Fast, 3D isotropic imaging of whole mouse brain using multi-angle-resolved subvoxel SPIM

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Abstract

The recent integration of light-sheet microscopy and tissue-clearing has facilitated an important alternative to conventional histological imaging approaches. However, the in toto cellular mapping of neural circuits throughout an intact mouse brain remains highly challenging, requiring complicated mechanical stitching, and suffering from anisotropic resolution insufficient for high-quality reconstruction in three dimensions. Here, we propose the use of a multi-angle-resolved subvoxel selective plane illumination microscope (Mars-SPIM) to achieve high-throughput imaging of whole mouse brain at isotropic cellular resolution. This light-sheet imaging technique can computationally improve the spatial resolution over six times under a large field of view,
eliminating the use of slow tile stitching. Furthermore, it can recover complete structural information of the sample from images subject to thick-tissue scattering/attenuation. With Mars-SPIM, we can readily obtain a digital atlas of a cleared whole mouse brain (~7 × 9.5 × 5 mm) with an isotropic resolution of ~2 μm (1 μm voxel) and a short acquisition time of 30 minutes. It provides an efficient way to implement system-level cellular analysis, such as the mapping of different neuron populations and tracing of long-distance neural projections over the entire brain. Mars-SPIM is thus well suited for high-throughput cell-profiling phenotyping of the brain and other mammalian organs.

Introduction

The comprehensive understanding of complex cellular connections in the whole mammalian brain is one of the fundamental challenges in neuroscience. To unravel the various neuronal profiles of different physiological functions in the whole brain, three-dimensional (3-D) high-resolution imaging is required over a mesoscale sized volume\textsuperscript{1,2}. However, creating such a large-scale brain dataset has posed a big challenge for current 3-D optical microscopy methods, all of which show relatively small optical throughputs\textsuperscript{3,5}. Furthermore, light scattering and attenuation are outstanding issues for the turbid tissues that limit the extraction of signals from deep brain. To address these issues, 3-D tile stitching combined with brain sectioning has been a popular strategy for obtaining mammalian brain atlases, which can be a meaningful platform for mapping neuronal populations, activities, or connections over the entire brain\textsuperscript{6}. For example,
sequential two-photon (STP) microscopy can three-dimensionally image the brain at subcellular high resolution7-15, but at the cost of long acquisition times of up to several days and a high-maintenance system setup. The advent of light-sheet fluorescence microscopy16-22 (LSFM) in conjunction with tissue-clearing23-28 eliminates the need for complicated mechanical slicing of samples by instead applying nondestructive optical sectioning. Although LSFM still needs repetitive image stitching to achieve high lateral resolution over a large field of view (FOV), its use of wide-field detection results in higher imaging speeds compared with the point-by-point scanning of epifluorescence methods. A few well-known derivations of LSFM, for example, selective plane illumination microscopy (SPIM), have recently been used for mouse brain imaging with balanced speed and spatial resolution. However, the axial extent of the plane illumination in SPIM has to be compromised to its lateral illumination FOV so that an anisotropic axial resolution (typically of 5 to 20 μm, depending on the size of the mosaic patch needing to be illuminated) can be yielded for whole-brain scale imaging29,30. As a result, it is difficult to resolve fine neuronal structures and connections in three dimensions, as can be achieved by conventional epifluorescence methods such as micro-optical sectioning tomography13,14 (MOST) and STP excitation microscopy10-12. Furthermore, even with the much larger imaging depth enabled by tissue clearing30-33, photon absorption and scattering still occur in the clarified tissues of whole mammalian organs. These cause noticeable deterioration of signals from deep tissues. Recently, multi-view fusion techniques34-36, which have previously been used in the imaging of small live embryos37,38, have also been applied to excised mouse brains. These can
improve the relatively low axial resolution and suppress deep tissue scattering\textsuperscript{39}. However, for the direct imaging of mesoscale intact organs, the lateral resolution of SPIM systems, being 5 $\mu$m at its best, is insufficient to visualize single cells under a large FOV of over 5 mm. In such circumstances, multi-view techniques cannot overcome the lateral resolution limit determined by the detection optics. However, if multi-view techniques were to be combined with repetitive image stitching, the throughput advantage of LSFM would be significantly reduced, as well as the photon utility.

Here, we present a whole-brain mapping pipeline, termed multi-angle-resolved subvoxel SPIM (Mars-SPIM), which can image the whole mouse brain at an isotropic voxel resolution of $\sim$1 $\mu$m with a high throughput rate of half an hour per brain. This imaging strategy combines our sub-voxel-resolving computation\textsuperscript{40} with multi-view Bayesian deconvolution\textsuperscript{36,41} to achieve fast and accurate reconstruction of a whole brain with isotropically improved resolution. Unlike conventional whole-brain imaging methods that use stepwise z-scanning and 3-D tile stitching, Mars-SPIM directly records low-resolution whole-brain images using a continuous non-axial scanning method. This unique scanning mode provides a high acquisition rate, and meanwhile encrypts sub-resolution shifts into raw images, which could be further processed by the multi-view sub-voxel-resolving computation. Furthermore, this computation pipeline is parallelized with multi-GPUs to achieve a high reconstruction throughput (gigavoxels per minute) that matches the fast image acquisition. By reconstructing a single-cell-resolution whole-brain atlas, we demonstrate successful brain-wide tracing
of single neural projections and the counting of different neuronal populations over the entire brain. Mars-SPIM shows spatial-temporal performance superior to the other available techniques for large-scale cell profiling, where sample size, spatial resolution, and imaging throughput are all highly valued. It is therefore suitable for system-level cellular analysis of brain or other whole organs.

**Results**

**Mars-SPIM setup and characterization**

We developed a Mars-SPIM system, with a low-profile setup with wide-FOV illumination and detection sufficient to cover the entire mouse brain (see Methods and Supplementary Figures 1 and 2). Under a certain view, the brain sample to be imaged is continuously scanned across the laser-sheet along a non-axial direction. The camera is synchronized to sequentially record a stack of plane images with a step-size significantly smaller than the laser-sheet thickness. This non-axial scanning mode of the Mars-SPIM encodes the low-resolution (LR) raw image stack with sub-voxel spatial shifts, which are then used to reconstruct high-resolution (HR) images through the application of an sub-voxel-resolving (SVR) algorithm (Supplementary Figure 3). The sample is then rotated along the y-axis and non-axially imaged under multiple views (Figure 1a). After the SVR computation has generated a series of HR images with anisotropic volumes for all the views, a neuron-feature-based registration followed by a multi-view Bayesian deconvolution (MVD) procedure is applied to accurately align the multiple SVR volumes, compute their conditional probabilities, and finally produce
an output with recovered complete signals and isotropic super-resolution in three dimensions. This SVR-MVD fused computation pipeline is further illustrated in Figure 1b and Supplementary Figure 3.

We used fluorescent microbeads with a diameter of ~500 nm as a point source to characterize the Mars-SPIM system (Figure 1c and Supplementary Figure 4). For each view, the microbeads were scanned by a ~12-μm thick (full width at half maximum value; FWHM) laser-sheet with 280 nm step-size, and were detected by a 4×/0.16 objective. This non-axial scanning process (10 degrees to the x-z and y-z planes) generated sub-resolution shifts of 48 and 272 nm in the lateral and axial directions respectively. Thirty-four groups of LR image volumes representing the standard resolution of the system optics (voxel size: 1.625 × 1.625 × 4.5 μm) were extracted from the raw image sequence to compute the HR images. The raw LR, single-view SVR, conventional MVD, and SVR-MVD results are compared in Figure 1c. The line intensity profiles of the resolved beads are plotted in Figure 1d and e, to indicate the lateral and axial resolutions of these methods. The achievable lateral and axial FWHMs of the SVR-MVD are improved from ~4.2 μm and 12 μm in the raw image to isotropic values of ~1.4 μm, which are superior to both the single-view SVR (1.7 μm and 4.5 μm) and conventional MVD (isotropic 3.4 μm).

We then demonstrated the imaging capability of the Mars-SPIM using clarified brain tissue from a transgenic mouse (thy1-GFP-M). The brain sample was optically sectioned by a 15-μm laser-sheet and imaged using a 4×/0.28 objective and a high-speed camera (Hamamatsu Flash 4.0 v2) at a rate of 50 frames per second. The brain
sample was translated at a non-axial step-size of 280 nm (4 × 4 × 2 enhancement) and
rotated 45 degrees for each new view. Eight different view image stacks were rapidly
recorded in a total time of around 20 minutes. The raw image volume of each view was
acquired at the limited resolution of the system optics, and hence the densely-packed
neuronal fibers remained unresolvable (Figure 2a). The SVR procedure for each view
was then started with an initial guess, which was simply a ×4 interpolation of one of
the subdivided LR groups, and the process iteratively converged to the HR solution
(data not shown). Then, in the multi-view registration step, the neuronal cell bodies
were recognized as features to establish correspondences, instead of the beads. This
cell-based registration was verified to be as accurate as the bead-based one
(Supplementary Figures S5 and 6), while at the same time producing a cleaner
visualization (Supplementary Videos 1 and 2). Figure 2c shows the final Mars-SPIM
result with a reconstructed voxel size of 0.4 μm. This result is further compared with
conventional multiview SPIM (Mv-SPIM, Figure 2b), high magnification SPIM
(20×/0.45 with ~6.5-μm laser-sheet, Figure 2d) and confocal microscopy (10×/0.4,
Figure 2e). The linecuts through the transverse plane of the neuron dendrite (Figure 2a-
d) using each method reveal significantly improved resolution with the Mars-SPIM,
which surpasses both the 20×-SPIM result with anisotropic resolution in the
longitudinal direction, and the Mv-SPIM result with insufficient overall resolution
(Figure 2g). With the substantially enhanced isotropic resolution, two giant pyramidal
neurons could be finely segmented across a large volume (≈400 gigavoxels for the
entire sample), as shown by the blue and red colors in Figure 2f and g. We note that
besides the increased space-bandwidth product (SBP; volume size divided by resolution)\textsuperscript{4, 5}, the Mars-SPIM also shows an improvement in the signal-to-noise ratio (SNR), which can help to discern weak signals from the strong background signal of thick tissue (Supplementary Figure 7). Furthermore, we tested different numbers of views to verify that eight views formed a good balance between the data size/throughput and performance (Supplementary Figure 8). Mars-SPIM can thus be considered as a light-sheet microscope that is less vulnerable to spherical aberration and light scattering in thick tissue, and combines a large FOV with high-resolution advantages that are difficult to achieve with previous methods. From another perspective, the stitching-free continuously-scanning mode exhibits a higher acquisition throughput than other stitching-based methods, as well as lower photobleaching. In Figure 2h, we rate the imaging performances of standard 4× SPIM, 20× SPIM, 10× confocal and our 4× Mars-SPIM through comparisons of the system complexities, imaging speeds, photobleaching rates, and spatial resolutions (also see Supplementary Figure 9 and Supplementary Table 1). Compared with the confocal microscope (10×), the Mars-SPIM gains advantages in imaging depth and axial resolution (Supplementary Figures 10 and 11). Besides the well-balanced volumetric resolution, the Mars-SPIM yields the highest effective throughput at ~400 gigavoxels SBP in a 20-minute acquisition. Mars-SPIM also eliminates the need for mechanical stitching, slicing, high-maintenance optics, and precisely-modulated illumination, instead using a relatively simple light-sheet setup and fast GPU-based computation to address the general challenge of high-throughput high-resolution 3-D microscopy that was originally coupled to the physical
limitations of a system’s optics. In the following whole-brain applications, this underlying robustness allows the Mars-SPIM prototype to image the entire thick organ with high spatial-temporal performance while maintaining a simple set-up.

High-throughput, in toto imaging of whole mouse brain at high resolution

An 8-week-old whole mouse brain (Tg: thy1-GFP-M) was optically cleared using the a-uDISCO method\(^4\), before being imaged by the Mars-SPIM. The brain shrank in size from \(\sim 9.3 \times 14 \times 7.1\) mm to \(\sim 7 \times 9.5 \times 5\) mm after clearing (Figure 3a). Despite the use of tissue clearing, light attenuation/scattering from deep tissue remained a big challenge for the complete imaging of the whole brain (Supplementary Figure 13). However, brain-wide biomedical applications such as cell population mapping and neuronal projection tracing intrinsically need high spatial resolution across a large area, hence highlighting the significance of the Mars-SPIM method. Experimentally, the whole brain was imaged under a low-magnification setup of 25-\(\mu\)m light-sheet illumination plus \(\times 2.2\) wide-field detection, and therefore only required to be stitched twice because of the large FOV. The brain was then rotated for each view of the nonaxial scanning (950 nm step-size, 5500 frames in 110 s), with a total of 16 views of raw stacks being rapidly obtained in around half an hour. Using the above mentioned SVR-MVD procedure, we successfully reconstructed the entire brain at an isotropic voxel size of 0.975 \(\mu\)m. Figure 3b shows a reconstructed volume rendering of the whole brain (400 gigavoxels, MIP mode). The horizontal planes (x-y) at different depths (Figure 3c-e) and coronal planes (x-z) at different heights (Figure 3f-h) are extracted from the
reconstructed brain volume and compared with the conventional SPIM results. It is
obvious that the Mars-SPIM shows remarkable improvements in resolution, contrast,
and signal integrity. Vignettes of high-resolution Mars volumes of five selected areas
(Figure 4a), including the left and right cortex, hippocampus, thalamus and cerebellum,
are shown in Figure 4b-f respectively. The strong efficacy of the neural signal recovery,
as well as the resolution enhancement by Mars-SPIM, are further illustrated in
Supplementary Figure 13, in which a full coronal plane acquired by conventional SPIM
and experiencing strong scattering from both illumination (x) and emission (z) is
compared with the same plane acquired by Mars-SPIM. By quickly creating a cellular-
resolution brain atlas encompassing 400 gigavoxels across a large volume of over 300
mm$^3$ (post-computation time ~12 hours), the Mars-SPIM enables high-throughput
analysis of massive neurons at the whole brain level, which are otherwise spatially or
temporally more challenging using regular light sheet microscopes. (Figure 2h,
Supplementary Figure 14).

Whole-brain visualization and segmentation

Using the Mars-SPIM reconstruction of the whole brain (8-week-old mouse), we
explored the neuronal cyto-structures in various brain sub-regions, and precisely traced
the interregional long-distance projections of neurons which is crucial for
understanding the functionality of the brain (Figure 5, Supplementary Videos 3, 4 and
5). After the Mars-SPIM reconstruction of the whole brain, we used an adaptive
registration method$^{43-45}$ to three-dimensionally map the brain to the standard Allen
Brain Atlas (ABA). The brain was first re-orientated from horizontal view to coronal view and automatically pre-aligned to the ABA using Elastix\textsuperscript{43}. This pre-aligned brain was then resized into LR and HR groups, as shown in Figure 5a step 2. Next, we finely registered the LR group to the ABA, and obtained the transform correspondence (step 3), which was then applied to the HR group to obtain a registered and transformed HR brain (step 4). This mapped brain atlas was finally visualized in Imaris to facilitate the neuron analysis (Figure 5a).

With the creation of the atlas, the neurons localized to different encephalic regions (such as cortex, hippocampus, cerebellum and midbrain) could be identified (Figure 5b), and in toto mapped out at a whole-brain scale (Figure 5c). Then, the neuron population and the density in different encephalic regions were quantified by calculating the volume of the regions and counting the identified cell bodies within them (Figure 5d). The results show that among the 12 primary regions, the hippocampus formation had the highest neuron density of 4600 cells/mm\textsuperscript{3}, which is consistent with prior knowledge\textsuperscript{46}. It should be noted that the current low-number counting results were obtained using a \textit{thy1-GFP-M} transgenic mouse, in which GFP signal is expressed by less than 10\% of all motor axons, retinal ganglion cells, lumbar dorsal root ganglia, and cortex\textsuperscript{47}. According to the registered HR images, we could trace the neuron projections passing through different brain regions. The whole brain data were volumetrically rendered with several sub-regions being segmented in different colors. Figure 5e shows horizontal and coronal views of the volume renderings. The trajectories of six long-distance (LD) projection neurons were successfully traced and
annotated in the digital whole-brain, revealing how they were broadcast across the
different regions of the brain (Figure 5f). Given the fact that this quantitative analysis
was implemented using a thy1-GFP-M mouse with a large number of neurons being
labeled, this procedure should be more efficient if the mouse brain were to be labelled
more specifically, such as with a virus tracer. Through the above mentioned
demonstration, we have shown the potential of our strategy for imaging-based
quantifications of the whole-brain, or other whole-organ-level analyses, which are
crucial for a variety of applications in histology, pathology, and neuroscience.

Discussion

Mars-SPIM can computationally surpass the resolution limit of a regular light-sheet
microscope and suppress the light scattering/attenuation that often exists in thick-tissue
imaging. Unlike mechanical slicing and tile stitching, which require complicated
operations, this strategy provides a simple and efficient way to achieve high-throughput
whole-brain mapping at a single-cell resolution. The use of simple optics in the Mars-
SPIM offers an ultra-large FOV of hundreds of mm³, facilitating direct coverage of the
whole brain (or other whole organs). Its stitching-free continuous scanning mode
greatly reduces the acquisition time for such tissue volumes from several hours with
traditional methods to several minutes. Complementing the rapid data acquisition, a
highly GPU-parallelized SVR-MVD computation flow is followed to reconstruct the
super-resolved 3-D brain atlas at a high throughput time of a few hours. In our
demonstration, the quickly reconstructed digital mouse brain acquired by Mars-SPIM
presents an isotropic cellular resolution (~2 μm) with three- to ten-fold improvement in
resolution compared with conventional macro-view SPIM. It should also be noted that
this Mars-SPIM strategy can be applied to most existing light-sheet microscopes using
a simple retrofit, and can expand the optical throughput for fast, high-resolution
mapping of whole biological specimens without necessarily increasing the system
complexity. Thus, it can be characterized as a high-throughput 3-D imaging method
with a simple and cost-effective setup. Furthermore, the Mars-SPIM imaging in
conjunction with efficient brain registration can form a pipeline for creating an isotropic
whole-brain atlas, with which brain-wide quantitative analysis (e.g., neuron populations,
densities, and long-distance neuronal projections) could be easily implemented. In
combination with recent advances in specimen preparation techniques, such as
fluorescence-friendly tissue clearing, virus-based sparse labelling and transgenic
animal models, the Mars-SPIM could be more powerful in enabling various cellular
analyses of neural systems. Besides whole-brain mapping, we believe the Mars-SPIM
method could improve the efficiency of imaging other mammalian organs, such as lung,
kidney, and heart, and be of benefit for a wide variety of biomedical applications.
Furthermore, its ability to readily accomplish cellular imaging of mesoscale organisms
at hundreds of gigavoxel SBP renders Mars-SPIM a widely applicable tool for cellular
profiling, phenotyping, or sample screening assays in histology, pathology, and
developmental biology, in which both large-scale statistics and cellular details are often
desired for whole-tissue-level study.
Methods

Mars-SPIM imaging setup

A fiber-coupled DPSS laser (CNI laser, 488 nm, single-mode fiber) was used for excitation source. The laser was first collimated into a Gaussian beam with diameter ~3.3 mm (Thorlabs, F280FC-A). Then a sandwich structure containing a convex lens (f = 50 mm, Thorlabs, AC254-050-A) and two cylindrical lenses (f = 30 mm and 150 mm, Thorlabs, LJ1212L1, LJ1934L1) was designed to transform the round beam into an elliptical shape. The expansion ratio in short (x) and long (y) axis was ×0.6 and ×3, respectively, forming an elliptical beam with size of 10 by 2 mm (Supplementary Figure 2). A pair of adjustable mechanical slits (0-8 mm aperture, Thorlabs, VA100C/M) were placed orthogonally to further truncate the beam and thereby tune the height and thickness of the laser-sheet. The modulated elliptical beam was equally split into two parts via a 50/50 prism (Thorlabs, CCM1-BS014/M), to form two opposite beams, which will be further used to illuminate the sample from dual sides. A dual-side optical sectioning of the whole brain sample was finally formed by using two symmetric combination of cylindrical lens (Thorlabs, LJ1695RM) and illuminating objective (Olympus, 4×/0.10). The laser sheet had a widely tunable range from 5 to 50 μm in thickness and 0.5 to 10 mm in height.

Unlike regular detection setup applied in SPIM, we specially used a 4×/0.28 objective in conjunction with an ED Plan 1× tube lens to construct an infinity-corrected, wide-field, and large-aperture detection path (equivalent magnification ×2.2). Compared to the conventional infinity-corrected low-magnification detection, e.g. 2× Nikon
objective plus 200 mm focal length tube lens, this setup can collect much more fluorescent signals under a large illumination range due to the larger aperture (Supplementary Figure S9). A four-degree-of-freedom motorized stage (x, y, z translation and rotation around the y-axis, Thorlabs and Phidgets Inc.) integrated with a pair of customized tilting plates (10° inclined surface) was constructed for sample mounting, rotating at multiple angle of views, and scanning across the laser sheet in an off-detection-axis direction (Supplementary Figure S1). A digital camera (Hamamatsu Orca Flash 4.0 v2 plus, or Andor Zyla 5.5) continuously records the images from the consecutively illuminated planes at a high speed up to 50 full frames per second.

Sample preparation

Tissue clearing is an essential procedure before imaging. Here we used a-uDISCO method to clear the brains of 8-weeks thy1- GFP mice (line M, Jackson Laboratory). In the brain block experiment, to preserve the fluorescence and avoid photobleaching, the cleared brain was embedded into a specific formulated resin, the refractive index of which was equal to the index-matched immersion. For conducting bead-based registration, fluorescent beads (Lumisphere, 1%w/v, 500 nm, SiO₂) were mixed around the sample in the resin. 10 μl of bead stock solution was centrifuged at 1200 rpm for 3 minutes with the water-phase supernatant being removed and replaced with 20 μl methanol. Then the methanol-based bead solution was mixed into the resin to form the bead-resin mixture, which was finally poured into a tube mold with the brain specimen embedded. The tube containing beads, resin and sample was stored in a dark
place for 2-3 days till it was solidified for LSFM imaging. For the cell body-based registration of brain block, the sample was directly embedded in the resin without the procedure of mixing bead. For whole brain imaging, the brain was dissected with keeping an ~5 mm long spinal cord (Figure 3a). After optical clearing, the sample could be mounted to the stage via connecting the harden spinal cord with the beam shaft of the rotating motor (Figure 3a).

Multi-view imaging acquisition

The brain samples were scanned under eight views. Each times of scanning was executed along a non-axial direction with a step-size significantly smaller than the thickness of light sheet. Under continuous scanning mode, this value was determined by the scanning velocity and camera frame rate, varying from 0.3 μm (for brain block) to 1 μm (for whole brain), depending on the optical configuration. The corresponding acquisition time for total 8 views was around 20 and 30 minutes, respectively. The high magnification SPIM images for comparison were obtained using 20× objective plus a thinner light sheet of ~6.5 μm. Finally, hundreds of gigabyte raw images were transferred to a high-capacity SSD RAID of the workstation via the camera link cable.

SVR-MVD reconstruction

SVR computation combined multi-view Bayesian deconvolution was implemented to achieve isotropic high-resolution reconstruction of whole mouse brain. Under each view, a series of low-resolution image stacks were extracted from the oversampled raw
All low-resolution stacks were correlated with each other in terms of sub-voxel-resolution displacements and spatially registered to a high-resolution image stack with an oblique, sub-voxel shift. The multiple low-resolution images and an initial guess of high-resolution image were input into a maximum-likelihood-estimation based computation model to iteratively obtain a converged high-resolution image. This high-resolution estimate was corrected into the final reconstruction by a voxel re-alignment, which recovers the accurate shape of the sample. In practice, the SVR algorithm was applied in parallel to quickly obtain resolution-enhanced results for all the views.

After SVR processing for each view, the resolution-enhanced results were regarded as input for multi-view reconstruction in Fiji program. Similar with the bead-based registration method, here the neuron cell bodies were recognized as fiducial markers to establish the correspondences between each two views. Then all the SVR views could be precisely registered using these correspondences. A multi-view Bayesian deconvolution was applied at the final step to rationally gather the information from all the registered SVR views, and generate an output image with containing complete sample information as well as enhanced isotropic resolution. An improved Richardson-Lucy deconvolution was used to obtain the final deconvolved image with faster convergence. Furthermore, this SVR-MVD computation procedure could be highly parallelized with GPU-based acceleration. The whole processing time for an entire brain atlas (400 gigavoxels) was ~12 hours on a workstation equipped with dual E5-2630 CPU, quad GeForce 1080Ti GPU and 1T memory. This time consumption could be further reduced with employing more powerful computation units.
Confocal microscope and UltraMicroscope imaging

The confocal images are taken by Olympus FV3000 under 10× objective, with a voxel size of 0.8 × 0.8 × 2 μm at 0.5 Hz (Supplementary figure 9, 10 and 11). The whole brain images taken by commercial light-sheet microscope (UltraMicroscope, LaVision BioTec) are acquired at 1.6× and 8× magnification, which take about 20 minutes and 450 minutes, respectively (Supplementary figure 14).

Software

The synchronization of scanning and acquisition was accomplished by LabVIEW (National Instruments). The SVR processing was implemented by customized code and computed with CUDA acceleration. The multi-view registration was processed in Fiji. The planar x-z planes of PSFs were performed using ImageJ. The 3-D rendering of PSFs were visualized by Amira (Visage Imaging). The visualizations of neuron imaging, including planar projections, 3-D renderings, neuron tracing, were performed by Imaris (Bitplane).

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Author contributions

P.F. initiated the investigation, designed the project. J.N., S.L., Y.P., T.Y., and F.Z. developed the programs, carried out the imaging experiments and implemented quantifications. Y.L., W.M. and T.Y. prepared the samples and helped to analyze the data. P.F., D.Z., and S.Z. contributed, refined the concept and wrote the paper.

Additional information

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Conflict of interest

The authors declare that they have no conflict of interest.

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**Figure 1. Illustration and characterization of Mars-SPIM.** (a) The schematic of Mars-SPIM. A low NA objective generates a relatively thick light-sheet and a low magnification objective collects fluorescence with large FOV. The operation of the sample includes four degrees of freedom as: x axis, y axis, non-axial direction s and rotation (along y axis). Unlike standard z-scan SPIM, here the 3-D scanning vector \( s \) (red) has a certain angle \( \theta \) with respect to the x, y, z axes. This off-z-axis scanning strategy in conjunction with a small step size encrypts the raw image stack with lateral and axial sub-voxel-size shifts, which can be used to reconstruct a resolution-enhanced volumetric image via SVR procedure. To suppress the light scattering from the deep tissues and achieve isotropic 3-D resolution, the whole brain sample is rotated and imaged under eight views. (b) The
work flow of SVR-MVD procedure which can *in toto* reconstruct the whole brain at isotropically enhanced resolution. It majorly includes: first, the SVR computation for multi-view, sub-voxel-scanned raw images; second, feature-based registration of SVR-processed images; and third, a Bayesian-based deconvolution that generates the final output based on multi-view SVR images. (c)

The resolution comparison between single-view raw image, SVR only, MVD only and SVR-MVD, via resolving sub-resolution fluorescent beads (~500 nm diameter). x-z images show the lateral and axial extents of the resolved beads (red circles). (d)-(e) The intensity plots of the linecuts through the resolved beads along the lateral and axial directions, respectively. The SVR-MVD shows an obviously highest isotropic resolution at ~1.4 μm, which is compared to ~4.2 (lateral) and 12 μm (axial) in raw image. Scale bars: 5 μm in (c).
Figure 2. Mars-SPIM demonstration on thy1-GFP-M brain block. (a) Visualization of the neurons at cortex area by conventional SPIM using 4×/0.28 objective plus a 15 μm laser-sheet. The voxel size is 1.625 by 1.625 by 6 μm. (b) Conventional multiview SPIM (Mv-SPIM) results with an isotropic voxel size of 1.625*1.625*1.625 μm. (c) The Mars-SPIM results of the same neurons, with an isotropic reconstructed voxel size of 0.41*0.41*0.41 μm. (d) Comparison from high-resolution SPIM using 20×/0.45 air objective plus 6.5 μm light-sheet. Due to the increasing...
spherical aberration under higher magnification, the SNR of the images is obviously decreased. (e)

The intensity plot of the dash lines transversely across a few neural fibers in (a) - (d). It shows Mars-

SPIM has the narrowest peaks which indicate highest resolving power in practice. (f) The SVR-

MVD reconstruction of the entire brain block with size around 3 by 3 by 3 mm. As a result of finer

reconstruction, two pyramidal neurons with dendrites and axons are finely segmented, shown as (i)

and (ii). (g) Vignette high-resolution view of the segmented neuron (ii) in (f), showing the clearly

resolved fibers. (h) The radar map that compares the system simplicity, imaging throughput, photo-

bleaching, and spatial resolutions of four methods. The values are outputted by the logarithm and

normalized. Scale bars are 50 μm in (a)-(e).
Figure 3. Comparison of whole mouse brain image by conventional SPIM and Mars-SPIM.

(a) The photographs of an adult mouse brain (8 weeks) before and after a-uDISCO clearing. (b) The 3D reconstruction of cleared whole mouse brain. With obtaining optically cleared brain for light-sheet imaging, our Mars-SPIM system rapidly provides 3D visualization of entire brain via SVR-MVD reconstruction (400 gigavoxels). Under each view, the sample is imaged using $2.2\times$ magnification plus $\sim 25 \, \mu m$ laser-sheet. The sub-voxel scanning step size is $\sim 1 \, \mu m$. The final result is recovered from raw images of eight views, with reconstructed isotropic voxel size of 1 by 1 by 1.
μm. The imaging throughput here is ~30 minutes per whole brain, and the post-processing time is ~12 hours with employing quad NVIDIA graphical cards. (c)-(e) compare the transverse (xy) planes at 500, 2500 and 4000 μm z-depth, by conventional SPIM and Mars-SPIM. Mar-SPIM shows more uniform image quality at the deep of tissue. (f)-(h) correspondingly compare the reconstructed coronal (xz) planes at the height of 1500, 4000 and 8000 μm. The completely blurred parts by tissue scattering/attenuation are discarded in conventional SPIM images. Besides the reconstruction integrity of whole brain, the insets in (c)-(e) and (f)-(g) further compares the achieved lateral and axial resolutions of regular SPIM images and Mars-SPIM images with using the same optics. Scale bars: 500 μm in (b)-(g) and 100 μm in insets.
Figure 4. High-throughput, whole-brain imaging at isotropic cellular resolution using Mars-SPIM. (a) The reconstructed whole mouse brain by Mars-SPIM. Five selected volumes (I-V) at left cortex (blue), right cortex (cyan), hippocampus (red), thalamus (purple) and cerebellum (yellow) are shown in (b)-(f), respectively, with each one containing the transverse (x-y), sagittal (y-z), coronal (x-z) planes and 3-D rendering of the selected volume. The neuronal cell bodies together with the projecting fibers can be identified as a result of significantly enhanced resolutions by Mars-SPIM. Scale bars: 20 μm in (b)-(f).
Figure 5. Quantifications of a thy1-GFP-M mouse brain based on Mars-SPIM image. (a) The quick creation of whole brain atlas. Step 1: re-orientation of the Mars-SPIM image from horizontal to coronal view and pre-alignment to standard Allen brain atlas (ABA) using Elastix. Step 2: resizing the pre-aligned coronal images into low-resolution (LR) and high-resolution (HR) groups. Step 3: registration of LR group to ABA to obtain the transformation matrix. Step 4: Application of the transformation matrix to HR group to obtain the registered HR images. Step 5: 3D visualization of the ABA-registered brain. Step 6: Segmentation of the brain regions in Amira. (b) With isotropic single-cell resolution at whole-brain scale, 3D detection of single neurons can be readily achieved at various brain regions. As a result, the neuron distribution at different regions of the whole brain
can be mapped out, as shown in the coronal and transverse views in (c). Each color represents a brain region. (d) The neuron population and density calculated at 12 primary brain regions. CB, cerebellum; CTS, cortical subplate; HPF, hippocampal formation; HY, hypothalamus; ICX, isocortex; MD, medulla; MB, midbrain; OFL, Olfactory areas; PAL, pallidum; Pons, pons; STR, striatum; TH, thalamus. (e) Horizontal and coronal views of the traced neuron long-distance projections shown in volumetric rendering. (f) The pathway annotations of 6 long-distance projection neurons. Abbreviations: AAD, anterior amygdaloid area, dorsal part; Acbsh, accumbens nucleus, shell; Astr, amygdalostrial transition area; BLA, basolateral amygdaloid nucleus, anterior part; cp, CPu, caudate putamen (striatum); DM, ic, LH, lateral hypothalamic area; MCPO, magnocellular preoptic nucleus; mfb, medial forebrain bundle; MGP, medial globus pallidus (entopeduncular nucleus); SI SNL, substantia nigra, lateral part; SNR, substantia nigra, reticular part; Tu, olfactory tubercle; ICj, islands of Calleja; LAcbsh, lateral amygdaloid nucleus; VP, ventral pallidum; Pir, piriform cortex; CxA, cortex-amygdala transition zone; MCPO, magnocellular preoptic nucleus; SI, substantia innominata; BMA, basomedial amygdaloid nucleus, anterior part; MeAD, medial amygdaloid nucleus, anterior dorsal; ic, internal capsule; cp, cerebral peduncle, basal part; PSTh, parasubthalamic nucleus; LH, lateral hypothalamic area; SNC, substantia nigra, compact part; vsc, ventral spinocerebellar tract; A1, A1 noradrenaline cells; IRt, intermediate reticular nucleus; RVL, rostroventrolateral reticular nucleus; C1, C1 adrenaline cells; 7N, facial nucleus; 7DI, facial nucleus, dorsal intermediate subnucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; PL, paralemniscal nucleus; DpMe, deep mesencephalic nucleus; InCo, intercollicular nucleus; DMPAG, dorsomedial periaqueductal gray; S1FL, primary somatosensory cortex, forelimb region; CPu, caudate putamen; ec, external capsule;
S2, secondary somatosensory cortex; scp, superior cerebellar peduncle; ml, medial lemniscus; sumx,
supramammillary decussation; LH, lateral hypothalamic area; mfb, medial forebrain bundle;
SLEAM, sublenticular extended amygdala, medial part; acp, anterior commissure, posterior; AcbSh,
accumbens nucleus, shell; vp, ventral pallidum;