design and system maintenance. L.A.C. and W.-C.W. performed data visualization. S.-W.C. performed the simulation. P.C., Y.-K.H., T.-K.L., A.-S.C., and R.-C.C. planned and managed the project. L.-A.C., C.-H.L., B.-C.G., and A.-S.C. wrote the paper.

Competing interests
The authors declare no competing interests.

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