A hybrid open-top light-sheet microscope for versatile multi-scale imaging

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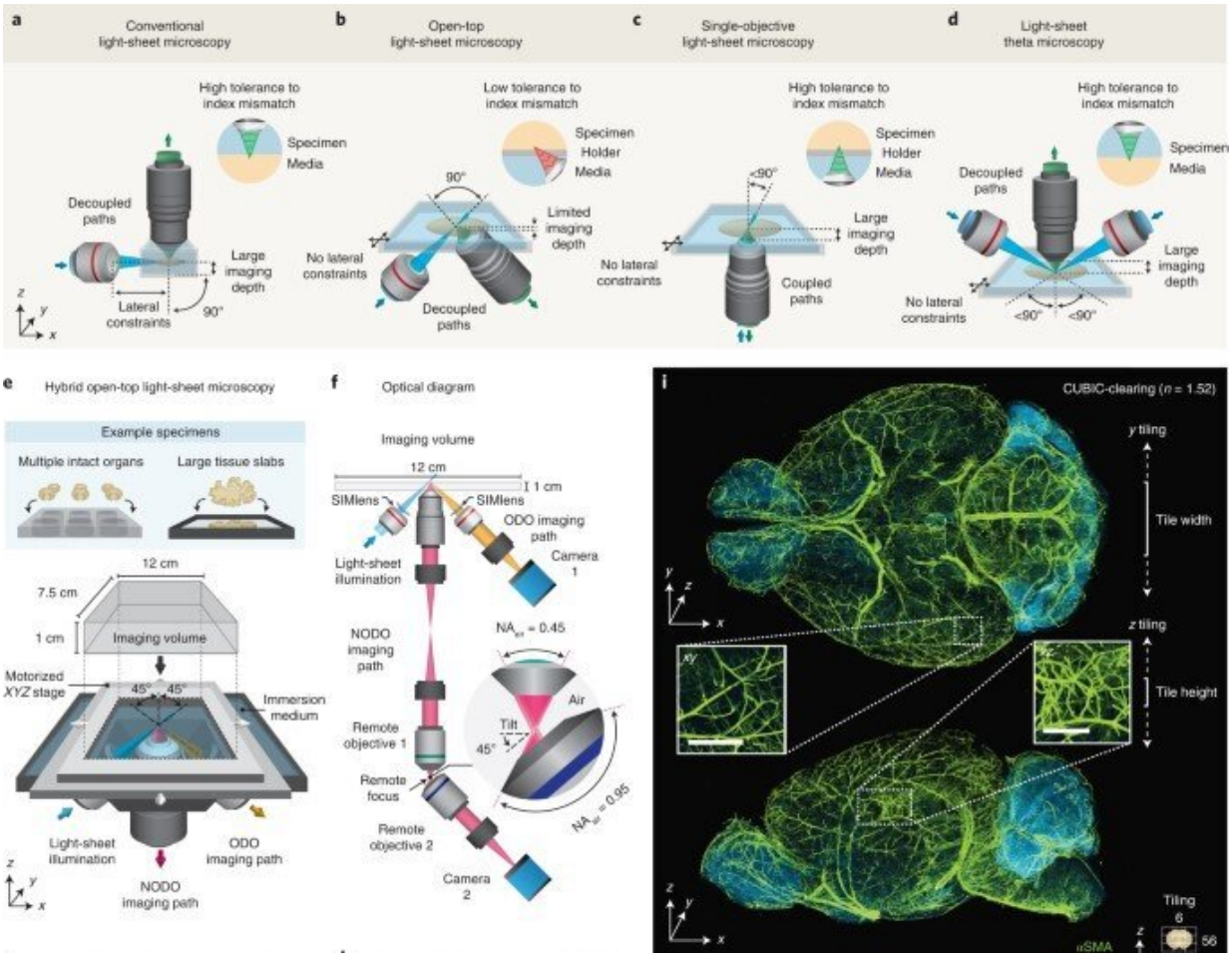


(a–d) Optical layouts of conventional, open-top, single-objective, and theta light-sheet microscopy systems. Aspects of each microscope architecture, including number and orientation of objectives, lateral constraints on specimen size, maximum imaging depth, and tolerance to index mismatch are highlighted. (e–f) Our hybrid microscope architecture consists of three objectives positioned below

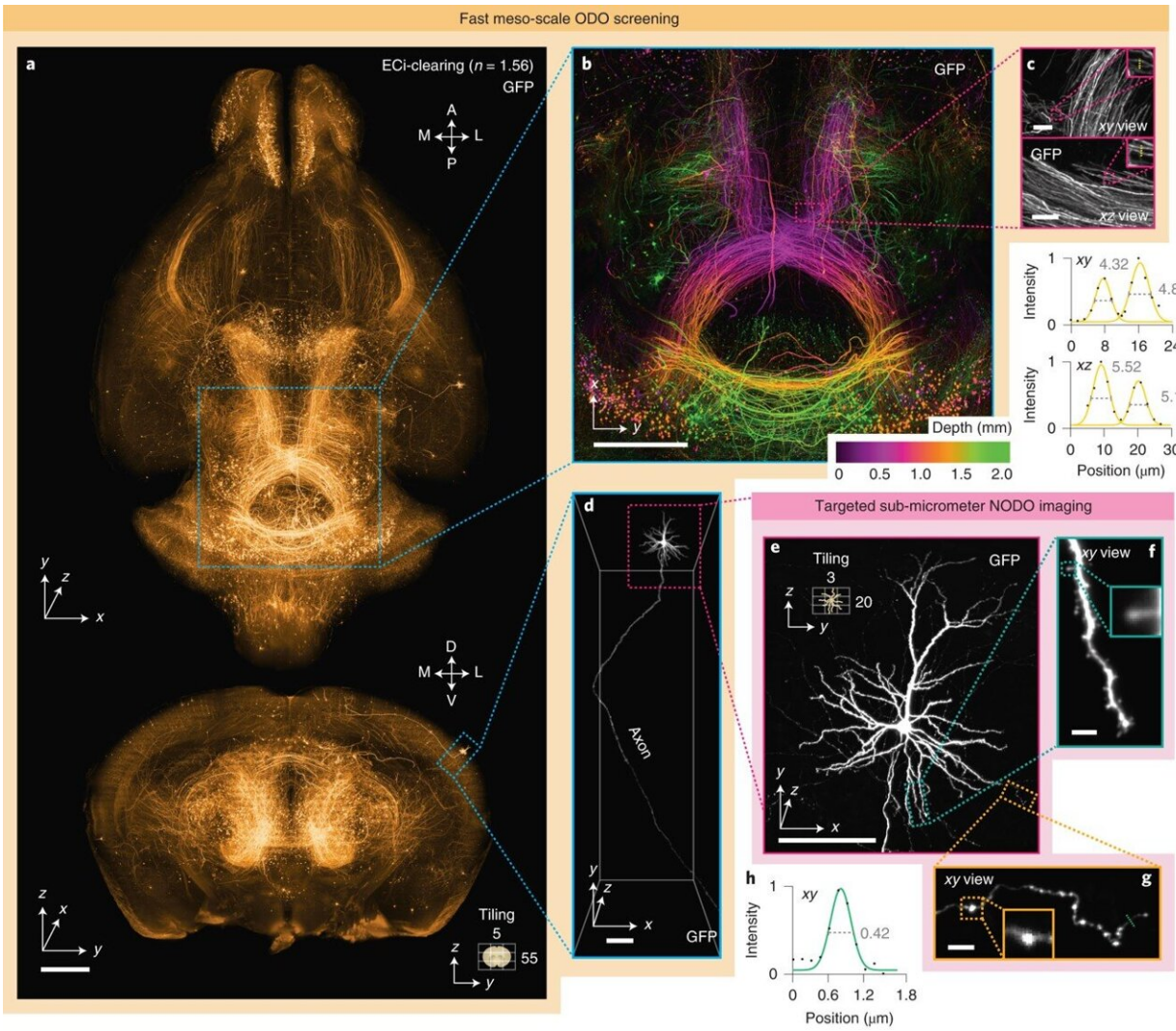
the specimen. One objective is used for light-sheet illumination, and the other two objectives are used for orthogonal dual-objective (ODO) and non-orthogonal dual-objective (NODO) imaging. By using a motorized stage, tiled imaging is possible with both paths over a large 12 × 7.5 × 1 cm (XYZ) imaging volume, accommodating multiple intact cleared organs and large tissue slabs mounted in an array of specimen holders. (g) In combination, the ODO and NODO paths enable imaging over a tunable lateral resolution range of 0.5–10.7 μm at imaging speeds of ~5 mm3 to 10 cm3 per hour. The current set points of the system are highlighted. (h) At these set points, the mean ± standard deviation full-width half-maximum (FWHM) resolutions (xyz) for the ODO path are 4.09 ± 1.07, 4.41 ± 0.83, and 5.48 ± 1.08 μm (n = 109 beads), and for the NODO path are 0.45 ± 0.07, 0.46 ± 0.06, and 2.91 ± 0.31 μm (n = 437 beads). (i) Representative ODO imaging results of an entire intact CUBIC-cleared mouse brain with arterial (αSMA) and nuclear (SYTOX-G) staining. The ODO imaging path is able to clearly resolve vasculature in both the xy and xz planes (insets). The size and direction of tiling is annotated. (j,k) Targeted NODO imaging of a sub-region centered on a branching arteriole resolves individual smooth muscle cells and sub-nuclear features that are not resolved by ODO imaging. Scale-bar lengths are as follows: i, 1 mm (insets, 500 μm); j, 100 μm; and k, 10 μm. All images are displayed without deconvolution. The imaging data in i–k were acquired from a single mouse brain in a single experiment. Credit: *Nature Methods* (2022). DOI: 10.1038/s41592-022-01468-5

During image analysis, researchers use light sheet microscopy of cleared tissue as a preferred method for high throughput volumetric imaging. A flexible system can provide a range of sizes, resolution and tissue-clearing protocols. In a new report now published in *Nature Methods*, Adam K. Glaser and a team of interdisciplinary scientists in mechanical engineering, bioengineering, and synthetic biology in the U.S. and Japan presented a new hybrid imaging system. Using the new method, the team combined non-orthogonal dual-objective and conventional open-top light-sheet microscopy for multi-scale volumetric imaging to visualize an intact, cleared mouse brain at the sub-micron scale. The team achieved

high-throughput automated imaging of multiple specimens, and compared the outcomes with existing light-sheet microscopy systems to show a unique combination of versatility and performance in the hybrid setup.

Open-top light-sheet microscopy (OTLS)

Tissue clearing protocols aim to reduce optical scattering, aberrations and background fluorescence for deep-tissue imaging with high resolution and contrast. The approach is used in many fields including neuroscience, developmental biology and anatomic pathology. The new method is a high-resolution volumetric technique to image cleared tissues with unrivaled speed and low photobleaching. Academic researchers and commercial entities have previously developed a diverse array of light-sheet microscopes; however, most light-sheet microscopes at present cannot satisfy all requirements of user-friendly standards, compatibility across protocols, large imaging depth and multi-scale imaging capabilities at the sub-micron scale. To address these shortcomings, Glaser et al developed an orthogonal dual-objective (ODO) system with tilted objectives. The open-top light-sheet microscopy (OTLS) method provided an easy technique alongside potential accessory devices including microfluidics, electrophysiology and microdissection. The team implemented a single-objective architecture and formed a hybrid open-top light-sheet microscope to open the door for advanced experiments at the sub-micron scale.

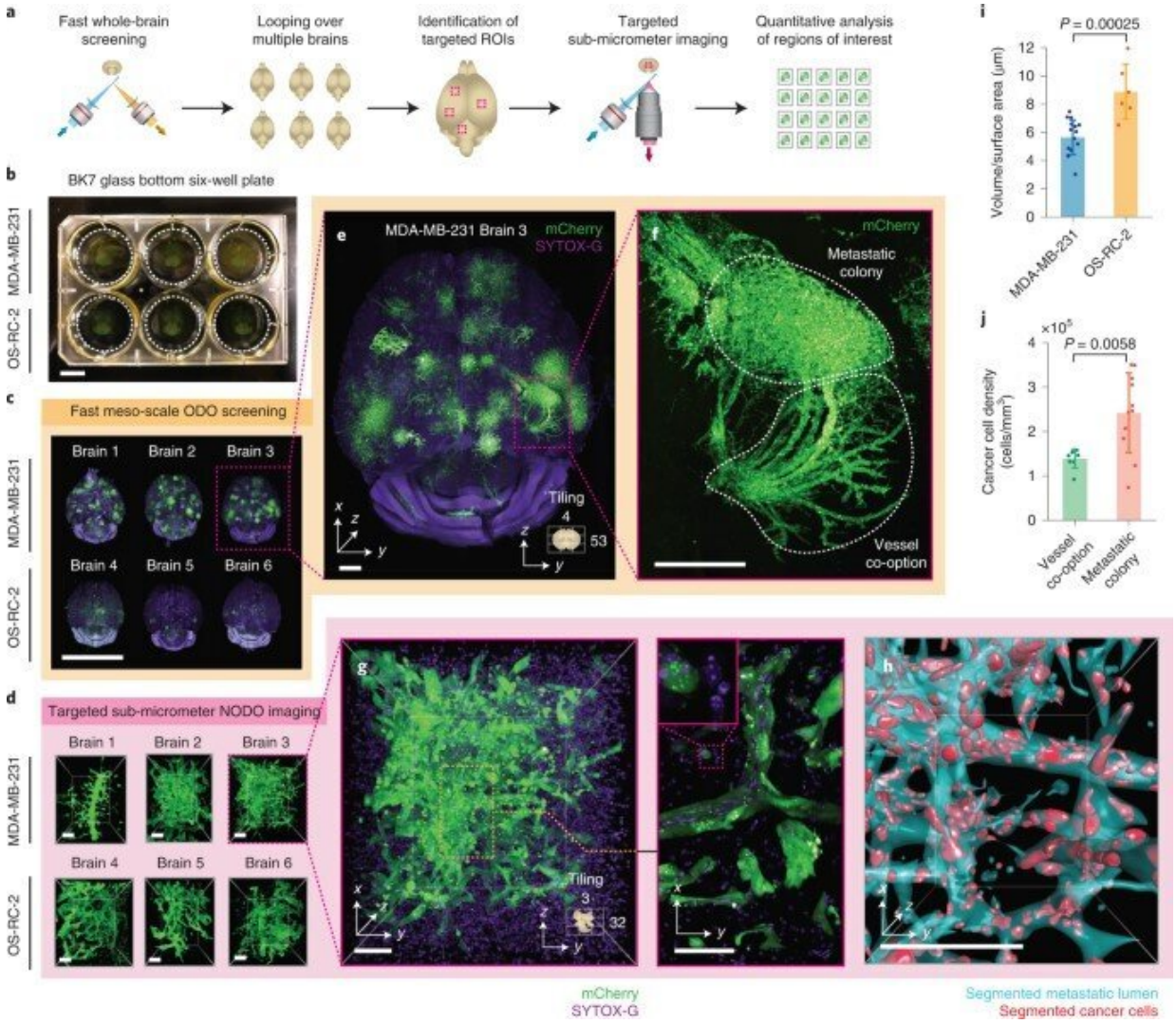


Fast meso-scale screening and targeted sub-micrometer imaging in cleared tissues. (a) Fast meso-scale screening is performed of an entire intact ECi-cleared Slc17a7-Cre mouse brain with brain-wide axonal projections. (b) A depth-coded region of interest shows dense projections in the midbrain. (c) xy and xz zoom-in views illustrate the near-isotropic resolution of the hybrid OTLS microscope. Line profiles through individual axons demonstrate an ODO lateral and axial resolution of 4–5 μm at a large depth within the cleared specimen. (d,e) Targeted sub-micrometer imaging is performed of a region of interest around a cortical pyramidal neuron. (f,g) Zoom-ins of a dendrite and axon demonstrate sufficient lateral resolution to visualize individual spines and varicosities. (h) A line profile through an individual axon demonstrates a NODO lateral resolution of 0.42 μm within the cleared specimen. Scale-bar lengths are as follows: a,b, 1

mm; c, 10 μm; d,e, 100 μm; and f,g, 5 μm. All images are displayed without deconvolution. The imaging data in a–g were acquired from a single mouse brain in a single experiment. Credit: *Nature Methods* (2022). DOI: 10.1038/s41592-022-01468-5

System design

Glaser et al designed the new system to feature three main objectives, which they positioned below the specimen to provide an unobstructed open top for three-dimensional imaging. The researchers included all three objectives into a monolithic imaging chamber via direct immersion and used a solid immersion meniscus lens to expand the range of refractive indices. They then developed a new non-orthogonal dual-objective light-sheet configuration alongside remote refocusing to rectify the imaging plane. The new architecture provided superior resolution to that of a single-objective light-sheet system, with broader applications for cleared-tissue imaging. The team then used the hybrid system in two example studies to represent multiscale imaging enabled time- and data-efficient experimental workflows.



Multi-scale OTLS microscopy for quantitative analysis of brain metastases. (a) Hybrid OTLS microscopy workflow for multi-scale imaging of multiple specimens. (b) n = 6 whole mouse brains containing metastatic lesions from two different cancer cell lines (MDA-MB-231 and OS-RC-2) were placed into a glass-bottom 6-well plate and mounted onto the hybrid OTLS microscope system for multi-scale imaging. (c) The ODO imaging path was used to rapidly screen multiple intact mouse brains containing metastatic colonies. (d) n = 34 total metastatic ROIs across all brains were identified and subsequently imaged at sub-micrometer resolution using the NODO imaging path (only one ROI per brain is shown for illustrative purposes). (e–g) Visual inspection of a single brain with MDA-MB-231 metastases in e revealed multiple colonies distributed throughout the brain, with signs of vessel co-option (f,g) that were not observed for OS-

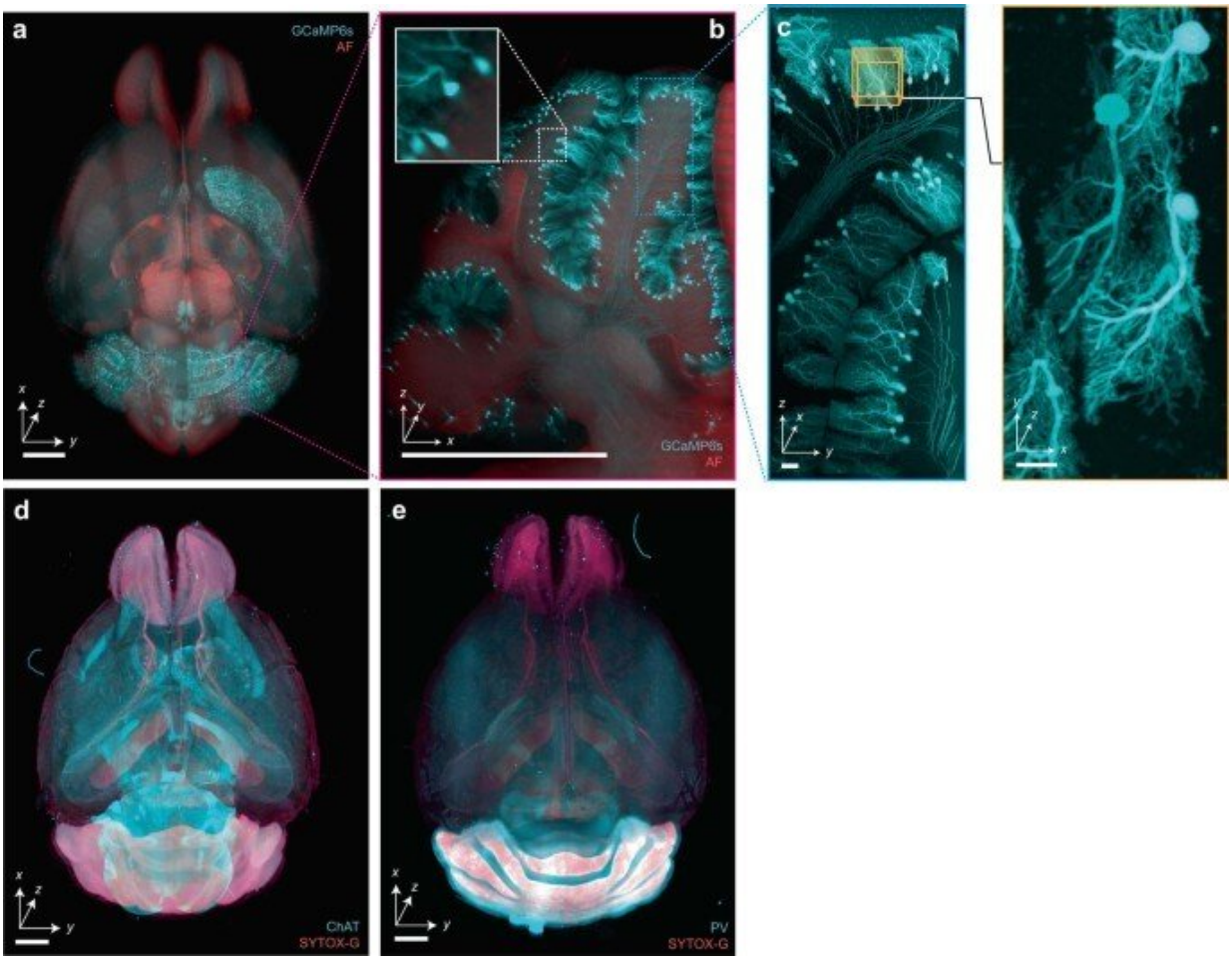
RC-2 metastases. (h) To quantify these phenotypic differences between the MDA-MB-231 and OS-RC-2 metastases, the lumens spaces within the metastatic regions (cyan) and cancer cells (red) were computationally segmented and analyzed. (i) The volume to surface area ratio was computed for MDA-MB-231 ROIs (n = 16) and OS-RC-2 ROIs (n = 6). Quantification of the resulting segmentation masks revealed statistically different 3D growth patterns (P value = 0.00025) between the two cancer cell lines, consistent with a previous report (i,j)42. (j) For MDA-MB-231 ROIs, the cancer cell density in ROIs of metastatic colonies (n = 11) and vessel co-option (n = 8) was found to be statistically different (P = 0.0058). The center line and error bars in i and j denote the mean and s.d., with the associated data points plotted. P values in i and j were calculated using a two-sample t-test. Scale-bar lengths are as follows: b,c, 1 cm; d, 100 μm; e, 1 mm; f, 500 μm; g,h, 100 μm. All images are displayed without deconvolution. The imaging data in c were acquired from in a single experiment. Credit: *Nature Methods* (2022). DOI: 10.1038/s41592-022-01468-5

Targeted imaging: Sparse axons

During the first experiment, Glaser et al imaged axons in an intact mouse brain. Tracking the axons of individual neurons presented a challenging problem, since axons are very thin and span very large distances, the researchers therefore relied on bright labeling of a few neurons with high resolution and high contrast imaging to capture the entire brain. The scientists generated large datasets of sub-micron level imaging for computationally intensive downstream pipelines for data handling, storage and processing. Using the hybrid multiscale imaging system, Glaser et al simplified the process to screen an entire mouse brain at low resolution and identify target regions of neurons, followed by high-resolution imaging, to categorize a specific region of interest.

Multiscale imaging metastatic colonies

During further experiments, the researchers performed broad multiscale imaging across multiple specimens. For this, they used the hybrid OTLS (open-top light-sheet microscopy) system, and studied metastatic colonies from two cancer cell lines within intact mouse brains. Typically, it is difficult to identify the sparse and unpredictable spatial distribution of brain metastases in whole brains due to the absence of a low resolution screening method. Glaser et al therefore first identified the metastatic sites and then aimed to conduct high-resolution quantitative analysis of these regions thereafter. While studies in the past have relied on the individual manual transfer of specimens between different microscopic systems, in this case the team analyzed six intact mouse brains via the hybrid system during a single imaging session. The hybrid method reduced overall hardware costs to simplify multiscale datasets and allowed automation of multiscale imaging experiments to prevent the need to manually transfer specimens between different imaging systems. For example, while previous research required two separate microscopic systems, and weeks of tedious imaging, in addition to manual specimen transfer, the hybrid OTLS system allowed the entire experimental imaging process to be completed in two days.



Imaging immunostained and endogenously fluorescent mouse brains. Hybrid OTLS imaging of whole mouse brains. (a-c) with endogenously preserved GCaMP6s fluorescence, and immunostained with (d) anti-ChAT antibody + SYTOX-G or (e) anti-Parvalbumin (PV) antibody + SYTOX-G by CUBIC-HistoVision. Scale bar lengths are as follows: (a-b) 1 mm, (c) 10 μm, (d-e) 2 mm. The imaging data in (a-e) was acquired from in a single experiment, which was not repeated. Credit: *Nature Methods* (2022). DOI: 10.1038/s41592-022-01468-5

Additional experiments

Aside from the experiments conducted as proof of concept, the device is

also suited for an additional array of imaging studies. The team highlighted the role of the system in several projects, including multi-scale non-destructive 3D pathology of prostate cancer, whole-brain imaging of endogenous fluorescent proteins, 3D imaging of mouse embryos, imaging plants and pigmented animal models as well as large-scale imaging of human brain slices. As with all light-sheet microscopy methods, the researchers highlighted various factors underlying the process, including system design, sample preparation and sample mounting that contributed to imaging quality.

Outlook

In this way, Adam K. Glaser and colleagues showed how axial and lateral resolution of an imaging system can be improved via a hybrid open-top light-sheet microscopy (OTLS) system. In the present setup, the team chose three separate objectives; however, they point out that it is also possible in theory to achieve improved performance with an optimized non-orthogonal dual-objective, or with a single high numerical aperture with a large field of view and working distance. In summary, the hybrid OTLS experimental setup provided a practical platform with commercially available optical components to achieve an impressive balance of performance and versatility for cleared tissue imaging experiments. The team also envision the possibility of integrating high-speed voice coil actuators and tunable mirrors as alternate devices integrated to the system in the future.

More information: Adam K. Glaser et al, A hybrid open-top light-sheet microscope for versatile multi-scale imaging of cleared tissues, *Nature Methods* (2022). DOI: 10.1038/s41592-022-01468-5