Dual-slit confocal light sheet microscopy for \textit{in vivo} whole-brain imaging of zebrafish

Zhe Yang,\textsuperscript{1,2} Li Mei,\textsuperscript{1,2} Fei Xia,\textsuperscript{1,2} Qingming Luo,\textsuperscript{1,2} Ling Fu,\textsuperscript{1,2,3} and Hui Gong\textsuperscript{1,2,*}

\textsuperscript{1}Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan 430074, China
\textsuperscript{2}MoE Key Laboratory for Biomedical Photonics, Department of Biomedical Engineering, Huazhong University of Science and Technology, Wuhan 430074, China
\textsuperscript{3}Correspondence: lfu@mail.hust.edu.cn
\textsuperscript{*}huigong@mail.hust.edu.cn

Abstract: \textit{In vivo} functional imaging at single-neuron resolution is an important approach to visualize biological processes in neuroscience. Light sheet microscopy (LSM) is a cutting edge \textit{in vivo} imaging technique that provides micron-scale spatial resolution at high frame rate. Due to the scattering and absorption of tissue, however, conventional LSM is inadequate to resolve cells because of the attenuated signal to noise ratio (SNR). Using dual-beam illumination and confocal dual-slit detection, here a dual-slit confocal LSM is demonstrated to obtain the SNR enhanced images with frame rate twice as high as line confocal LSM method. Through theoretical calculations and experiments, the correlation between the slit’s width and SNR was determined to optimize the image quality. \textit{In vivo} whole brain structural imaging stacks and the functional imaging sequences of single slice were obtained for analysis of calcium activities at single-cell resolution. A two-fold increase in imaging speed of conventional confocal LSM makes it possible to capture the sequence of the neurons’ activities and help reveal the potential functional connections in the whole zebrafish’s brain.

© 2015 Optical Society of America

OCIS codes: (180.2520) Fluorescence microscopy; (110.0110) Imaging systems; (170.3880) Medical and biological imaging; (170.2945) Illumination design; (180.1790) Confocal microscopy.

References and links
1. B. B. Averbeck, P. E. Latham, and A. Pouget, “Neural correlations, population coding and computation,” Nat. Rev. Neurosci. 7(5), 358–366 (2006).
2. J. N. D. Kerr and W. Denk, “ imaging in vivo: watching the brain in action,” Nat. Rev. Neurosci. 9(3), 195–205 (2008).
3. M. M. Churchland, J. P. Cunningham, M. T. Kaufman, J. D. Foster, P. Nuyujukian, S. I. Ryu, and K. V. Shenoy, “Neural population dynamics during reaching,” Nature 487(7405), 51–56 (2012).
4. V. Gilja, C. A. Chestek, P. Nuyujukian, J. Foster, and K. V. Shenoy, “Autonomous head-mounted electrophysiology systems for freely behaving primates,” Curr. Opin. Neurobiol. 20(5), 676–686 (2010).
5. B. A. Wilt, L. D. Burns, E. T. Wei Ho, K. K. Ghosh, E. A. Mukamel, and M. J. Schnitzer, “Advances in light microscopy for neuroscience,” Annu. Rev. Neurosci. 32(1), 435–506 (2009).
6. B. A. Flusberg, E. D. Cocker, W. Piyawattanametha, J. C. Jung, E. L. M. Cheung, and M. J. Schnitzer, “Fiber-optic fluorescence imaging,” Nat. Methods 2(12), 941–950 (2005).
7. C. J. Engelbrecht, R. S. Johnston, E. J. Seibel, and F. Helmchen, “Ultra-compact fiber-optic two-photon microscope for functional fluorescence imaging \textit{in vivo},” Opt. Express 16(8), 5556–5564 (2008).
8. J. Lecoq, J. Savall, D. Vučinić, B. F. Grew, H. Kim, J. Z. Li, L. J. Kitch, and M. J. Schnitzer, “Visualizing mammalian brain area interactions by dual-axis two-photon calcium imaging,” Nat. Neurosci. 17(12), 1825–1829 (2014).
9. M. B. Ahrens, M. B. Orger, D. N. Robson, J. M. Li, and P. J. Keller, “Whole-brain functional imaging at cellular resolution using light-sheet microscopy,” Nat. Methods 10(5), 413–420 (2013).
10. N. Vladimirov, Y. Mu, T. Kawashima, D. V. Bennett, C. T. Yang, L. L. Looger, P. J. Keller, J. Freeman, and M. B. Ahrens, “Light-sheet functional imaging in fictively behaving zebrafish,” Nat. Methods 11(9), 883–884 (2014).

11. L. Silvestri, A. L. A. Mascaro, J. Lotti, L. Sacconi, and F. S. Pavone, “Advanced Optical Techniques To Explore Brain Structure and Function,” J. Innov. Opt. Health Sci. 06(01), 1230002 (2013).

12. T. F. Holekamp, D. Turaga, and T. E. Holy, “Fast three-dimensional fluorescence imaging of activity in neural populations by objective-coupled planar illumination microscopy,” Neuron 57(5), 661–672 (2008).

13. A. Ertürk, K. Becker, N. Jähring, C. P. Mauch, C. D. Höjer, J. G. Egen, F. Hellal, F. Bradke, M. Sheng, and H. U. Dott, “Three-dimensional imaging of solvent-cleared organs using 3DISCO,” Nat. Protoc. 7(11), 1983–1995 (2012).

14. A. Ertürk, C. P. Mauch, F. Hellal, F. Förster, T. Keck, K. Becker, N. Jähring, H. Steffens, M. Richter, M. Hübener, E. Kramer, F. Kireilhoff, H. U. Dott, and F. Bradke, “Three-dimensional imaging of the unsectioned adult spinal cord to assess axon regeneration and glial responses after injury,” Nat. Med. 18(1), 166–171 (2011).

15. L. Silvestri, A. Bria, L. Sacconi, G. Iannello, and F. S. Pavone, “Confocal light sheet microscopy: micron-scale neuroanatomy of the entire mouse brain,” Opt. Express 20(18), 20582–20598 (2012).

16. R. Tomer, K. Khaire, and P. J. Keller, “Shedding light on the system: studying embryonic development with contrast imaging of entire embryos with Digital Scanned Laser Light Sheet Fluorescence Microscopy,” Curr. Opin. Genet. Dev. 21(5), 558–565 (2011).

17. M. Weber and J. Huisken, “Light-sheet microscopy for real-time developmental biology,” Curr. Opin. Genet. Dev. 21(5), 566–572 (2011).

18. J. Huisken and D. Y. R. Stainier, “Selective plane illumination microscopy techniques in developmental biology,” Development 136(12), 1963–1975 (2009).

19. B. C. Chen, W. R. Legant, K. Wang, L. D. Milkie, M. W. Davidson, C. Janetopoulos, X. S. Wu, J. A. Hammer Jr, Z. Liu, B. P. English, Y. Mimori-Kiyosue, D. P. Romero, A. T. Ritter, J. Lippincott-Schwartz, L. Fritz-Laylin, R. M. Silas, W. T. Blevins, D. M. Mitchell, J. N. Bement, J. T. Wang, G. Seydoux, U. S. Tulu, D. P. Kiehart, and E. Betzig, “Lattice-sheet light-microscopy: Imaging molecules to embryos at high spatiotemporal resolution,” Science 346(6208), 1257998 (2014).

20. U. Krzic, S. Gunther, T. E. Saunders, S. J. Streichan, and L. Hufnagel, “Multiview light-sheet microscope for rapid in toto imaging,” Nat. Methods 9(7), 730–733 (2012).

21. R. Tomer, K. Khaire, F. Amat, and P. J. Keller, “Quantitative high-speed imaging of entire developing embryos with simultaneous multiview light-sheet microscopy,” Nat. Methods 9(7), 755–763 (2012).

22. J. Huisken, J. Swoger, F. Del Bene, J. Wittbrodt, and E. H. K. Stelzer, “Optical sectioning deep inside live embryos by selective plane illumination microscopy,” Science 305(5668), 1007–1009 (2004).

23. T. A. Planchon, L. Gao, D. E. Milkie, M. W. Davidson, J. A. Galbraith, C. G. Galbraith, and E. Betzig, “Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination,” Nat. Methods 8(5), 417–423 (2011).

24. T. Vettenburg, H. I. C. Dalgarno, J. Nyík, C. Coll-Lladó, D. E. K. Ferrier, T. Čižmár, F. J. Gunn-Moore, and K. Dholakia, “Light-sheet microscopy using an Airy beam,” Nat. Methods 11(5), 541–554 (2014).

25. P. J. Keller, A. D. Schmidt, A. Santella, K. Khairy, Z. Bao, J. Wittbrodt, and E. H. K. Stelzer, “Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy,” Nat. Methods 7(8), 637–642 (2010).

26. P. J. Keller and E. H. K. Stelzer, “Quantitative in vivo imaging of entire embryos with Digital Scanned Laser Light Sheet Fluorescence Microscopy,” Curr. Opin. Neurobiol. 18(6), 624–632 (2008).

27. F. O. Fahrbach, F. F. Voigt, B. Schmid, F. Helmchen, and J. Huisken, “Rapid 3D light-sheet microscopy with a tunable lens,” Opt. Express 21(18), 21010–21026 (2013).

28. N. Chen, S. Rehman, and C. J. R. Sheppard, “Advanced optical microscopy methods for in vivo imaging of subcellular structures in thick biological tissues,” J. Innov. Opt. Health Sci. 07(05), 1440001 (2014).

29. T. Breuninger, K. Greger, and E. H. K. Stelzer, “Lateral modulation boosts image quality in single plane illumination fluorescence microscopy,” Opt. Lett. 32(13), 1938–1940 (2007).

30. J. Mertz, “Optical sectioning microscopy with planar or structured illumination,” Nat. Methods 8(10), 811–819 (2011).

31. E. Baumgart and U. Kubitscheck, “Scanned light sheet microscopy with confocal slit detection,” Opt. Express 20(19), 21805–21814 (2012).

32. A. Lueczak, P. Barthé, S. L. Marguet, G. Buzsáki, and K. D. Harris, “Sequential structure of neocortical spontaneous activity in vivo,” Proc. Natl. Acad. Sci. U.S.A. 104(1), 347–352 (2007).

33. G. Cox and C. J. R. Sheppard, “Practical limits of resolution in confocal and non-linear microscopy,” Microsc. Res. Tech. 63(1), 18–22 (2004).

34. C. J. Engblom and E. H. Stelzer, “Resolution enhancement in a light-sheet-based microscope (SPLM),” Opt. Lett. 31(10), 1477–1479 (2006).

35. C. J. Sheppard, X. S. Gan, and M. Gu, “Signal-to-Noise Ratio in Confocal Microscopes,” in Handbook of Biological Confocal Microscopy, P. James, ed. (Springer, 2006).

36. T. Wilson, P. Török, and P. D. Higdon, “The effect of detector size on the signal-to-noise ratio in confocal polarization light microscopy,” J. Microsc. 189(1), 12–14 (1998).

37. R. Gauderon and C. J. Sheppard, “Effect of a finite-size pinhole on noise performance in single-, two-, and three-photon confocal fluorescence microscopy,” Appl. Opt. 38(16), 3562–3565 (1999).
1. Introduction

Brain function relies on the activity of multiple neurons and their communication [1]. A full understanding of when, where and how neurons interact with others at cellular level can reveal how the brain works [2]. Conventional electrophysiological [3,4] and optical [5] strategies have been designed to record the activities of neurons. Electrophysiological methods can record activities in high temporal resolution, but the quantity of neurons these techniques recorded is limited by the density of electrodes. Thanks to the advent of calcium fluorescent label methods, in vivo imaging approaches such as laser scanning confocal microscopy (LSCM) [6], multiphoton microscopy (MPM) [7,8] and light sheet based microscopy (LSM) [9,10] have been developed to better visualize neuron’s calcium activities in living animals [2,11].

In last decade, LSM has attracted increasing attention in life sciences, including neuroscience [9,10,12–15] and developmental biology [16–21]. In LSM, only one plane of the sample is illuminated with a thin sheet of light, and the fluorescence is collected by a wide-field microscope, whose optical axis is orthogonal to the excited plane [22]. Generally, there are two approaches to generate the light sheet. One is focusing a Gaussian beam with a cylindrical lens. The other is sweeping the focused Gaussian, Bessel or Airy beam [23,24] across the imaging plane in digital scanned light sheet microscopy (DSLM) [25]. The use of light sheet can reduce the photo-bleaching by only exciting the imaging plane rather than the large volume in conventional confocal microscopy. Therefore, LSM is especially suitable for long-term in vivo imaging [26]. Another advantage of LSM is that, the wide-field detection scheme allows for the high imaging speed of LSM and therefore enables observation of instantaneous events such as blood cell flow [27] and neuron activity [9].

However, the image degradation such as blur and low contrast hinders the application of LSM in turbid biological specimens like an intact brain. This degradation is mainly caused by the reduction of SNR and can be analyzed from two aspects: illumination and detection. First, the illumination beam is scattered by the turbid medium, which leads to the thickening of the light sheet. Consequently, more out-of-focus fluorophores are excited, giving rise to an increase in background noise. Second, before the fluorescence is collected by the wide-field detection system, it has been scattered by the turbid medium [28]. The heavily scattered fluorescent photons received by the detector lose the position information of corresponding fluorophores, and increase image noise.

Previous researches have proposed several methods to solve the above mentioned problems, which can be summarized as either illumination-based or detection-based strategies. The illumination-based approach known as structure illumination (SI) method has been introduced to LSM to eliminate scattered light (DSLM-SI [25], SPIM-SI [29]). This kind of method modulates the light sheet with shifted spatial pattern, then differentiates fluorescence signal from the weakly modulated scattered background and improves the SNR. Whereas, multiple raw images (three in general) with different illumination patterns need to be acquired to compose one final image, resulting in the limited imaging speed in practice. Moreover, in the illumination-based methods, the fluorescent photons are collected by the camera without distinction. In the case of imaging the strong scattering samples, most of the dynamic range may be occupied by the heavily scattered photons, limiting the effective dynamic range of the microscope [30], which may reduce the detectability of the transients during functional imaging.
In comparison, the detection-based strategy provides an alternative way to eliminate the scattered fluorescence. Confocal LSM utilizes a mechanical slit as a spatial filter in front of the camera [15]. Like the line confocal microscopy, the slit conjugates with the scanned illumination beam and blocks the out-of-focus and scattered fluorescent photons to improve the SNR. In confocal approach, the reduction of the detected scattered photons makes it possible to take better advantage of dynamic range of the detector, which meets the need of the functional imaging. Nevertheless, the descanning structure used to restore the spatial information of each line in the detection path hampers efficient detection due to the attenuation of fluorescence signal.

An alternative approach to achieve more efficient line confocal detection is using a scanning slit that conjugates with the illumination beam and is practically the rolling shutter on a sCMOS camera [31]. The exposed pixel rows serve as one slit and move with the scanned illumination beam where the most heavily scattered photons are blocked by the unexposed areas of the sensor. But, limited by the moving speed of slit, the frame rate is inadequate to capture the precise time-resolved calcium transients to describe the activity sequence of the functionally connected neurons whose latencies are typically within 50 to 100 ms [32]. As such, it is a crucial issue to address, how to acquire cell-resolved calcium transients with a higher frame rate using confocal LSM.

Aiming to achieve precise calcium transient with cellular resolution throughout the whole brain of a zebrafish, here we demonstrate a dual-slit confocal LSM that takes advantages of the characteristics of a rolling shutter mode in a state-of-the-art sCMOS camera. In this system, two focused beams scan the sample in two opposite directions and are synchronized with two “electronic slits” on the camera. This method improves the SNR by blocking the scattered fluorescence with slits and increases the imaging speed by simultaneously acquiring two halves of an image. To clearly visualize the structure and calcium transients in a whole brain, we theoretically and experimentally analyzed the effect of the slit on SNR improvement. In vivo structural and functional imaging of GCaMP5G transgenetic zebrafish embryos were obtained. The SNR improvement of images is demonstrated by a comparison with the conventional LSM. After reconstruction, the 3D structure of a brain of zebrafish can be resolved in cellular resolution in whole brain. Meanwhile, the calcium signal transients of individual neurons in one slice were recorded from the time-lapse functional imaging. The experimental results prove that our system can image the whole brain in cellular resolution at the imaging speed twice as fast as previous confocal LSM. One frame at 2048 by 1024 pixels can be captured within 50 ms with 1 ms exposure time for every pixel by our system comparing to 100ms in same imaging conditions by the conventional LSM. The latency of spontaneous activity within 100ms has been observed to describe the activity sequence of the functionally connected neurons in the zebrafish’s brain. Additionally, the possible junction problem introduced by the illumination is also discussed.

2. Materials and methods

To obtain confocal images through LSM at higher speed, an effective approach is acquiring two halves of the image independently and simultaneously. A dual-slit confocal LSM was designed and established to implement this approach. Two critical problems have to be solved in order to build up the dual-slit confocal LSM. One challenge is how to generate two separately scanned beams in the same plane to excite fluorescence. The other is how to apply two slits as spatial filters in an optically conjugate plane in front of the camera.

2.1 Optical setup

The first problem can be solved by generating two asymmetrically scanned beams from two opposite illumination directions. The schematic diagram of the dual-slit confocal LSM is shown in Fig. 1. A 488 nm wavelength DPSS laser (Sapphire HP, Coherent) is employed as the excitation light source. The beam is intensity-modulated by an acousto-optic tunable filter.
(AOTF, Crystal Technology) and expanded by a telescoped beam expander. Subsequently, a galvanometer mirror (6215H, Cambridge) scans the beam in the lateral plane. After a scan lens, the beam is separated by a 50/50 beam splitter. The galvanometer mirror plane is imaged onto the back focus plane of two aligned illumination objectives (LMPLFLN 5 ×, NA = 0.13, Olympus), respectively. The effective NA of the illumination objective is approximately 0.08 in our system. For the illumination beams, the tilt angle caused by galvanometer mirror is translated into a lateral displacement, which generates a light sheet in the lateral plane of the sample volume. Since the existence of the relay optics in one of the illumination paths, the beams from two illumination objectives scan in the opposite directions as shown in Fig. 1(b). These two beams form one light sheet that excites fluorescence in one plane of the sample. By this illumination strategy, two halves of sample are excited at the same time, so it is possible to improve the imaging speed for confocal LSM.

The sample is mounted on a four-axis sample scanner, including three orthogonal stacked translation stages and one rotatory stage (M111-1 and M116, PI). The sample is immersed into a custom designed chamber that is filled with water and attached to a water dipping detection objective (W “N-Achromat” 10 × /0.3, Carl Zeiss). Fluorescence is detected orthogonally to the light sheet with a fast sCMOS camera (Orca Flash 4.0, Hamamatsu Photonics). The excitation light is blocked from the imaging pathway by an optical band pass filter.

2.2 System Synchronization and data acquisition

The second problem mentioned above can be settled by generating two activated areas as slits shifting on the sCMOS sensor. We take advantage of the “rolling shutter” mode in sCMOS camera, where one image is formed by sequentially reading out the data of exposed rows that move across the sensor. In sCMOS, owing to its split readout scheme, the top and bottom halves of the sensor are readout independently. At the beginning of frame exposure, two rows in the middle of the sensor is activated. Then the neighboring rows are activated sequentially.
and symmetrically from middle to top and bottom of the sensor with a time delay (readout interval $t_r$, shown in Fig. 2(a)). When a row finishes its exposure, the data are then readout and stored. The activated area of the sCMOS sensor is composited by the exposing rows. This process looks like two “mechanical slits” scanning in the opposite directions from the middle to top and bottom on the sensor.

Fig. 2. Principle of slits generation and controlling waveform of dual-slit confocal LSM system. (a) Principle of the slit generation by the sCMOS sensor. The bars mean the pixel rows on the sensor. The activate rows move from middle to the both side of the sensor. Arrows in readout sequence plot mean the time sequence of activated rows’ readout on the sensor. Neighboring rows start exposing with a delay. (b) Controlling waveforms of the AOTF, galvanometer mirror and sCMOS. When the AOTF switches the laser on, the galvanometer scans the beam from middle to both sides of the field of view. The sCMOS works at level trigger mode and starts exposing when the galvanometer mirror starts scanning.

As two scanned beams and two slits are implemented, their synchronization is the prerequisite to generate the light sheet and block the scattered photons. In the system, the AOTF and the camera were synchronized to make sure that the sample was excited during the sCMOS’s exposure process. The galvanometer mirror was driven by an asymmetric triangular wave shown in Fig. 2(b), which can control two beams scanning in the opposite direction to generate light sheet continuously. The sCMOS worked at the level trigger mode and was synchronized with the galvanometer mirror. After initialization, two beams are in the middle of the field of view (FOV). At the same time, pixel rows in the middle of the sCMOS sensor were activated. As the beams scanned from middle to the two boundaries of FOV, the “slits” on the sensor moved simultaneously with them. After finishing one frame, galvanometer mirror was driven back to the central position rapidly. By repeating the procedure above, image stacks were acquired.

2.3 Sample preparation

To test our system’s performance, two types of samples were prepared for imaging. The embedded fluorescent beads were prepared for quantitative analysis of spatial resolution and SNR of our dual-slit confocal LSM. The transgenic zebrafish samples were prepared for structural and functional imaging to test this system’s performance on in vivo imaging.

Both 10 $\mu$m (90287, 490/525, Fluka) and 0.2 $\mu$m (F-8811, 505/515, Molecular Probes) fluorescent beads were embedded using low melting point agarose respectively. The diluted beads solution was oscillated in an ultrasonic cleaner for 5 min to prevent the beads from aggregation. The low melting point agarose (A-4018, Sigma-Aldrich) was dissolved at 40 °C (0.5% w/v). Then the beads solution and agarose solution were mixed at 1:1000 and drawn into glass micropipettes (B150-86-10, Sutter Instruments) with a plunger. After polymerization for 20 min at room temperature, embedded sample can be extruded from the micropipette and immersed into the chamber of the microscope.
5-day-old zebrafish larvae of the HuC-GCaMP5G line in albino background were used for in vivo imaging. Low-melting-point agarose was diluted in phosphate buffered saline (PBS) (1.0% w/v). The zebrafish larvae were placed in liquid agarose at 37 °C, and drawn into a glass micropipette slowly with a plunger waiting for polymerization. Then the micropipette was fixed at sample stage and the sample was extruded out to right height with the plunger. The temperature was about 25 °C during the whole imaging process. All animal experiments were performed according to the animal experiment guidelines of the Animal Experimentation Ethics Committee of HUST.

3. Results

3.1 Spatial resolution of dual-slit confocal LSM

3D point spread function (PSF) was obtained to measure the spatial resolution. The embedded sub-diffraction fluorescent beads (200 nm) were imaged with different slit widths. For each slit width, several stacks of images were acquired from different beads. The length of z-step of these stacks is 1 μm. 3D PSF was extracted by reconstructing the image stacks using ImageJ (NIH, MD), as shown in Fig. 3(a) and 3(c). The scale was calibrated by using standard 10 μm fluorescence beads sample. After fitting the xy and yz profile of the beads with Gaussian curves, the lateral and axial resolution are quantified by the averaged full width at half maximum (FWHM) values of these curves (N = 5 for each data point). Figure 3(b) and 3(d) illustrate the lateral and axial resolution according to different slit’s width.

![Fig. 3](image)

As shown in Fig. 3(b) and 3(d), when the slit width changes from 6.45 μm to 500 μm, the lateral and axial resolution of microscope keep around 0.95 μm and 3.41 μm, respectively. It means that the existence and the width of the slits has no significant effect on the spatial resolution of dual-slit confocal LSM. The result of lateral resolution is consistent with the...
conclusion of previous research in confocal microscopy [33]. For the axial resolution, this result shows difference from conventional concept about the sectional ability of confocal microscopy. But in LSM, the sectioning capability is mainly determined by the thickness of light sheet, especially in the condition that the NA of the detection objective is low (0.3 in our system), because the axial extent of detection system’s PSF is larger than the thickness of light sheet [34]. Therefore, the axial resolution does not change with various slit widths. The fluctuation of axial resolution values is mainly caused by the gradual change in thickness of the light sheet in the FOV.

3.2 SNR of dual-slit confocal LSM

3.2.1. SNR enhancement in dual-slit confocal LSM

Although the slits of dual-slit confocal LSM does not introduce significant enhancement or decay in the resolution, we find the SNR of images changes obviously for the different slit width. To test our system’s SNR, in vivo images on zebrafish larvae are obtained with conventional LSM and dual-slit confocal LSM, respectively. The conventional LSM images were acquired by using the simulated global-shutter mode of sCMOS, which was achieved by setting a delay for excitation beams until all the pixel rows start exposure through AOTF. The slit confocal images were acquired with the slit width of 64.5 μm, equaling to 10 activity pixel lines. The exposure time in both conventional and dual-slit confocal LSM were 10 ms. The agarose-embedded zebrafish larvae were placed into the imaging chamber filled with PBS and was moved to the focal plane of the detection objective. Room temperature was maintained throughout the imaging acquisition.

Fig. 4. Comparison of conventional LSM and dual-slit confocal LSM images of the zebrafish’s brain in vivo. (a) Image acquired by conventional LSM. (b) Image acquired by dual-slit confocal LSM. (c) Normalized intensity values of plots as indicated by the line in (a) and (b). (d, e) Higher magnification of the mid-brain region indicated in (a) and (b). (f) Normalized intensity values along the lines in (d) and (e), respectively. Scale bars, 100 μm for (a, b), 10 μm for (d, e).
From the imaging results, it is found that the SNR of the images acquired by dual-slit confocal LSM is higher than conventional LSM, especially in deep tissue. As can be seen in Fig. 4(b) and 4(e), the images acquired by our system are clear enough to distinguish almost each neuron cells through their boundary, which is hard to resolve by conventional LSM as shown in Fig. 4(a) and 4(d). To quantify the effect of image quality improvement, the normalized intensity along the lines labeled in images was analyzed. The plot in Fig. 4(c) and 4(f) demonstrate that, the background rejection ability of dual-slit confocal LSM is obvious and results in a higher contrast to resolve individual cells in deep tissue.

3.2.2. SNR optimization

Through the comparison between our system and conventional LSM, it is found that the SNR of images improves because the existence of conjugate slits. According to the previous research on confocal microscopy, the SNR is determined by the shape and extent of the conjugate spatial filter. Strictly speaking, the absolute line confocal images should be acquired with an infinitesimally narrow slit, however resulting in extremely weak signal and limited imaging speed. On the other hand, a large slit width will make the system lose the ability to reject the scattered photons [35]. To obtain the best imaging quality, we need to analyze the relationship between the SNR and slit width in our system.

Our dual-slit confocal LSM could be considered as two equivalent systems for both illumination and detection. For either of them, the illumination could be seen as an orthogonal line illumination from an objective and the fluorescence detection could be seen as a line detection with finite length, which is different from the conventional line-scanned confocal microscopy. Following the noise model described in previous research on confocal microscopy [36,37], the SNR is defined as the ratio of the signal from a point object and the noise from background in 3D space. In confocal LSM, it can be calculated by the following equation:

$$\frac{S}{N} = \frac{F(\nu_d)}{2\nu_s\nu_s}$$

$F(\nu_d)$ is the fraction of incident signal light on the detector, $\nu_s$ is the length of the slit. In our experiment, $\nu_s$ equals to the length of the sCMOS sensor in the direction that is perpendicular to the slits. $\nu_d$ is the radius of the detector in normalized coordinates [36]:

$$\nu_d = \frac{2\pi r_d \sin \alpha}{M \lambda_f},$$

Where $r_d$ is the real radial coordinates, $\sin \alpha / M$ is the NA of the system in detecting space, $M$ is the magnification of the detection system, and $\lambda_f$ is the wavelength of the fluorescence.

In our system, fluorescence is excited by line illumination, whose waist is comparable with the width of the slit detector. If the slit width is $2\nu_d$, the fraction of incident signal light on the detector should be described as

$$F(\nu_d) = \frac{\pi^{1/4}}{2} \frac{\mathbf{H}_1(2\nu)}{\nu^2} d\nu,$$

where $\mathbf{H}_1(x)$ is first order Struve function

$$\mathbf{H}_1(x) = \frac{x}{\sqrt{\pi \Gamma(3/2)}} \int_0^\infty \sin(x \cos \tau) \sin^2(\tau) d\tau.$$
The theoretical relationship between the SNR and slit width has been obtained according to the numerical calculation result of Eq. (1). In order to verify this relationship, we calculated the SNR of images acquired with various slit width. Assuming that the noise follows the Poisson distribution, the SNR of images can be get by the following equation [31]:

\[
\frac{S}{N_{\text{exp}}} = \frac{I_s}{\sigma_b}.
\]

\(I_s\) is the signal amplitude and \(\sigma_b\) is the noise level of the image. \(I_s\) is decided by the peak value of the measured PSF. \(\sigma_b\) is measured by the standard deviation of the background selected as the region without signal. We acquire the experimental SNR values by averaging 10 data sets acquired from different areas. Since the illumination strategy’s difference, it is found that the trend of the SNR’s variation is similar with conventional line-scanned confocal microscopy [38], while the optimized slit width is different in our confocal LSM. Based on the calculation and measurements above, the relationship among SNR, frame rate and slit width is plotted as shown in Fig. 5. The theoretical and experimental SNR values are normalized, respectively.

Figure 5 indicates that the SNR changes with different slit’s widths. When the slit width is far less than the width of the illumination beam in detection space, most of the effective signal is also blocked by the slit. Therefore, the SNR is low in this situation. As the increase of the slit’s width, more ballistic photons can be received by the camera and most scattered photons are blocked by the spatial filter. Therefore, the SNR is improved greatly, and reaches the optimum value when the slit width is about 60 \(\mu\)m. With the further expansion of the slit’s width, the SNR decays slowly because of the increase in number of the scattered photons received by the camera.

We also imaged the same zebrafish by our system with different slit width. The results are shown in Fig. 6. The slit widths in Fig. 6(a) to 6(c) are 6.45 \(\mu\)m, 64.5 \(\mu\)m and 322.5 \(\mu\)m, respectively. The difference on SNR can be seen by comparing these images. The SNR of...
image acquired with 64.5 μm is better than the rest of the two conditions, and the trend of the changes coincide with the theoretical calculation shown in Fig. 5. Combined with the results in Section 3.1, it is concluded that the introduction of dual slits in LSM can obtain higher SNR than conventional LSM without influencing the spatial resolution.

Fig. 6. Zebrafish imaging by dual-slit confocal LSM with different slit width. The slit width of (a), (b) and (c) are 6.45 μm, 64.5 μm and 322.5 μm respectively. The exposure time are all 10 ms. The scale bars is 20 μm. (d) is the normalized intensity along the lines labeled in (a) to (c).

3.3 Imaging speed of dual-slit confocal LSM

In our system, the camera works at rolling shutter mode. It means that one frame can be acquired after all the pixel rows finish their readout sequentially. If the frame center is in the middle of the sensor, the duration of forming one frame can be calculated as

$$T = \frac{t_{\text{exp}} \times H}{d_{\text{slit}}},$$

(6)

where $t_{\text{exp}}$ is the exposure time as shown in Fig. 2(a), $d_{\text{slit}}$ is the slit’s width and $H$ is the total width of the frame.

According to the Eq. (6), it is denoted that the dual-slit confocal SLM only consume half-frame scanning time to acquire one complete frame compared with the conventional LSM. As for single slit confocal LSM, the frame rate is one half that of dual-slit situation, as illustrated in Fig. 5(f) where the exposure time is set to be 1 ms for each pixel row. The highest imaging speed is up to 90 frames per second at 2048 by 2048 pixels, while the single slit condition is just 45 frames per second. In the imaging experiments, the frame rate recorded by the software (HCimage Live, Hamamatsu Photonics) is identical with the theoretical calculation. Therefore, dual-slit confocal LSM can improve the frame rate by twofold compared with the previous single slit confocal LSM.
3.4 In vivo imaging of zebrafish’s brain

3.4.1. 3D structural imaging

To acquire the whole brain structure of the zebrafish’s brain, fluorescently labeled zebrafish (HuC-GCaMP5G, albino) was imaged by our dual-slit confocal LSM. The sample was moved by sample stage along detection axis (z-axis) with 1 μm step size, then image stacks were acquired and stored. 300 slices were acquired for each stack. We reconstructed the 3D structure of zebrafish’s brain with Amira 5 (Visage Imaging), as shown in Fig. 7 and Media 1.

![Fig. 7. 3D structure of zebrafish’s brain. (a) to (d) are raw images from in vivo imaging showing four different sections. The scale bar is 100 μm in (a), (e) to (h) show enlarged views of four regions labeled in (a) to (d), demonstrating cellular resolution at different depths in the brain. The scale bar is 20 μm in (e). (i) is one slice from lateral view (xz) of zebrafish’s brain reconstructed by the raw images. The structure of optic tectum, epencephala and hindbrain are apparent. The scale bar is 100 μm. (j) and (k) are the enlarged views of regions labeled in (i), demonstrating the cellular resolution in the whole-brain. The scale bar is 20 μm in (j).](image)

Figure 7(a) to 7(d) show the raw images acquired by dual-slit confocal LSM in different depth (15 μm, 55 μm, 95 μm, and 135 μm). To demonstrate the cellular resolution in the whole brain, enlarged views of four regions labeled in (a) to (d) are shown in (e) to (h). Owing to the optimized slit’s width to restrain the background noise, the different structures are apparent with cellular resolution in hindbrain (e), epencephala (f), optic tectum (g) and forebrain (h). One reconstructed slice in lateral view is shown in Fig. 7(i). The position and structure of the different brain regions can be identified and the distribution of cells in the different brain regions is obvious. The enlarge view showed in (j) and (k) show the different structure of hindbrain (j) and optic tectum (k) in lateral view, demonstrating the cellular resolution in axial direction.

As demonstrated in previous results [21], single-sided illumination introduces the contrast gradients along the illumination direction in each image. In the case of the dual-slit confocal LSM, the top half of the sample is illuminated by the beam from one direction, and the bottom half is illuminated from the other direction. Since the illumination direction switches frame by frame, the trends of these gradients in two sequential images are swapped, and may introduce artifacts of intensity fluctuations in image sequences. However, the decay of the contrast is weak in our experiments, because we used the pigments inhibited albino fishes in 5 days past fertilization that are transparent. As can be seen in Fig. 4, Fig. 7 and Media 1, the gradient of image contrast is not severe for resolving individual neurons. The artifacts introduced by the switching illumination can therefore be negligible in the structural and functional analysis.
From the 3D imaging result, cellular resolution in lateral and axial directions can be acquired in whole brain of zebrafish by the dual-slit confocal LSM, which is hard to be achieved in conventional LSM. The cellular resolution is benefit to segment individual cells in three dimensions all over the whole brain. Furthermore, the precise segmentation of cells helps to record each neuron’s activities correctly.

3.4.2. Time lapsed functional imaging

To demonstrate the capability of our dual-slit confocal LSM for functional measurements of multiple neural activities, one slice of the brain of a transgenetic fish (HuC-GCaMP5G, albino) was imaged in vivo. Giving consideration to the SNR and imaging speed, the camera was cropped to a subregion of 1024 × 2048 pixels, and the slits width was set to be 64.5 μm. 380 seconds are taken to acquire the image sequences with a frame rate of 20 Hz. The exposure time for every pixel row was 1 ms. The movie of these image sequence is shown in Media 2.

Fig. 8. Single-neuron resolved activity of zebrafish’s midbrain and hindbrain recorded by dual-slit confocal LSM (Media 2). (a) Brain image of zebrafish larvae (HuC-GCaMP5G, albino, 5 days old). Neurons were segmented manually and colored by different area of brain. Scale bar is 100 μm. (b) Activity traces of all neurons marked in (a). The label denotes the corresponding brain regions in: Left Midbrain (LMB, red), Right Midbrain (RMB, green), Left Hindbrain (LHB, blue) and Right Hindbrain (RHB, yellow). (c) Fluorescence (ΔF/F) traces of different neurons indicated in (b). (d) Close-up views of the calcium transients indicated by the box in (c).

Spontaneous fluorescence intensity fluctuations are observed in both midbrain and hindbrain, as observed in Fig. 8. Because of the optimized SNR, almost all the neurons of the imaging plane can be distinguished. 973 individual cells were segmented manually (Fig. 8(a)).
and their activity traces were plotted according to corresponding brain regions as the thermal plot shown in Fig. 8(b). By calculating the correlation coefficient between neurons’ activities from each brain region, we found out positive relationship between some neurons in left-midbrain and right-midbrain, as well as negative relationship between some neurons in left-hindbrain and right-hindbrain.

The calcium signals of several individual cells were picked up from the thermal plot (Fig. 8(b)). Five calcium signal traces of neurons were expressed as relative fluorescence intensity changes ($\Delta F/F$) after background subtraction (Fig. 8(c), 8(d)). The slow oscillations typically lasted for 4–6 seconds. The raise time of calcium signal is shorter than the decay time, which is in agreement with previous *in vivo* studies on GCaMP5G labeled zebrafish [9,39]. Some fast oscillations about at 1 Hz are observed in some hindbrain regions.

To observe the potential functional connections of neurons in zebrafish’s brain, the temporal relationships of the neurons were analyzed by measuring the fluorescence intensity of individual cells in the temporal space. According to the previous research, the latencies of the sequential activation of neurons are typically within 50 to 100 ms [32]. Since our system’s frame rate is 20 Hz, the sequence of the neurons’ activities can be captured. By contrast, this sequence will be ignored by the conventional confocal LSM, because its frame rate is only 10 Hz under the same imaging condition with our dual-slit confocal LSM.

![Image](a-b.png)

Fig. 9. The temporal relationship of the neurons in optic tectum of zebrafish’s brain. (a) is the imaging result by dual-slit confocal LSM, as shown in Media 3. Marked A and B are neurons that have transients sequentially. The scale bar is 40 $\mu$m. (b) is the temporal plot of neurons marked in (a). The latency can be observed and the value is measured to be 90 ms. The fluorescence intensity has been normalized.

As shown in Fig. 9 and Media 3, two neurons in different regions of optic tectum have been analyzed. The cell A is at the anterior optic tectum and the cell B is at the posterior optic tectum. It is found that the cell A fires earlier than cell B. Owing to the sampling frequency of the dual-slit confocal LSM, the latency of these neurons’ transients can be observed and measured. The interval of transients is 90 ms, which will be ignored if the imaging duration of one frame is longer than this interval. The possible functional connections as well as its upstream and downstream relationship can be inferred, according to the temporal sequence of these two neurons’ activities.

4. Conclusion and discussion

We developed a dual-slit confocal LSM for capturing the calcium images of zebrafish’s brain *in vivo*. In our system, we used dual-beam illumination scheme and two detection slits generated by sCMOS’s rolling shutter mode. An optimized SNR was achieved based on theoretical analysis and experimental verification. The results of the imaging experiments with 200nm fluorescent beads denote that our system could obtain images with both enhanced...
SNR and contrast compared with the conventional LSM and with a frame rate twice as high as previous reported confocal LSM. Upon these characteristics, our dual-slit confocal LSM is suitable for in vivo whole brain imaging of zebrafish. Through the structural and functional imaging results, apparent whole-brain structures with cellular resolution and the calcium activities of one layer are observed. Furthermore, we can expect to acquire 3D structural and functional information if combining this dual-slit confocal LSM technique with the existed fast depth-scan technique of LSM [27]. This indicates potential applications in revealing more subtle processes in living organism and related neurobiological studies.

The problem of junction mentioned in previous research [31] has also been considered. As the excitation beams from two directions excite the sample at the same time, the junction will appear in the middle of the imaging area. We carefully analyzed the inhomogeneous area in the middle of the images and found the inhomogeneous area of each frame depends on the width of the slit and the excitation beams. In our system the inhomogeneous areas are typically about 60 pixel lines in the middle of the images, which is merely 2.9% of the whole frame. We also analyzed the grey values, resolution and SNR of these areas by imaging the embedded fluorescence beads. It is found that the grey values varies in these areas, but the resolution and the SNR keep similar with the rest of the frame areas. This can be explained by the fact that the signal and the background are linearly superposed by the two excitation beams in the inhomogeneous areas. In addition, in the functional imaging process, when the relative intensity (\(\Delta F/F\)) is analyzed, the variation of the grey values in different areas will be removed in the temporal analysis.

Acknowledgments

This work is supported by Science Fund for Creative Research Group of China (Grant No. 61421064) and National Natural Science Foundation of China (Grant No. 61178077). The authors thank Jiulin Du, Chunfeng Shang and Yufan Wang from Institute of Neuroscience, Chinese Academy of Science for the supply of fluorescently labeled zebrafish samples. Additionally, the authors acknowledge Hamamatsu Photonics Beijing for a free trial of the ORCA-Flash 4.0 sCMOS camera and relevant technical supports. Authors also thank the Optical Bioimaging Core Facility of WNLO-HUST and the Analytical and Testing Center of HUST for the facility support.