Axially Overlapped Multi-Focus Light Sheet with Enlarged Field of View

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Light sheet fluorescence microscopy provides optical sectioning and is widely used in volumetric imaging of large specimens. However, the axial resolution and the lateral Field of View (FoV) of the system, defined by the light sheet, typically limit each other due to the spatial band product of the excitation objective. Here, we develop a simple multi-focus scheme to extend the FoV, where a Gaussian light sheet can be focused at three or more consecutive positions. Axially overlapped multiple light sheets significantly enlarge the FoV with improved uniformity and negligible loss in axial resolution. By measuring the point spread function of fluorescent beads, we demonstrated that the obtained light sheet has a FoV of 450 μm and a maximum axial FWHM of 7.5 μm. Compared with the conventional single-focus one, the multi-focus Gaussian light sheet displays a significantly improved optical sectioning ability over the full FoV when imaging cells and zebrafish.

Light sheet fluorescence microscopy (LSFM) has become an indispensable tool in volumetric imaging, with the advances in high spatiotemporal resolution and low photo-toxicity to the fluorescent sample. The open-source design with detailed instructions encourages DIY setups, which has significantly accelerated the wide-range adoption and applications of LSFM. Pioneering works, including OpenSPIM [1], OpenSpin [2], and the recent mesoSPIM [3], provide the detailed protocols for building and using the microscopes. These joint efforts further allow the biology labs to build their LSFM system for a specific application, including more complex schemes, such as multiview excitation or detection configurations [4-6].

The key of LSFM is to generate a thin light sheet with a large field of view (FoV). The use of an illumination objective with a higher NA will generate a thinner light sheet, better for improving the axial resolution, as well as protecting the specimen from photobleaching due to the reduced illumination thickness. However, this is at the price of a smaller FoV. To solve this conflict between the axial resolution and FoV, the community has proposed various approaches.

The self-reconstructing beams, such as Bessel beams [7-9], Airy beam [10], attenuation-compensated propagation-invariant beam [11], have been used to maintain the thin sheet over a long propagation distance. Despite their superiority over Gaussian, these beams bring more sidelobes and photobleaching. To minimize the sidelobes, the combinations of light sheet illumination and confocal detection [12, 13], multi-photon excitation [14-17], field modulation [18], phase filters [19], coherent [20-22] and incoherent [23, 24] superpositions have been proposed. Significantly, the dithered lattice light sheet has attracted much attention due to its high resolution and minimal photobleaching [20]. However, there are still different opinions doubting the superiority of self-reconstructing beams over Gaussian beams in light sheet microscopy [25, 26]. Besides, the tradeoff between NA and FoV remains to be the bottleneck for large-scale volumetric imaging with high resolution.

The other approach is to generate the light sheet with multiple beams. For example, the mSPIM [27] or SiMView [4] applied the dual-objective excitation configuration to double the FoV with similar axial resolution. Successive focus can be generated by axial scanning with tunable acoustic gradient-index (TAG) [28, 29] lens, electrically tunable lens (ETL) [30, 31], spatial light modulator (SLM) [32, 33], multi-configuration [34] and remote focusing optics [35, 36]. Synchronization with a rolling shutter further increases the axial resolution [35]. However, during the process of creating the virtual thin light sheet, the actual exposure time in the focus region (Rayleigh region) is limited, and specimens beyond the in-focus area suffers from unnecessary photodamage. In a recent effort of the tiled light sheet, thin and small light sheet illumination was spliced to form a large FoV by focus shifting and sequential image acquisition at consecutive positions [32]. However, this approach is at the cost of reduced speed. Besides, all these approaches require precise synchronization, which significantly increases the imaging system's complexity.
Inspired by the remote focusing mechanism [37, 38], here, we design an axially overlapped multi-focus system to break the tradeoff between resolution and FoV, obtaining a high-resolution LSFM with extended FoV. Only one simple component is required: a multi-layer beam splitter (MBS), to generate multiple focal planes at different focal distances. The overlapped light sheets extend the FoV for over an order-of-magnitude, leading to high-resolution LSFM imaging at homogeneous illumination and sharp optical sectioning capability at a large scale. The easy implementation of the MBS makes our LSFM system low-cost, compact, and largely compatible with other LSFM systems. Following the open-source culture, we demonstrate the MF modular design on OpenSPIM, one of the most widely used light sheet system.

Fig. 1. Schematics of axially overlapped multi-focus light sheet (MF-LS) microscopy. The illumination beam from the laser source (473 nm, bandwidth 0.2 nm, MBL-III-473, CNJ) passes through a 3× beam expander, a slit aperture (SA), a cylindrical lens (CL, ACY254-50-A, Thorlabs) and then is guided into the MF component. Finally, three foil are generated after the illumination objective (Obj. Olympus 10×/NA. 03W). The detailed information of the MF component is illustrated in the right part. It includes one polarization beam splitter (PBS, CCM1-PBS251, Thorlabs), a quarter-wave plate (QWP, WPA4420-450-650, Union Optics), and the multi-layer beam splitter (MBS), laid between a pair of lenses (L1, AC254-50-A, Thorlabs, and L2, AC254-150-A). The detection path, which is composed of one detection objective (Olympus, 40×/NA. 0.8 W), one emission filter (550LP, Thorlabs), the tube lens (Olympus, 0.5×), and the detector (Hamamatsu ORCA-Flash 4.0), is in the orthogonal direction with a total magnification of 20×.

The axially overlapped multi-focus light sheet (MF-LS) setup integrated into the OpenSPIM system is shown in Fig. 1. The axially overlapped MF-LS can be achieved with one polarization beam splitter (PBS), one quarter-wave plate (QWP), and MBS. The beam before PBS is horizontally polarized. After passing the PBS and 45° placed QWP, the rays reflected by MBS go through QWP with polarization changed to vertical, and then be reflected by PBS to lens L2 and then the objective. The MBS consists of several beam-splitters and one mirror (Fig. S1), which produces consecutive beams with different divergence, resulting in multiple focusing on the illumination plane. Spacers with variable thickness can adjust the focus drift between neighboring beams, which is proportional to the distance between reflective layers by the ratio of the square of the magnification. Both the reflectivity of each beam-splitter and the distance between neighboring reflectors should be well designed to generate the appropriate light sheet (Supplementary Note 1). This work focuses on the MBS setup with two beam splitters, which produces three primary foil.

We firstly conduct theoretical simulations to study the design and performance of the MBS. Considering Gaussian beam with intensity distribution as follows,

\[ \text{I}(z, x) = I_0 \left( \frac{w_0}{w(x)} \right)^2 \exp \left( -\frac{2z^2}{w^2(x)} \right) \]  

where, \( w(x) = w_0 \sqrt{1 + \left( \frac{x}{x_0} \right)^2} \), \( x_0 = \frac{\pi w_0^2}{\lambda} \), and \( w_0 = \frac{n \lambda}{\pi \text{NA}} \). \( z \) is the axial distance, and \( x \) is the axial distance (propagation direction) from the center of the beam. \( x_0 \) is the Rayleigh length, and \( w_0 \) is the beam waist. In a first approximation [39], \( w_0 \) can be calculated as in Eq. (4).

Generally, the in-focus depth of a single Gaussian beam light sheet (FoV) is considered to be twice the Rayleigh length, with the average axial full-width at half maximum (FWHM) equal to

\[ \text{FWHM}_{\text{FOV}} = \frac{1}{\pi} \int_{-\pi}^{\pi} \text{FWHM}(x) \, dx \approx 1.35 w_0 \]  

where \( \text{FWHM}(x) = \sqrt{2 \text{ln}2 \text{w}(x)} \) according to its definition. Thus, the focus drift is also set to be two Rayleigh lengths in the MF model for simplicity (Supplementary Note 2). We use NA=0.064 in the simulation, which is consistent with our experimental setting. This N.A. results in a Rayleigh length of ~65 μm, covering FoVs of 130 μm with cellular resolution (FWHM=4.2 μm).

Besides, in our experiments, the axial shift between neighboring beams is much larger than the coherence length of the laser source (Supplementary Note 3). Therefore, the intensity profile of multiple shifted beams would be the sum of each beam under incoherent conditions,

\[ I_{\text{MF}}(z, x) = \sum_{i} I_i \left( \frac{w_0}{w(x - x_i)} \right)^2 \exp \left( -\frac{2z^2}{w(x - x_i)^2} \right) \]  

where \( x_i \) is the axial shift of each beam.

Fig. 2. Principle of axially overlapped MF-LS. (a) simulated image of the excitation beam for single-focus LS, MF-LS, and swept focus LS with the same NA. (NA=0.064). For MF-LS, the same Gaussian beams are focus drifted with doubled Rayleigh lengths (~130 μm). Scale bar: 50 μm. (b) The graph shows the FWHM of the simulated light sheet at different positions along the illumination axis with NA=0.064. (c) Both the FoV and FWHM change with the illumination NA. The diagram shows the triple-focus LS has a much larger effective FoV than the single-focus LS.
~570 μm, enlarging the FoV by 2.6 times. Meanwhile, the performance of the MF model is comparable to the swept focus model, showing more advantages at the edge of a larger FoV. Furthermore, we compared the average resolution within the Rayleigh regions between single-focus LS and triple-focus LS in Fig. 2c. When requiring an effective FoV of 500 μm, the average resolution maintains at 5.5 μm in the triple-focus model, while it falls to 8.3 μm in the single-focus model.

Fig. 3. Experimental measurements of the single-focus LS and triple-focus LS. (a) Profile of line beam imaged in the single-focus model and (c) in the triple-focus model over propagation (from left) length of 665 μm. The focus drift in the triple-focus experiment is around 160 μm. (b) Maximum intensity projection (M. I. P) of microspheres (500 nm) embedded in 2% agarose in the x-z plane under single- and (d) under triple-focus LS illumination. The inserted scale bar is 100 μm. (e) Comparison of the FWHM of the images of the line beam with scattering light (line-plot) and the axial FWHM of fluorescent images of microspheres (dot-plot) over the FoV.

The simulation assumes that multiple Gaussian beams are of the same intensity. To experimentally achieve a uniform light sheet, the reflectivity of every beam splitters should be well designed. With calculation, the optimum reflectivity of two beam splitters should be 23.5% and 39.5% for three foci (Supplementary Note 1). To reduce the cost, we use off-the-shelf beam-splitters with reflectivity of 27% and 40%. Besides the primary focus spots, there also exist some weaker focus spots due to multiple reflected beams, which can also be observed in experimental results. The detection path was set with a magnification of 20× with single focus image (from left) length of 665 μm. The effective N.A. was set to be around 0.064 in the experiments by adjusting the slit aperture (SA) shown in the schematics (Fig. 1). In the MBS, the thickness of spacers is set to be 2 mm, and the thickness of the beam splitters is 3 mm, resulting in the focus shift of ~160 μm, slightly larger than the doubled Rayleigh lengths.

To experimentally implement our MF-LS scheme, firstly, we imaged the line beam with scattered light in the single-focus model (Fig. 3a) and triple-focus model (Fig. 3c), respectively. The cylindrical lens (CL) shown in Fig. 1 was temporarily removed, and the SA is replaced with a circular aperture to adjust the effective N.A., in the single-focus scheme, the laser power was ~1.9 mW in the pupil plane and increased to ~5.4 mW in the triple-focus model with an exposure time of 15 ms. From the FWHM distributions of the measured beams (Fig. 3e), it can be seen that, though the single-focus light sheet achieved a higher resolution at the center (~3 μm), the resolution dropped rapidly when the FoV became larger, e.g., ~7.5 μm at the FoV of ~150 μm in diameter. In contrast, in the triple-focus light sheet, the FoV could be extended to approximately ~400 μm in diameter with a consistent resolution better than 7.5 μm.

The calibration experiments with fluorescent microspheres (500 nm) fixed in 2% agarose were carried out under single-focus versus triple-focus illumination, respectively. In the calibration experiment, we changed the detection objective with a low N.A. one (Olympus, 10×/N.A. 0.3 W), in which the axial resolution is determined by the thickness of the light sheet. Fig. 3b shows the Maximum Intensity Projection (M. I. P) image of the fluorescent beads under single-focus LS illumination, and Fig. 3d shows that under triple-focus LS. The single-focus LS covers a FoV of ~150 μm in diameter with axial resolution beyond 7.5 μm. In contrast, the triple-focus LS covers an FoV of ~450 μm, consistent with line beam imaging analysis (Fig. 3e).

Fig. 4. Experimental data with biological specimens for single-focus (a, c, f) and triple-focus light sheet (b, d, e, g). (a-d) M. I. P of Hela cells labeled by Nile red for single-focus LS (a, c) and triple-focus LS (b, d) in the x-y plane (a, b) and in the x-z plane (c, d). (e) 3D imaging (Δx × Δy × Δz = 665 × 1100 × 460 μm³) of juvenile zebrafish illuminated by MF-LS. (f-g) M. I. P of fixed juvenile Zebrafish labeled SYTOX™ Green Nucleic Acid Stain in the x-z plane (Δx × Δz = 665 × 460 μm²) for triple-focus light sheet (f) and for single-focus light sheet (g). (h) The dot-marked in the M. I. P of biological specimens shows the significant resolution and brightness increment at the edge of the FoV in triple-focus light sheet compared with single-focus light sheet. Scale bar: 100 μm.

Furthermore, we evaluated the performance of MF-LS on different biological specimens. For single focus light sheet illumination, the laser power was set to ~0.06 mW with an exposure time of 25 ms, and then turned up to ~0.18 mW in the triple-focus model. Hela cells and juvenile zebrafish were fixed in 2% agarose inside a glass capillary (1 mm diameter) and then pushed out for imaging. Nile-red labeled Hela cells were imaged with single-focus LS and triple-focus LS. The triple-focus illumination showed better illumination uniformity in the x-y perspective (Fig. 4a, b). In the x-z perspective, the cells on the border appear to be elongated and blurred in the single-focus image (Fig. 4c) due to increased FWHM of the light sheet, while those cells are still in round shape in the triple-focus images (Fig. 4d). Afterward, we imaged a volumetric biological specimen. Fixed ~3-day-old juvenile zebrafish were labeled with SYTOX™ Green Nucleic Acid Stain and imaged. As shown in the x-z M. I. P image in single-focus LS (Fig. 4f) and triple-focus LS (Fig. 4g), the shape of cells was elongated along the z-axis.
8. F. O. Fahrbach, P. Simon, and A. Rohrbach, Nature Photonics 4, 780-785 (2010).
9. J. Yang, L. Gong, Y. Shen, and L. V. Wang, Appl Phys Lett 113, 181104 (2018).
10. T. Vettenburg, H. I. Daigorno, J. Nytk, C. Coll-Llado, D. E. Ferrier, T. Cizmar, F. J. Gunn-Moore, and K. Dhoklaik, Nat Methods 11, 541-544 (2014).
11. J. Nytk, K. McCluskey, M. A. Preciado, M. Mazilu, Z. Yang, F. J. Gunn-Moore, S. Aggarwal, J. A. Tello, D. E. K. Ferrier, and K. Dhoklaik, Sci Adv 4, eaar4817 (2018).
12. F. O. Fahrbach, and A. Rohrbach, Nat Commun 3, 632 (2012).
13. E. Baumgart, and U. Kubitscheck, Opt Express 20, 21805-21814 (2012).
14. S. C. Lau, H. C. Chiu, L. Zhao, T. Zhao, M. M. T. Loy, and S. Du, Rev Sci Instrum 89, 043701 (2018).
15. A. Escobet-Montalban, F. M. Gasparoli, J. Nytk, P. Liu, Z. Yang, and K. Dhoklaik, Opt Lett 43, 5484-5487 (2018).
16. N. A. Hosny, J. A. Seforyth, G. Spickerman, T. J. Mitchell, P. Almada, R. Chesters, J. S. Mitchell, G. Chennell, A. C. Vernon, K. Cho, D. P. Sivastava, R. Forster, and T. Vettenburg, Biomed Opt Express 11, 3935-3955 (2020).
17. F. O. Fahrbach, V. Gurchenkov, K. Alessandri, P. Nassoy, and A. Rohrbach, Opt Express 21, 13824-13839 (2013).
18. X. Xu, J. Chen, B. Zhang, L. Huang, Y. Zheng, K. Si, S. Duan, and W. Gong, Opt Lett 45, 4851-4854 (2020).
19. S. Ryu, B. Seong, C. W. Lee, M. Y. Ahn, W. T. Kim, K. M. Choe, and C. Joo, Biomed Opt Express 11, 3936-3951 (2020).
20. B. C. Chen, W. R. Legant, K. Wang, L. Shao, D. E. Milkie, M. W. Davidson, C. Janopolous, X. S. Wu, J. A. Hammer, 3rd, Z. Liu, B. P. English, Y. Mimori-Kiyosue, D. P. Romero, A. T. Ritter, J. Lippincott-Schwartz, L. Fritz-Laylin, R. D. Mullins, D. M. Mitchell, J. N. Bembrnnek, A. C. Reymann, R. Bohme, S. W. Grill, J. T. Wang, G. Seydou, U. S. Tulu, D. P. Kiehart, and E. Betzig, Science 346, 1257998 (2014).
21. T. A. Planchon, L. Gao, D. E. Milkie, M. W. Davidson, J. A. Galbraith, C. G. Galbraith, and E. Betzig, Nat Methods 8, 417-423 (2011).
22. L. V. Nhu, H. Hoang, M. Pham, and H. Le, Eur Phys J Plus 135 (2020).
23. B. J. Chang, M. Kittisopikul, K. M. Dean, P. Roudot, E. S. Welf, and R. Fiolka, Nat Methods 16, 235-238 (2019).
24. L. Gao, L. Shao, C. D. Higgins, J. S. Poulton, M. Peifer, M. W. Davidson, X. Wu, B. Goldstein, and E. Betzig, Cell 151, 1370-1385 (2012).
25. B. J. Chang, K. M. Dean, and R. Fiolka, Opt Express 28, 27052-27077 (2020).
26. E. Remacha, L. Friedrich, J. Vermot, and F. O. Fahrbach, Biomed Opt Express 11, 8-26 (2020).
27. J. Huiskens, and D. Y. Stainier, Opt Lett 32, 2608-2610 (2007).
28. W. Zong, J. Zhao, X. Chen, Y. Lin, H. Ren, Y. Zhang, M. Fan, Z. Zhou, H. Cheng, Y. Sun, and L. Chen, Cell Res 25, 254-257 (2015).
29. K. M. Dean, and R. Fiolka, Opt Express 22, 26141-26152 (2014).
30. F. O. Fahrbach, F. F. Voigt, B. Schmid, F. Helmchen, and J. Kiehart, Opt Express 21, 21010-21026 (2013).
31. P. N. Hedde, and E. Gratton, Micr Ross Tec 81, 924-928 (2018).
32. L. Gao, Opt Express 23, 6102-6111 (2015).
33. L. Gao, W. C. Tang, Y. C. Tsai, and B. C. Chen, Opt Express 27, 1497-1506 (2019).
34. K. M. Dean, P. Roudot, E. S. Welf, T. Pohlkamp, G. Garrelts, J. Herz, and R. Fiolka, Optica 4, 263-271 (2017).
35. K. M. Dean, P. Roudot, E. S. Welf, G. Danuser, and R. Fiolka, Biophys J 108, 2807-2815 (2015).
36. S. Deng, Z. Ding, D. Yuan, M. Liu, and H. Zhou, J Opt Soc Am A Opt Image Sci Vis 38, 19-24 (2021).
37. E. J. Botcherby, R. Juskaitis, M. J. Booth, and T. Wilson, Opt Lett 32, 2007-2009 (2007).
38. E. J. Botcherby, R. Juskaitis, M. J. Booth, and T. Wilson, Opt. Commun. 283, 880-887 (2008).
39. O. E. Olarte, I. Andilla, E. J. Gualda, and P. Loza-Alvarez, Adv Opt Photonics 10, 111-179 (2018).