Line-scanning microscopy with laterally symmetric imaging using simultaneous cross-line illumination

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Using an on-the-fly scanning scheme, line confocal microscopy can obtain complex structures of large biological tissues with high throughput. Yet, it suffers from lateral imaging asymmetry and thus introduces the potential deformations of the observation results. Here, we propose cross-line illumination microscopy (cLIM) that acquires the imaging data of two perpendicular directions simultaneously through the same objective lens in a line scanning and utilizes two-direction deconvolution fusion to achieve lateral symmetric imaging performance. Imaging fluorescence beads indicates that cLIM reduces lateral resolution asymmetry from 46.1% to 2.5% and improves lateral resolution by 31.0%, compared with traditional line-scanning imaging. Compared with commercial point-confocal microscopy, the cLIM has a 25.84× increase in imaging speed and 1.93× better background-suppressing ability when imaging an 11,306 μm × 7783 μm × 100 μm mouse kidney slice. We also show the advantages of the cLIM in observing direction-sensitive texture features by imaging a muscular tissue slice. cLIM offers a novel solution to achieve laterally symmetric line-scanning imaging with simple modifications while maintaining high throughput and accuracy for imaging large-scale samples. © 2024 Chinese Laser Press

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1. INTRODUCTION

Optical microscopy is a powerful tool to quickly obtain fine structures of large samples [1,2]. Due to the limited field of view (FOV) of a single objective lens, we need to employ scanning to cover the whole imaging region of interest (ROI) of the specimen. The on-the-fly scanning scheme adopts continuous-motion scanning and thus obtains higher throughput than step scanning [3]. Benefiting from this, line confocal microscopy (LC) [4,5] is superior to both point confocal [6,7] and wide-field microscopy [8,9] using step scanning in large-sample imaging, and the advantages become more obvious for the larger sample. The combination with multi-line parallel detection further improves the optical sectioning ability [10] and the spatial resolution [11] of the LC. However, the LC has an inherent problem of lateral asymmetric imaging performance due to only achieving a confocality in one direction [12,13]. High-frequency information along the non-focusing direction has a bigger amplitude decrease than that along the focusing direction, thus being more easily submerged by the background. It may introduce the potential deformations of the observation results.

To eliminate or weaken the impact on the imaging results due to the asymmetric point spread function (PSF) of the imaging system, a simple operation is to employ post-image processing methods such as the deconvolution algorithm [14,15]. However, the post-image processing methods cannot restore the high-frequency information that has already been submerged in the background. Therefore, obtaining raw data imaged from different directions is necessary to achieve symmetric lateral imaging performance. The current solution is to use sequential imaging along different lighting directions [16,17]. However, the imaging time of these methods was inevitably more than doubled due to the start-up and braking time of the scanning device.

Here, we propose cross-line illumination microscopy (cLIM), which fuses two sets of line-scanning imaging data orthogonal to each other simultaneously captured with a single objective lens to achieve symmetric imaging performance in
the LC. We demonstrate the symmetric lateral imaging performance of the cLIM on both fluorescent beads and biological specimens. By numerical simulation of a spoke-like sample and biological imaging of the muscle tissue, we show that the cLIM can restore accurate and complete information distributed along different directions, which is unavoidably affected by the asymmetric lateral imaging performance in the observation result of the LC. Therefore, we confirm that the cLIM, as a high-throughput, powerful, and versatile imaging approach, potentially promotes the widespread application of line scanning in biomedical experiments. All the animal experiments followed procedures approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology.

2. METHOD

A. System Configuration

The optical configuration of the cLIM is presented in Fig. 1. An expanded 488 nm laser beam (Genesis MX STM, Coherent, Santa Clara, USA) is adjusted in terms of polarization by a half-wave plate (HWP, WPH10ME-488, Thorlabs, Newton, USA). Then the beam is divided by a polarized beam splitter PBS1 (CCM1-PBS251/M, Thorlabs) into two beams with equal intensity. The cylindrical lenses CL1 and CL2 (\( f = 150 \text{mm} \), ACY254-150-A, Thorlabs) converge two beams into a linear shape distributed along the \( X \) and \( Y \) axes, respectively. Another polarized beam splitter PBS2 combines these two beams to generate a cross-line illumination without energy loss. This illumination is projected by L1 (\( f = 150 \text{mm} \), AC254-150-A, Thorlabs) and an objective lens (Obj, 20 \( \times \), NA 1.0, XLUMPLFLN 20XW, Olympus, Tokyo, Japan) to the focal plane of Obj in the sample. A filter set including a dichroic mirror (DM, ZT488rdc, Chroma, Vermont, USA), an excitation filter F1 (ZET488/10\( \times \), Chroma), and an emission filter F2 (AT525/30m, Chroma) is employed to split the excitation and emission light and suppress stray light.

The fluorescent signals that pass through Obj and a tube lens (TL, \( f = 180 \text{mm} \), TTL180-A, Thorlabs) are divided into two beams by a beam splitter (BS, CCM1-BS013, Thorlabs) and detected by sCMOS1 (ORCA Flash 4.0, Hamamatsu Photonics K.K., Hamamatsu, Japan) and sCMOS2 cameras separately. Both detectors operate in a sub-array mode and are aligned with each illumination direction to achieve the confocality of the corresponding direction and avoid interference from another direction.

To capture two orthogonal directions of the specimen simultaneously, we have designed a diagonal scanning method. This method employs a translation stage (\( X \) axis, ABL20020; \( Y \) axis, ANT130; \( Z \) axis, AVL125, Aerotech, Pittsburgh, USA) to shift the specimen diagonally along the cross linear beams, namely the fast axis (\( F \)-axis). Two strip-shaped images can be captured during a single scanning, each representing the imaging results of \( x \)-line confocal (\( X \)-LC) and \( y \)-line confocal (\( Y \)-LC) imaging systems. To cover the whole imaging range of the imaged section of the sample, multiple strip-shaped images are required. To make full use of the scanning range, the sample is rotated 45° horizontally and the direction of inter-strip displacement is set along the slow axis (\( S \)-axis), perpendicular to the \( F \) axis.

Our diagonal-scanning method can also improve the spatial sampling rate of the system by \( \sqrt{2} \) times compared with conventional line-scanning approach since the detection array is at an angle of 45° with the scanning direction:

\[
P_S = P_D \times \sin 45°,
\]

where \( P_D \) is the pixel size of the detector projected back to the focal plane of Obj under the conventional line-scanning mode, and \( P_S \) is the scanning pixel size under the diagonal scanning mode.
B. Image Processing Flow

We demonstrate the image processing flow of the cLIM by imaging a fluorescent calibration slide (Argo-HM V2, Argolight, Pessac, France), as illustrated in Fig. 2. Raw data were pixel reassigned to eliminate the misalignment due to the diagonal scanning [Fig. 2(a)]:

\[
\begin{align*}
I_X^R(i, i + 1: i + M) & = I_{Raw}^X(i; i:M) \\
I_Y^R(i, N - i + 1; N - i + M) & = I_{Raw}^Y(i; i:M),
\end{align*}
\]

(2)

where \( M \) is the frame number captured in a diagonal scanning; \( N \) is the FOV width; \( i = 1, 2, 3, ..., N \) is the column number of the strip image; \( I_{Raw}^X \) and \( I_{Raw}^Y \) are the raw data of the \( X \)-LC and \( Y \)-LC, respectively; and \( I_X^R \) and \( I_Y^R \) are the corresponding results after the pixel reassignment. Since the scanning speed matches the scanning pixel size and exposure time, there is no need for numerical interpolation during the pixel reassignment. The redundancy at the beginning and end of the diagonal scanning after pixel reassignment is negligible compared to the scanning range and can be trimmed before being written into the disk.

Subsequently, we perform the combination of the nonlinear registration algorithm [18] and the linear registration algorithm for \( I_X^R \) and \( I_Y^R \) to balance alignment accuracy, image resolution, and speed. The nonlinear algorithm can effectively correct complex misalignment due to the asymmetric distribution of high-order aberrations but at the risk of blurring the image. On the other hand, the linear algorithm we adopted involves only rotation and translation. It can maintain the resolution of the image but lacks the ability to deal with complex misalignments. Therefore, we first use the nonlinear registration algorithm to obtain a preliminary registration in the whole FOV with a width of 2048 pixels. Then, the linear registration algorithm is performed sequentially on each local area of \((256 + 10) \times (256 + 10)\) pixels with an overlap of 10 pixels within the FOVs. The residue misalignment of the \( I_X^R \) and \( I_Y^R \) is shown in the pseudo-color image by merging the imaging results of the \( X \)-LC (magenta) and \( Y \)-LC (green), as shown in Fig. 2(b). The enlarged views of Regions I, II, and III from the middle to the edge of the FOV before and after registration are shown in Fig. 2(c). As indicated by the green arrowheads, the aberrations can be effectively corrected. The increase of the values of normalized cross-correlation (NCC) coefficients of \( I_X^R \) and \( I_Y^R \) after the registration also confirms effective registration, from 0.87, 0.51, and 0.43 to 0.94, 0.93, and 0.82, respectively, from the center to the edge of the FOV.

The final process is the effective fusion of the \( X \)-LC and \( Y \)-LC imaging results. Here, we extend the classic Richardson–Lucy deconvolution algorithm [19] into a two-direction fusion method (De2dir). The key point of the Richardson–Lucy deconvolution algorithm is to minimize the error between the imaging result \( I \) and the convolution result of the imaging system PSF and the intensity distribution of the object \( O \):

\[
O = \arg \min_O \{ \text{PSF}^X \ast O - I_X^R \}^2 + \{ \text{PSF}^Y \ast O - I_Y^R \}^2,
\]

(3)

where \( \ast \) denotes convolution operation.

Specifically, the question in the cLIM becomes

\[
O = \arg \min_O [\text{PSF}^X \ast O - I_X^R]^2 + [\text{PSF}^Y \ast O - I_Y^R]^2,
\]

(4)

where \( \text{PSF}^X \) and \( \text{PSF}^Y \) are corresponding PSFs of the \( X \)-LC and \( Y \)-LC, respectively.

To avoid the ringing effect, we further employ the sparse distribution of signals of the sample as prior knowledge [20]:

\[
O = \arg \min_O \{ \text{PSF}^X \ast O - I_X^R \}^2 + \{ \text{PSF}^Y \ast O - I_Y^R \}^2 + \lambda \sum \omega |F \ast O|^2,
\]

(5)

where \( F \) is a second-order Laplace operator serving as a derivative filter, \( \omega \) is a weight factor with the value of the reciprocal of the norm of \( F \), and \( \lambda \) is a coefficient of the image prior taking a value of 0.001 based on experience. This non-convex minimization problem can be easily solved by the iterative reweighted least square method [21], and the framework of the iterative calculation is based on the commonly used joint-deconvolution [22]. Considering both the enhancement of image resolution and the continuity of biological structure, two or three iterations of the De2dir are generally adopted. For an image containing \( 2048 \times 2048 \) pixels processed with three iterations, the computation time is about 6 s using MATLAB 2017a on a desktop (Intel Core i7-7700 CPU@3.60 GHz, Lenovo).

\( I_{De2dir} \), the fusion result of De2dir, is the final iterative calculation result of \( O \). Figure 2(d) shows the enlarged views of the

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Fig. 2. Image processing flow. (a) Pixel reassignment of \( X \)-LC and \( Y \)-LC results. (b) Merged pseudo-color strip image of imaging results by the \( X \)-LC (magenta) and \( Y \)-LC (green). (c) Registration results of three ROIs in (b). (d) Fusion result of the De2dir. The insets in (c) and (d) are the intensity profiles along the corresponding color lines. NCC, normalized cross correlation (green values in the top left corner). FWHM, full width at half maximum (blue values; in \( \mu \)m).
fusion results. The insets in Figs. 2(c) and 2(d) are the intensity profiles along each line in Regions I, II, and III. The values of the full width at half maximum (FWHM) of the lines in Regions I, II, and III reduce from 0.70 μm, 0.75 μm, and 0.70 μm in the X-LC to 0.35 μm, 0.30 μm, and 0.30 μm in the cLIM, showing that the cLIM achieves about a 1-fold improvement in the spatial resolution values due to the deconvolution.

3. EXPERIMENTS AND RESULTS

A. Simulation Test on a Spoke-Like Sample

To illustrate the necessity of the cLIM, we perform a simulation test on a spoke-like sample using a 60x, NA 1.1 Obj. Figures 3(a) and 3(b) show the raw images and corresponding spectra of the X-LC and Y-LC, respectively. Figures 3(c) and 3(d) show the deconvolution results and corresponding spectra of the X-LC and Y-LC, respectively. Compared to the raw data, the deconvolved results have a higher definition and stronger signal of high frequency in both groups. However, the resolution of the reconstructed result and their spectral distribution still show obvious asymmetry in both the X-LC and Y-LC. The simulation indicates that high-frequency information cannot be effectively and symmetrically restored by deconvolving single-direction data. In contrast, the cLIM fuses the imaging results from two orthogonal directions, and thus restores symmetric and enhanced high-frequency information, as shown in Fig. 3(e). This simulation demonstrates the necessity of collecting raw imaging data from two orthogonal directions to overcome the resolution asymmetry.

B. Comparison among Different Fusion Methods

We compare the effects of different fusion algorithms with De2dir by imaging bovine pulmonary artery endothelial (BPAE) cells (F36924, ThermoFisher Scientific, Massachusetts, USA). We compared four classic fusion algorithms, including the maximum intensity projection algorithm (Max_I), the minimum intensity projection algorithm (Min_I), the maximum frequency projection algorithm (Max_F), and the intensity production algorithm (Prod) [23–25]:

\[
\begin{align*}
I_{\text{Max},I} &= \max(I^X, I^Y) \\
I_{\text{Min},I} &= \min(I^X, I^Y) \\
I_{\text{Max},F} &= \text{FFT}^{-1}\{\max[\text{FFT}(I^X), \text{FFT}(I^Y)]\} \\
I_{\text{Prod}} &= I^X \times I^Y / C
\end{align*}
\]

where \( I_{\text{Max},I}, I_{\text{Min},I}, I_{\text{Max},F}, \) and \( I_{\text{Prod}} \) are the corresponding fusion results of the Max_I, Min_I, Max_F, and Prod, respectively; FFT and FFT\(^{-1}\) denote the Fourier transform and inverse Fourier transform, respectively; and \( C \) is the maximum value of \( I^X \) and \( I^Y \). Note that the Prod can numerically double the dynamic range of images. For a fair comparison, the fusion result of the Prod is divided by a constant value of \( C \). We use the Max_I or Max_F to fuse the deconvolution images in directions \( X \) and \( Y \) to generate the estimated image in each iteration of the De2dir.

Due to the uneven PSF distribution of the imaging system in the three-dimensional (3D) space, the information contained in the imaging results is different while imaging the same sample from different views. Figure 4(a) displays a pseudo-color merged image of the imaging results of the X-LC (magenta) and Y-LC (green) to observe the signal distinctions between these two results. In detail, the filamentous actins (F-actins) along the \( X \) axis are finer and brighter in the imaging result of the Y-LC than that of the X-LC, indicated by a white arrowhead. On the other hand, the F-actins along the \( Y \) axis are finer and brighter in the imaging result of the X-LC than that of the Y-LC, indicated by a blue arrowhead.

The integrity of the information contained in the fusion result and the improvement of the image quality are used as the criteria to evaluate the quality of the fusion algorithm. The normalized fusion results processed by the Max_I, Min_I, Max_F, Prod, and De2dir are presented in Fig. 4(b), and the related enlarged views are exhibited in Fig. 4(c). First, we discuss information integrity, where the Max_I and Min_I show deficiency. The fusion result of the Max_I fails to distinguish the three F-actins in the blue dashed region due to information overlapping. The microfilament, marked by the yellow arrowheads in the green dashed rectangle region, is nearly obscured in the fusion result of the Min_I.

![Fig. 3. Simulated results of a spoke-like sample. (a), (b) Raw images and corresponding spectra of the X-LC and Y-LC, respectively. (c), (d) Deconvolved images and corresponding spectra of the X-LC and Y-LC, respectively. (e) Fusion result and its spectrum of the cLIM. Region size, 4.7 μm × 4.7 μm; pixel size, 13.5 nm.](image-url)
We further analyze the image contrast by plotting the intensity profiles along the corresponding colored lines in the green dashed region. We define the contrast as the ratio of the greatest and smallest intensity along the line. The contrast values for the fusion results of the $\text{Max}_I$, $\text{Min}_I$, $\text{Max}_F$, Prod, and De2dir are 2.75, 1.88, 3.68, 4.84, and 578, respectively. The De2dir greatly enhances the image contrast compared to the others. Finally, we examine the image resolution by determining the FWHM along the intensity profiles. The FWHM values in the fusion results of the $\text{Max}_I$, $\text{Min}_I$, $\text{Max}_F$, Prod, and De2dir are 0.92 μm, 1.21 μm, 0.92 μm, 0.87 μm, and 0.58 μm, respectively, indicating De2dir increases image resolution by nearly 1-fold. These results demonstrate that the De2dir can reconstruct signals as much as possible, enhance image contrast, and improve image resolution. Thus, we employ the De2dir as the fusion algorithm in the cLIM.

**C. Improved Symmetric Imaging Performance of the cLIM**

To evaluate our system performance, we compared the spatial resolution of the $X$-LC, $Y$-LC, and cLIM by imaging fluorescent beads (F8811; excitation, 505 nm; emission, 515 nm; ThermoFisher Scientific) with a 60×, NA 1.1 Obj (LUMFLN 60XW, Olympus). We calculate the FWHM values of the intensity profiles via Gaussian fitting of the intensity distribution curves along the $X$ and $Y$ directions, as shown in Fig. 5(a).

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**Fig. 4.** Comparison of different dual-direction fusion algorithms by imaging BPAE cells. (a) Pseudo-color-merged image of the X-LC (magenta) and Y-LC (green). Blue and white arrowheads indicate the differences between the signals contained in these two imaging results. (b) Fusion results by the Min_I, Max_I, Max_F, Prod, and De2dir. Dynamic ranges for displaying images are shown by the bars at the bottom right corners. (c) Corresponding enlarged views in (b). Intensity profiles along the colored lines are also shown at the bottom, including the maximum and minimum intensities, their ratios, and FWHM values. The yellow hollow and solid arrowheads indicate the signal distributions of the same microfilament in the different fusion algorithms. The scale bars in (a) and (c) are 50 μm and 2 μm, respectively.

**Fig. 5.** Lateral resolution measurement of the cLIM by imaging fluorescent beads. (a) Typical images of a fluorescent bead by the X-LC, Y-LC, and cLIM and the corresponding lateral resolution measurement by Gaussian fitting analyses. The statistical lateral resolution values are labeled ($n = 10$). (b) Asymmetry of lateral resolution of the X-LC, Y-LC, and cLIM. Scanning pixel size, 0.077 μm.
We selected 10 beads at equal intervals within the FOV, and the statistical FWHM values of the corresponding imaging results of the X-LC, Y-LC, and cLIM are labeled. In the X-LC, the FWHMs in the X and Y directions were 0.245 ± 0.019 μm and 0.358 ± 0.045 μm, respectively. In the Y-LC, the FWHMs in the X and Y directions were 0.347 ± 0.042 μm and 0.261 ± 0.030 μm, respectively. In the cLIM, the FWHMs in the X and Y directions were 0.241 ± 0.010 μm and 0.247 ± 0.020 μm, respectively, near the diffraction limit. Meanwhile, the cLIM successfully reduced the lateral resolution asymmetry from 46.1% in the X-LC and 33.0% in the Y-LC to 2.5% as shown in Fig. 5(b), indicating that the resolution asymmetry of the cLIM is decreased by 18.4-fold over the LC. Compared to the Y-LC, X-LC has better resolution along the focusing direction and thus more severe asymmetry. This may result from the fact that the flatness of the dielectric film of the beam splitter in the detection path has a greater impact on the wavefront of the reflected light than on the one of the transmitted light. Compared to the non-focusing direction of the LC, the cLIM improves the resolution by 30.5% and 31.0% in the X and Y directions, respectively, due to the deconvolution.

To demonstrate the technical advantages of our method, we compare the cLIM with the point-confocal microscopy (CM) as a gold standard for biological optical-sectioning imaging. We compared the imaging speed, image contrast, and optical sectioning ability by imaging an 11,306 μm × 7783 μm × 100 μm β-actin-eGFP mouse (C57BL/6, The Jackson Laboratory, Maine, USA) kidney slice with a commercial confocal microscope (LSM710, 20×, NA 0.8, Plan-Apochromat, Zeiss) and our cLIM (20×, NA 0.7, UCPLFLN 20×, Olympus), as shown in Fig. 6(a). To ensure the same ROI under different imaging systems, we adjust the pitch angle of the sample by placing gaskets and rotate the sample by referring the sample contour. The total imaging time of CM is 35 min 47 s. In comparison, the LC and cLIM require only 1 min 20 s, 25.89-fold faster than CM, benefiting from the high-throughput advantage of line scanning. The extra time consumption is mainly used for splicing different FOVs. CM uses Mosaic scanning for multi-FOV imaging, and the movement of each adjacent FOV needs to start and stop the stage regardless of the X or Y direction. The line scanning adopted by the LC and cLIM also needs to start and stop the stage between adjacent lines, but the difference is that it
only needs to start and stop once for on-the-fly scanning and imaging under each line scanning. Therefore, with the increase of sample size, the more time saved by line scanning, the more obvious the advantage of imaging throughput. The enlarged views of the yellow rectangle region in Fig. 6(a) are shown in Fig. 6(b). Then, we analyze the image contrast by observing the axial cuts with 6 μm depth through the yellow dashed line [Fig. 6(c)] and the enlarged lateral views of the blue rectangle region [Fig. 6(d)] in Fig. 6(b). The enlarged ROIs are matched by axial scanning with the steps of 0.25 μm and 1 μm under the CM and cLIM, respectively, and rotating the imaging results of the cLIM with cubic interpolation. The axial cuts of the imaging results of the LC and cLIM are also processed with cubic interpolation. According to the intensity profiles [Fig. 6(e)] along the corresponding-colored lines in Fig. 6(d), the LC is incapable of distinguishing the structural details since there is little fluctuation in the intensity profile. The CM can barely distinguish these structures with slight ups and downs on the intensity profile. In contrast, the cLIM can distinguish them better than CM, increasing the peak-to-valley ratio from 1.07 to 1.36. The optical sectioning ability is quantified by a signal-to-background ratio (SBR):

\[ \text{SBR} = \frac{S}{B}, \]

where \( S \) is the peak intensity value of the signal of the image, and \( B \) is the mean intensity value of the background of the image. We calculated the SBR via the image histograms of Fig. 6(b), as shown in Fig. 6(f). We numerically normalize the value of \( S \) so that the SBRs of the imaging results by different imaging systems can be directly reflected by the value of \( B \). When the histogram of an image features two peaks, the valley between these two peaks defines the segmentation between the background and signal [26]. The histograms in Fig. 6(f) indicate that the values of \( B \) of the imaging results by the LC, CM, and cLIM are 1514, 1116, and 578, respectively. Thus, the value of SBR of the imaging result by the cLIM is 2.62 times that of the LC and 1.93 times that of the CM. The optical sectioning improvement of the cLIM results from the deconvolution adopted in the reconstruction process of dual-directional fusion, compared with CM. These results indicate that the cLIM has not only 25.84× higher throughput but also higher image contrast and 1.93× background-suppressing ability than CM. Therefore, our method compensates for the shortcomings of traditional LC that sacrifice imaging quality for high-throughput imaging and provides a powerful alternative solution for CM in imaging large samples.

D. Imaging of Direction-Sensitive Biological Tissue

To demonstrate the necessity of the cLIM in biological imaging, we image a muscular tissue slice of a 4692 μm × 8935 μm × 100 μm β-actin-eGFP mouse tongue in 1 min 21 s. The muscle cells crisscross the mouse tongue, presenting direction-sensitive texture features. It can be visually illustrated by the color changes of the structure tensor distribution in Fig. 7(a). The imaging result of the cLIM is reduced by 16 times, and the structure tensor (ST) is calculated based on the partial derivatives of the image [27]:

\[ \text{ST} = G \ast [(F_{2D} \ast I) \cdot (F_{2D} \ast I)^T], \]

where \( G \) is a 2D Gaussian kernel defining the local neighborhood of \( 3 \times 3 \) pixels over which the tensor is calculated, \( F_{2D} \) is a second-order Laplace operator serving as a derivative filter, \( I \) is the intensity distribution of the reduced image, and a superscript \( T \) means transpose. The peak orientation is extracted among the distribution of local orientations and determines the RGB value in the structure tensor image. The insets of Fig. 7(a) display all four types of intrinsic muscles, including the superior longitudinal muscle (SLM), inferior longitudinal muscle (ILM), vertical muscle (VM), and transverse muscle (TM) [28]. The asymmetric imaging quality of LC affects the contour recognition ability along different directions, which can be identified by the gradients of the X and Y directions. Figure 7(b) represents the gradients of the imaging results in Region 1 indicated by the blue box using the X-LC, Y-LC, and cLIM. In both X-LC and Y-LC results, the number of gradients with a high value along the focusing direction is greater than that along the non-focusing direction, as illustrated in the enlarged circle views. It demonstrates that the LC has a stronger contour recognition ability in the focusing direction than in the non-focusing direction. In comparison, in the same enlarged circle region of gradients with high value, the number of gradients along the X direction is nearly equal to that along the Y direction. This result demonstrates that the cLIM has an improved uniformly distributed contour recognition ability in both directions.

The asymmetric imaging quality of LC affects the observations of fine structures distributed along different directions. A typical example is to image the myofibrils and sarcomeres that are perpendicular to each other [29] in the muscle tissue. Figure 7(c) shows the enlarged views of the corresponding X-LC, Y-LC, and cLIM imaging results for Region 2 indicated by the yellow rectangle in Fig. 7(a). Both X-LC and Y-LC cannot simultaneously distinguish the myofibrils branching [enlarged views in Fig. 7(d)] and sarcomeres [enlarged views in Fig. 7(e)]. In detail, the branching of the myofibrils along the Y-direction indicated by the yellow arrowheads can be seen in the imaging results of the Y-LC, but is hidden in the imaging results of the X-LC. Meanwhile, as shown in Fig. 7(f) by the ups and downs on the intensity profiles along corresponding-colored lines in Fig. 7(e), the X-LC imaging system can discern the I-band, A-band, and Z-disk on the I-band along the X direction, which are the typical structures of the sarcomeres. However, the Y-LC imaging system fails to distinguish the Z-disk, as illustrated by the missing peaks of the Z-disk on the intensity profile. In contrast, the cLIM enables simultaneous distinction of the myofibril branching [yellow arrowheads in Fig. 7(d)] and extension of the Z-disk [presence of peaks on the intensity profile in Fig. 7(f)]. These results demonstrate the necessity of the cLIM to fuse two orthogonal directions for accurate observation of fine structures along different lateral directions.

4. CONCLUSION

We have proposed high-throughput, laterally symmetric line-scanning microscopy, cLIM, using simultaneous cross-line illumination and dual-directional deconvolution fusion. Non-interference cross-line illumination is generated by polarizing,
splitting, rotating, and combining the beam. The cLIM acquires two sets of line-scanning imaging results in orthogonal directions simultaneously by diagonal scanning. After effectively fusing these two sets of data, the cLIM achieves improved symmetric imaging performance in line-scanning microscopy without dual scanning.

Note that the overlapping part of the two linear beams causes sudden increases in the illumination intensity, which can be corrected by the shading correction algorithm [30]. In addition, separating the imaging results of the X-LC and Y-LC by the BS leads to the loss of half of the fluorescent signal in the current design. A customized mirror with a slit in the middle has the potential to solve this problem, separating the signal without extra loss. Simultaneous imaging of cross-line illuminations leads to 70% effective FOV compared with conventional line-scanning imaging at the same NA, while still faster than double scanning.

The cLIM offers lateral uniformed imaging performance with high throughput, suitable for all line illumination imaging systems. Our work provides a new solution for large-scale scanning imaging, especially for the acquisition of direction-sensitive signals.

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**Fig. 7.** Imaging results of a 100-μm-thick β-actin-eGFP mouse tongue slice. (a) Structure tensor distribution of the cLIM imaging result. The insets display four types of intrinsic muscles of the mouse tongue. (b) Gradients along the X and Y directions in Region 1 indicated by the blue box in (a) of the corresponding imaging results using the X-LC, Y-LC, and cLIM. (c) Enlarged views of the corresponding imaging results of Region 2 indicated by the yellow rectangle in (a) using the X-LC, Y-LC, and cLIM. (d) Enlarged views of myofibril branching (yellow arrowheads) indicated by the blue rectangle in (c). (e) Enlarged views of sarcomeres indicated by the brown rectangle in (c). (f) Intensity profiles along corresponding-colored lines in (e) show the typical structures of the sarcomeres, including I-band, A-band, and Z-disk. SLM, superior longitudinal muscle; TM, transverse muscle; VM, vertical muscle; ILM, inferior longitudinal muscle.
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Data Availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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