Tilt-invariant scanned oblique plane illumination microscopy for large-scale volumetric imaging

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Single front-facing microscope objective based light-sheet microscopy techniques are gaining traction for three-dimensional rapid imaging of biological samples. However, large-scale undistorted 3D visualization of biological samples has not yet been demonstrated with these techniques. Here, we present an improved tilt-invariant scanned oblique plane illumination (SOPi, /söpi/) microscope, which allows for stitching multiple volume scans (tiles) for large volume, rapid 3D fluorescent imaging. We show imaging results spanning complete zebrafish larvae, and over 1 mm³ volumes for thick mouse brain sections. Using one-photon SOPi, we demonstrate cellular resolution imaging at depth exceeding 330 µm in optically scattering mouse brain samples, and dendritic imaging in more superficial layers.

Light-sheet fluorescence microscopy (LSFM) has emerged as an indispensable tool in biology [1]. Among many developments in LSFM technology, the emergence of single front-facing microscope objective based tilted light-sheet techniques [2-4] for rapid volumetric imaging holds promise for applications in neurobiology, where fast imaging of neuronal activity and other dynamics is critical. These new techniques solve the issue of limited steric access to the sample, associated with classical LSFM arrangements [5]. Single objective based approaches use varied methods for remote scanning of the tilted light-sheet. OPM [3] relies on a piezo-electric actuator for on-axis movement of a remote second objective, while SCAPE [4] utilizes a polygon scan mirror arrangement. Although SCAPE approach simplifies the scanning architecture of OPM, it suffers from scan position dependent tilt of the light-sheet.

To overcome the existing drawbacks of single objective based light-sheet microscopy techniques, we recently introduced SOPi [6], which makes use of a single planar scan mirror to provide tilt-invariant scanning of the oblique light-sheet. Figure 1(a) illustrates the comparison of OPM, SCAPE, and SOPi light-sheet scanning orientations. In our previous work, we showed that this tilt-invariant scanning of SOPi is crucial for true perspective, 3D imaging of samples. Moreover, we demonstrated that two-photon (2p) light-sheet implementation with SOPi outperforms one-photon implementation for subcellular resolution in scattering samples. However, low numerical aperture (NA) illumination for light-sheet generation, coupled with low fluorescence cross section of 2p excitation, imply that superior resolution of 2p-SOPi comes at the cost of higher laser power, risking potential damage to live biological samples. The power requirement of 2p light-sheet also limits practical depth of imaging. Therefore, it is highly desirable to improve the resolution and depth penetration of one-photon (1p) based single objective light-sheet microscopy approaches.

In this letter, we show that single-objective based light-sheet microscopy can be extended to image much larger samples through multiple tile volume acquisitions. This is enabled by SOPi's tilt-invariant scanning geometry. We present a processing pipeline, provide examples of stitched SOPi acquired volume-tiles, and describe how to obtain true perspective 3D visualization in stitched datasets. Towards the goal of attaining large volume imaging capability, we also modify the 1p-SOPi system to be able to image deeper in scattering media.

The SOPi system working principles and optical layout have been described in detail elsewhere [6]. In our previous work, 1p-SOPi had lower resolution and shadow streak artifacts when compared to its 2p counterpart. In addition to a larger point-spread-function of 1p fluorescence excitation, there were several limitations on imaging performance in the original 1p-SOPi implementation. First, the use of a multimode, high divergence excitation source (laser diode) led to broadening of the light-sheet thickness, causing poorer optical sectioning. Second, like earlier attempts for this class of imaging techniques [2-4], the 1p light-sheet for SOPi was created by making use of a slit aperture and cylindrical lens in the illumination arm. From conventional LSFM approaches, we know that this leads to shadow streaks and degrades optical sectioning. Instead, a DSLM approach using a fast galvo scanner and a converging lens for a rapid movement of a micrometer thin beam of laser to generate the light-sheet should provide uniform illumination at improved power efficiency and better optical sectioning, due to reduced aberrations within the beam [7].
Therefore, here we implement the DSLM approach with 1p-SOPi system for improved imaging performance and depth penetration. The system layout is schematically presented in Fig. 1(b), largely similar in scanning geometry to earlier SOPi implementation. The system is arranged such that G1 (QS-12, 10 mm aperture, Nutfield Technology) and G2 (GVSM001, Thorlabs) rotation axes lie in conjugate planes of one other. Further, the rotation axis of G1 is in conjugate plane to back-focal planes (BFP) of both MO1 (20x, NA 1.0W, XLMPLFLN20XW, Olympus) and MO2 (20x, NA 0.75, UPLAPL020X, Olympus). This arrangement ensures that rotation of G1 and G2 provides tilt-invariant scanning as represented in Fig. 1(c). The illumination unit, unlike in previous SOPi implementation, consists of laser 1 (473 nm, violet/blue DPSS laser, Dragon Lasers) and laser 2 (532 nm, green DPSS laser, DJ532-40, Thorlabs), combined and co-aligned through a dichroic mirror/beam-splitter (FF495-DI03, Semrock). Fast scanning of G2 creates a light-sheet as shown in Fig 1(d). A multiband dichroic mirror (DI03-R405/488/532/635, Semrock) reflects the light-sheet illumination towards the sample and allows emitted fluorescence to pass. The amount of y-offset in the illumination beam determines the tilt of light-sheet in the sample space, and for our setup, it remains ~3.54 mm, corresponding to 45° tilt [6].

Fig. 1. (a) Comparison of OPM, SCAPE and SOPi light-sheet scanning. (b) Schematics for the experimental setup of SOPi. (c) Schematics showing the role of G1 and G2 in tilt-invariant scanning and creation of light-sheet, and (d) extended setup illustrating the dual laser arrangement for 2 color imaging. MO: microscope objective, G: galvo scanner, L: lens, MDM: multiband dichroic mirror.

The choice of converging lenses L1-L6 determines the effective magnification of the system. They must be chosen carefully, so that (1) lateral and axial magnification at intermediate image plane in front of MO2 are equal to the ratio of refractive indices of MO1 and MO2 immersion media; and (2) overall system magnification is optimized to the effective camera pixel size in sample space. The first requirement minimizes optical aberrations while imaging an oblique plane [2,8], while the second requirement optimizes resolution and field of view of the system. We used achromatic doublet lenses from Thorlabs with focal lengths f = 200 mm (L1, AC508-200-A-ML), f = 100 mm (L2, AC508-100-A-ML; L3, AC254-100-A-ML; L6, AC254-100-A-ML), f = 150 mm (L4, AC254-150-A-ML), and f = 80 mm (L5, AC254-80-A-ML). Value of L5 focal length was decided based on choice of MO3 (20x, NA 0.45, LUCPLFLN20X, Olympus), the SOPi system’s effective NA ≈ 0.34 [6] and camera’s pixel size 5.86 µm (1920×1200 pixels, GS3-U3-23S6M-C, Grasshopper3, FLIR). This resulted in system’s effective magnification of ~11.62x leading to pixel size in sample space of ~0.5 µm, which is approximately half of the microscope’s lateral resolution (0.61×λ/NA). This matching of focal length to camera pixel size and system NA optimizes resolution performance while maintaining a large field of view (here, ~950 µm along x-axis).

During imaging experiments, we used a manual 3-axis translation stage (PT3/M, Thorlabs) to position the sample within the field of view of SOPi system. A custom MATLAB graphical user interface (GUI) control software was used to send ramp voltage signals to galvo scanners via a data acquisition card (DAQ, PCIe-6321, National Instruments) on a Windows 10 PC. µManager was used for camera control and image acquisition [9].

A single sweep of oblique light-sheet from SOPi acquires an image stack corresponding to a sheared cuboid shaped volume, with its edges predictably misaligned relative to the translation stage Cartesian coordinates (x,y,z). Figure 2(a) illustrates the orientation of acquired volume tile. During processing of the image stack, Fiji/ImageJ [10,11] and other 3D reconstruction software manage the data in alternate coordinates (x′,y′,z′), so the raw 3D volume representation (left, Fig 2(b)) is incorrect. Nevertheless, the

Fig. 2. Orientation and processing of SOPi acquired tiles. (a) Relative orientation of sample (light grey cuboid) in lab, and SOPi acquired tile (green sheared cuboid) in light-sheet/image coordinates. (b) Geometrical transformations position the image stack in correct 3D orientation. (c) Processing pipeline for acquiring, stitching, and 3D visualization of multiple SOPi tiles.
reconstructed volume retains co-linearity, due to the tile-invariant scanning of SOPi. Therefore, the exact volume can be reconstructed by two simple geometrical transformations of scaling and shearing as described in Fig. 2(b). In practice, we use a single 4×4 Affine transformation matrix [6] to produce the combined geometrical transformation using transform plugin [12].

Next, we investigate how SOPi’s tilt invariant scanning can be used for stitching multiple tiles. Fig. 2(a) and 2(b) illustrate that even though Affine transformation places the tiles in correct orientation, two adjacent tiles can only be combined if they are stitched along x′ or y′ direction. Tiles along z′ direction would not merge after the transform, due to the extra corner padding (of blank pixels) added to each individual tile during transform. The simplest solution to this problem is to stitch raw tiles in their original form, i.e. pre-Affine transformation, as depicted in Fig. 2(c) workflow. All the tiles are acquired by moving translation-stage/sample and stitched together with existing stitching tools [13,14] to form large volume data. Tiles acquired along x, y and z axis in laboratory coordinates are stitched along x′, y′ and z′ axis in image coordinates, respectively. A single operation of Affine transformation on stitched volume data rearranges it into an exact 3D representation of sample volume. This large volume data, post transformation, is then passed to a plugin of choice for 3D visualization [15,16]. Note that no deconvolution or other post-processing is required, but could be implemented for further improvements.

Now, we present examples of stitching SOPi tiles along x, y and z axis. In the first example, we show stitching multiple tiles acquired by translation along x-axis. For this, we used an agar gel embedded 4 dpf (days post fertilization) zebrafish embryo from an olig2:GFP cross to mnx:Gal4; UAS:plTagRFP. Zebrafish was oriented with its length along the x-axis, and a total of six overlapping tiles were acquired with manual translation of the stage to cover ~ 4 mm length (brain and spinal cord) as illustrated in Fig. 3(a). Each SOPi tile was acquired at 50 fps, with G2 driven at 100 Hz, and light-sheet scanned to cover 400 µm along y-axis in 6 seconds. In the processing pipeline, each tile was first scaled down along x′ and y′ direction to half (to reduce data size). Tiles were stitched pairwise [13], Affine transformed [12], and visualized [16] using ImageJ plugins. Imaging was performed for green and red fluorescence channels separately, and 3D reconstruction of the entire zebrafish is presented in Fig. 3, and Visualization 1.

In the second example, we show stitching of multiple tiles acquired along the y-axis. We used 1 mm-thick, fixed, uncleared coronal Thy1-GFP mouse brain sections through the hippocampus (007788, Jackson Laboratory). The slice (Fig. 4(a)) was translated along y-axis in steps of ~200 µm to cover ~4.75 mm length through multiple overlapping tiles. Each tile spanning 250 µm was acquired in 5 seconds at 50 fps, with G2 driven at 100 Hz. We restricted scan range to 250 µm for uniform illumination throughout the y-sweep. We used BigSticher [14] to stitch the tiles (after rescaling each tile along x′ and y′ direction to half), transform [12] to Affine transform, and BigDataViewer [15] to visualize the volume. The stitched volume spans ~0.95 mm × 4.75 mm × 0.3 mm (~1 mm³). Figure 4(b) shows a virtual xy slice from stitched volume at a depth of 100 µm where all the cell bodies and dendrites are clearly visible. Visualization 2 shows oblique plane scan through the entire stitched volume.

In the final example, we demonstrate stitching along z-axis. Figure 5(a) illustrates how two connected SOPi tiles along depth of sample are acquired by simultaneous movement of sample along y and z axis. We acquired two overlapping tiles in the same mouse brain section by translating the sample diagonally by ~250 µm. Each tile was acquired at 50 fps, spanning 400 µm in 6 seconds. Tiles were scaled down (to half along x′ and y′), stitched pairwise, Affine transformed, and visualized with BigDataViewer. In this dataset, depth penetration capability of SOPi becomes apparent. Neurons are visible at greater than 330 µm depth, with dendritic processes...
well-resolved at more superficial depth in an optically scattering mouse brain section (Fig. 5(b), also see Visualization 3). This depth performance exceeds any published single objective one-photon light-sheet microscopy approach.

![Image](https://via.placeholder.com/150)

**Fig. 5.** Stitching tiles along sample depth (z-axis). (a) Placement and orientation of connected tiles along depth. Top region of tile highlighted by a rectangle. (b) Virtual z-slices along the depth of stitched volume of a thick mouse brain section. Some neurons at >350 µm depth (see Visualization 3) are resolved, with dendritic imaging at more superficial depths. (Scale bar: 100 µm)

In conclusion, we have improved the 1p-SOPi illumination architecture to obtain better optical sectioning capability to image deeper in scattering samples. In addition, we show that it is possible to obtain large-scale volumetric imaging by stitching multiple volumes scans together. These advances make SOPi suitable for in vivo imaging in mice as well as large sample imaging in other organisms. Moreover, the current implementation supports acquisition of high-quality imaging data at reasonable speed with basic, inexpensive cameras. The use of high-sensitivity sCMOS cameras would further speed up volume acquisitions [6]. Since scanning during a tile acquisition is done remotely by galvo scanners, and the sample remains motionless, there are no mechanical vibration artifacts. Thus, a simple manual translation stage, unlike in conventional light-sheet approaches, is sufficient for large volume stitching. With the help of an automated translation stage and a workstation for data processing experiments can be significantly scaled up, e.g., to image multiple zebrafish in parallel, or other large samples. In the future, the use of self-reconstructing beams [17,18] with SOPi should provide much deeper imaging capabilities yet. The already available choices of higher NA objectives and post-processing algorithms would enable future SOPi implementations to image at sub-dendritic and potentially molecular resolutions.

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**References**

1. R. M. Power, and J. Huisken. “A guide to light-sheet fluorescence microscopy for multiscale imaging.” Nat. Methods 14(4), 360-373, (2017).
2. C. Dunsby, “Optically sectioned imaging by oblique plane microscopy,” Opt. Express 16(25), 20306–20316 (2008).
3. M. B. Sikkel, S. Kumar, V. Maioli, C. Rowlands, F. Gordon, S. E. Harding, A. R. Lyon, K. T. MacLeod, and C. Dunsby, “High speed scCMOS-based oblique plane microscopy applied to the study of calcium dynamics in cardiac myocytes,” J. Biophotonics 9(3), 311–323 (2016).
4. M. B. Bouchard, V. Voleti, C. S. Mendes, C. Lacefield, W. B. Grueber, R. S. Mann, R. M. Bruno, and E. M. Hillman, “Swept confocally-aligned planar excitation (SCAPE) microscopy for high speed volumetric imaging of behaving organs,” Nat. Photonics 9(2), 113–119 (2015).
5. P. G. Patrone, J. Schindelin, L. Stuvenenberg, S. Preibisch, M. Weber, K. W. Elceirui, J. Huisken, and P. Tomancak, “OpenSPIM: an open-access light-sheet microscopy platform,” Nat. Methods 10(7), 598–599 (2013).
6. M. Kumar, S. Kishore, J. Nasenbeny, D.L. McLean, and Y. Kozorovitskii, “Integrated one- and two-photon scanned oblique plane illumination (SOPi) microscopy for rapid volumetric imaging,” Opt. Express, 26(10), 13027-13041 (2018).
7. P. J. Keller, A. D. Schmidt, J. Wittbrodt, and E. H. K. Stelzer, “Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy,” Science 322(5904), 1065–1069 (2008).
8. E. J. Botcherby, R. Juskaitis, M. J. Booth, and T. Wilson, “Aberration-free optical refrocusing in high numerical aperture microscopy,” Opt. Lett. 32(14), 2007–2009 (2007).
9. A. D. Edelstein, M. A. Tsuichida, N. Amodaj, H. Pinkard, R. D. Vale, and N. Stuurman, “Advanced methods of microscope control using µManager software,” J. Biol. Methods 1(2), e10 (2014).
10. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona, “Fiji: an open-source platform for biological-image analysis,” Nat. Methods 7(6), 676–682 (2012).
11. C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, “NIH Image to Image: 25 years of image analysis,” Nat. Methods 9(7), 671–675 (2012).
12. E. H. W. Meijering, W. J. Niessen, and M. A. Viergever, “Quantitative Evaluation of Convolution-Based Methods for Medical Image Interpolation,” Med. Image Anal. 5(2), 111–126 (2001).
13. S. Preibisch, S. Saalfeld, and P. Tomancak, “Globally optimal stitching of tiled 3D microscopic image acquisitions,” Bioinformatics, 25(11), 1463-1465 (2009).
14. D. Hörl, F. R. Rusak, F. Preusser, P. Tillberg, N. Randel, R. K. Chhetri, A. Cardona, P. J. Keller, H. Harz, H. Leonhardt, and M. Treier, “BigStitcher: Reconstructing high-resolution image datasets of cleared and expanded samples,” bioRxiv, p.343954 (2018).

15. T. Pietzsch, S. Saalfeld, S. Preibisch, and P. Tomancak, “BigDataViewer: visualization and processing for large image data sets,” Nat. Methods, 12(6), 481-483 (2015).

16. L. A. Royer, M. Weigert, U. Günther, N. Maghelli, F. Jug, I. F. Sbalzarini, and E. W. Myers, “ClearVolume: open-source live 3D visualization for light-sheet microscopy,” Nat. Methods 12(6), 480–481 (2015).

17. F. O. Fahrbach, P. Simon, and A. Rohrbach. “Microscopy with self-reconstructing beams,” Nat. Photonics 4(11), 780-785 (2010).

18. T. Vettenburg, H. I. Dalgarno, J. Nyk, C. Coll-Lladó, D. E. Ferrier, T. Čižmár, F. J. Gunn-Moore, and K. Dholakia, “Light-sheet microscopy using an Airy beam,” Nat. Methods, 11(5), 541-544 (2014).