Improved contrast in inverted selective plane illumination microscopy of thick tissues using confocal detection and structured illumination

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Abstract: Inverted selective plane illumination microscopy (iSPIM) enables fast, large field-of-view, long term imaging with compatibility with conventional sample mounting. However, the imaging quality can be deteriorated in thick tissues due to sample scattering. Three strategies have been adopted in this paper to optimize the imaging performance of iSPIM on thick tissue imaging: electronic confocal slit detection (eCSD), structured illumination (SI) and the two combined. We compared the image contrast when using SPIM, confocal SPIM (using eCSD alone), SI SPIM (using SI alone) or confocal-SI SPIM (combining both methods) on images of gelatin phantom and highly-scattering fluorescently-stained human tissue. We demonstrate that all the three methods showed remarkable contrast enhancement on both samples compared to iSPIM alone, and SI SPIM and the combined confocal-SI mode outperformed confocal SPIM in contrast enhancement. Moreover, the use of SI at high pattern frequencies outperformed confocal SPIM in terms of optical sectioning capability. However, image signal-to-noise ratio (SNR) was decreased at high pattern frequencies when imaging scattering samples with SI SPIM. By combining eCSD with SI to reduce background signal and noise, the superior optical sectioning performance of SI could be achieved while also maintaining high image SNR.

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1. Introduction

Fluorescence microscopy with optical sectioning ability provides unique benefits of high contrast, high molecular sensitivity, and depth information, which has proved useful in cell study [1], developmental biology [2], neuroscience [3], as well as clinical applications [4–6]. Among the common fluorescence optical sectioning techniques, such as confocal laser scanning microscopy (CLSM), two-photon (2P) microscopy, and structured illumination microscopy (SIM), light sheet fluorescence microscopy (LSFM) has become increasingly popular for imaging relatively large samples for its advantages over other methods [1,7,8]. By using two decoupled optical paths, only an in-focus slice of the sample is illuminated by a thin light sheet and imaged by a wide-field camera through an orthogonal imaging system. This selective illumination of LSFM creates less out-of-focus background than epi-illumination approaches and reduces photodamage to the fluorophores or living specimen [9]. Furthermore, the widefield detection yields fast data acquisition compared to other beam-scanning optical sectioning microscopes. In addition, compared to some traditional configurations of point detection approaches such as CLSM or 2P microscopy where a PMT is usually used, better signal to noise ratio (SNR) can be achieved at similar frame rates due to optically efficient widefield detection and the use of high QE, low-noise wide-field cameras [1]. All these features strongly benefit imaging of thick samples, such as embryos, brain tissue, and diagnostic cancer biopsies.

Since the first version of light sheet microscopy developed by Siedentopf and Zsigmondy to observe gold particles in glass, a variety of LSFM implementations has been accumulated and compared [10,11], most of which were built around samples embedded in agarose [12]. Some new LSFM configurations [13–15] have been created to apply to the conventional sample preparation, making it possible to image various samples with common sample holders. The dual-view selective plane inverted plane illumination microscope (diSPIM), adopted in our work, is one such configuration [16]. Moreover, by using stage-scanning instead of beam scanning to generate stacks of images for volumetric reconstruction, the Gaussian beam thickness does not vary over planes of the sample parallel to the stage scanning direction [17], and the sample size that can be imaged only depends on the moving range of the stage, which allows us to image samples in the range of squared centimeters.

Despite the ability to image large samples, the imaging performance of LSFM is often deteriorated by light scattering, especially in large, nontransparent samples. To demonstrate high-resolution features over wide fields of view, Dodt et al. [18] combined optical clearing and LSFM for the first time. An ideal optical clearing method should provide high transparency of sample, allow fluorescence labeling, preserve structures of sample, as well as considering speed, cost, safety and repeatability. However, in practice, one has to sacrifice some of these properties to preserve some other features to meet their own requirements [19].

From the aspect of imaging, several LSFM-based strategies have also been established to achieve higher imaging quality on scattering tissue. For example, two-photon inverted selective plane illumination microscopy (2PE-iSPIM) [20] showed background signal reduction and improved imaging quality while traveling deep in sample by the use of red-shifted wavelengths, which are less influenced by scattering.

Structured illumination (SI) has long been used to obtain optical sectioning in wide-field microscopy by projecting a single-spatial-frequency grid pattern onto the focus plane at multiple phase shifts [21], with higher frequencies providing better optical sectioning. Using SI in addition to LSFM can further lower the contribution of scattered photons to the image background [22–25]. However, there are two limitations when using SIM. Firstly, the SNR is lower when imaging deep in thick tissue because of the amplification of shot noise arising...
from the computationally rejected scattered background photons [26]. Secondly, the
recovered signal of the sectioned image $I_{\text{sectioned}}$ depends on the pattern modulation depth ($m$),
with increased pattern modulation depth resulting in more efficient recovery of the
contribution of photons generated in the in-focus plane [27]. The modulation depth ($m$)
decays with increasing pattern frequency due to the low-pass filter property of optical systems
[28], as well as with contamination by out-of-plane signal either by the extended illumination
field directly (widefield epi-illumination) or by broadening or steering of the illumination
beam (planar illumination). The use of planar illumination provides an inherent advantage in
the latter regard, in that less out-of-plane fluorescence is generated by confining the excitation
beam to the plane of interest; however, scattering can still cause broadening or steering of the
beam, which can degrade modulation contrast. The problem is exacerbated at high pattern
frequencies, as the contribution of the scattered (broadened) beam contributes more
prominently to modulation depth deterioration. Overall, the deteriorated modulation depth
results in decreased recovered signal intensity, and limits the practical use of high frequency
modulation patterns for incoherent SI, thus limiting its optical sectioning performance.

Several other groups used the rolling shutter mode of the camera sensor to generate an
effective confocal slit, a technique known as electronic confocal slit detection (eCSD) [17,29–
32]. In the global exposure mode, all rows of the camera sensor are exposed at the same time.
But in eCSD mode, by precisely synchronizing the translation of the excitation beam across
the field-of-view (FOV) with the exposed rows in rolling shutter mode, the out-of-focus light
in the direction of beam scanning can be removed.

Among all these different strategies, SI and eCSD are most readily achieved on iSPIM
without complicated and expensive hardware modification. However, for adding eCSD, one
challenge is to synchronize the beam scanning with the rolling shutter at the microsecond to
millisecond level. Due to the movement inertia of mechanical scanners, the starting point of
the camera trigger and the line readout time of camera sensor needs to be carefully and
precisely controlled, which can be troublesome when the mechanical movement feature of the
scanners is nonlinear. Even though SI and eCSD have been used and compared before [33],
there is no quantitative comparison over different parameters (slit size, pattern frequency)
between these two. Moreover, no prior studies have evaluated how eCSD can be used
synergistically with SI to enhance its performance by reducing some of the limitations of SI
(namely, degraded pattern modulation depth and amplification of background shot noise).

In this work, we use a straightforward way to implement eCSD into iSPIM system with
micromirror laser scanners by creating a look-up table between beam position on the sensor
and the input micromirror control voltages, to compensate for the nonlinear mechanical
movement of the micromirror scanners. Then we added SI to the same iSPIM, and achieved
the combined mode (eCSD + SI). We compared the imaging performance of four different
imaging methods, including SPIM, confocal SPIM (adding eCSD alone), SI SPIM (using SI
alone) and confocal-SI SPIM (combining eCSD and SI), with different parameters, on tissue
phantoms and millimeter-size, scattering human prostate biopsy samples. To understand how
eCSD influences the performance of SI SPIM, we also compared the illumination pattern
modulation depth on SI SPIM and confocal-SI SPIM. Moreover, we studied how pattern
frequency influences axial resolution in confocal-SI SPIM.

2. Materials and methods

2.1 Instrumentation

Figure 1(A) shows a schematic of the iSPIM system. It is constructed around a commercially
available diSPIM platform (Applied Scientific Instrumentation), incorporating two micro-
mirror beam scanners, scanner and imaging tube lens, filter cubes with multi-band filter sets
(Semrock), two detection cameras (Hamamatsu Orca Flash 4.0 v2), and two immersion
objectives (Nikon, 10X, 0.3NA) that are aligned vertically above the XY stage with each
having a 45° angle to the horizontal plane, enabling traditional sample preparation. 3D
imaging can be realized by using a motorized XY stage with sub-micron repeatability. The stage and scanners are controlled with Tiger controller (Applied Scientific Instrumentation). Multi-color imaging was achieved with two illumination diode lasers (Omicron Laserage) providing 488-nm and 647-nm illumination, respectively. The maximum voxel sampling rate in SPIM mode is 420-megavoxels per second in full-frame mode, and 205-megavoxels per second in full-frame mode for confocal mode. Adding SI will triple the imaging time required. However, for the data sets shown in this paper, we did not optimize imaging speed, and for consistency, 200 ms exposure time per frame was used. Compared to the traditional xyz coordinates, a new set of coordinates of x’y’z’ for iSPIM is used taking into account the 45° angle of the raw slices. x’ axis is along the illumination direction, which includes a part of the depth information. z’ is the same as the traditional x axis which is one of the directions that the stage moves to create a 3D stack. y’ is the same with y.

Fig. 1. Schematic of iSPIM system. A virtual light sheet generated by one of the micro-mirror scanners that scans in the y(y’) direction rapidly illuminates the sample at an oblique 45° angle from one objective. The emission signal from the sample is then collected by the other objective perpendicular to the illumination objective and is imaged with the camera at the same side with the detection objective. Compared to the traditional xyz coordinates, a new set of coordinates of x’y’z’ for iSPIM is used. x’ axis is along the illumination direction, z’ is the same as the traditional x axis which is the direction that the stage moves to create a 3D stack, and y’ is the same with y. BS: beam splitter. MM: micro-mirror scanner. TL: tube lens. DM + F: dichroic mirror + emission filter.

The beam coming from the laser is scanned back and forth by the micro-mirror scanner and forms a virtual light sheet (Fig. 1(C)). After going through the excitation filter (390/482/532/640 BrightLine Quad-band Bandpass Filter, Semrock) and the dichroic mirror (405/488/532/635 BrightLine Laser Dichroic Beamsplitter, Semrock), the beam is reflected down to the illumination objective. The emission fluorescent signal is then collected by the detection objective, and passing through the emission filter (446/510/581/703 BrightLine Quad-band Bandpass Filter, Semrock), reflected to the camera sensor. The data is then acquired and stored in four SSD (Samsung 850 EVO, 250GB) running at RAID 10 mode.

The system has a lateral resolution of 1.5 μm and a minimum beam waist of 9.6 μm (we use the diameter at which the beam intensity has fallen to 1/e² for this paper) at 488 nm illumination, with a single frame FOV of 1.3 mm x 1.3 mm, at 4.2-megapixel resolution (2,048 x 2,048 pixels). The theoretical confocal parameter for the beam with 9.6 μm waist is 289.4 μm, which only covers 22% of the whole FOV. However, the beam waist and confocal parameter are readily adjustable in our system by adjusting the iris opening in the scanners that control the beam diameter to the BFP. Uniform beam thickness over the frame (pencil beam) can be achieved by using the beam with 20.6 μm waist. For the data set shown in this
paper, we chose the beam with 12 µm waist that gave us an approximately 450 µm confocal parameter, considering the limited imaging depth of uncleared prostate samples. Although the system hardware makes dual-sided imaging (diSPIM) possible for isotropic resolution, for these sectioning mode comparisons, only one-sided imaging was used. System control was achieved via custom-developed LabVIEW code. Controlling signals, including the camera trigger signal, scanner drive voltages and beam modulation voltages, were generated by a National Instruments PCI 6255 data acquisition card.

2.2 Different modes of iSPIM

The sCMOS can run in either global exposure mode or eCSD (‘light sheet’) mode. In eCSD mode (confocal SPIM), to retain most beam light while rejecting scattered photons along the direction of beam scanning, the beam scanning needs to be synchronized with the camera’s rolling shutter. The beam scans once unidirectionally to form one image plane. The electronic slit size, which is the width of active rows on the camera sensor, can be adjusted to balance between signal collected and scattered light rejected. The slit size at the sample in unit of micrometers can be calculated by the multiplication of the number of active rows, the pixel size, and the detection system magnification.

Usually scan mirrors exhibit an acceleration stage at the initialization of scanning, a deceleration stage at the end, and a linear stage in between, where a camera delay and a linear fit of beam positions are needed for synchronization [29,30]. However, in our case, the scanning is slightly nonlinear over the whole scanning range (Fig. 2(A)), which could result from the type of the scanners we use and the relatively large volume we scan. Thus, a new method for calibration is required. We began by investigating the relation between input control voltages to scan the beam (V_i) and the corresponding beam positions (Row_i) on the sensor. We inputted 2,048 linear voltages (linear V_i), the range of which was chosen to be able to scan over the whole FOV. The corresponding 2,048 ‘parked’ beam images in fluorescent dye solution were obtained, and the beam positions were measured with MATLAB based on where the maximum intensity of the beam is. A look-up table containing the relation of input voltages to actual beam positions was generated. We selected those voltages that corresponded to the 1st, 2nd, 3rd…2,048th row of the sensor from the look-up table and created a list of nonlinear input voltages. The mock look-up table in Fig. 2(B) shows how we created a customized voltage from the results of the linear input voltages. For example, if the beam position remained stalled on row 1 for voltage “1” and “2” in linear V_i, we knew to skip voltage “2” for our customized V_i. On the contrary, if a row is skipped (i.e. row 5 in the mock table), we used a local linear fitting to accommodate that row. The general idea is to customize the voltages to compensate the nonlinearity of the beam scanning as shown in Fig. 2(A). The look-up table approach was validated by comparing fluorescent beads images obtained in eCSD mode and in SPIM mode as shown in the Results section. The line readout time of the camera was calculated by dividing the exposure time for one plane by the number of sensor rows (for example, 200 ms / 2,048 rows = 97.65 µs/row). The line exposure time was calculated by multiplying the line readout time by the number of active sensor rows (for example, 97.65 µs/pixel * 15 rows = 1.465 ms).
In SI mode (SI SPIM), instead of using the laser with constant intensity, an additional analog voltage can be used to modulate the beam intensity as the beam scans across the image plane, enabling grid patterns of arbitrary waveform to be formed on the focal plane (for SI, sinusoidal patterns are typically employed). The frequency and phase of the grid pattern can be readily adjusted in this manner. Three sequential images $I_1$, $I_2$ and $I_3$ were taken with 120° phase shift between each image. Optically sectioned images were then computed based on the square-law detection algorithm [21] given in Eq. (1). Camera trigger, beam scanning and beam modulation were synchronized and started simultaneously for each plane. To better analyze the effect of grid pattern frequencies on imaging performance and compare it with previous studies, we use the normalized grid frequency $v'$ given by $v' = v \frac{\lambda'}{NA}$ [28], where $v$ is the actual spatial frequency in cycles/μm, $\lambda'$ the wavelength in μm, and $NA$ is the numerical aperture of the detection objective. The maximum attainable normalized spatial frequency with the current objective lens/camera combination is 1.28.

$$I_{sectioned} = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}$$

Confocal SI-SPIM is then readily realized by combining eCSD and SI mode after these two have been achieved separately.

### 2.3 Phantom and tissue preparation

500 nm YG (yellow-green) fluorescent beads (Fluoresbrite, Polysciences) were added to 3% gelatin solution, and then several drops of the mixed solution were placed onto a coverslide. After curing at 4 °C for 5 minutes, the gelatin phantom was put into the water chamber and imaged.

Human prostate tissue was collected and processed under a Tulane University Biomedical Institutional Review Board approved protocol. The tissue with a total size of about 4 mm*2 mm*0.5 mm was fixed with formalin, then thoroughly rinsed with PBS, followed by staining with propidium iodide (PI), a fluorescent nuclear dye, for 30 seconds. Before mounting the
sample into PBS solution for imaging, we added several drops of 3% gelatin solution around the sample on a cover slide and cured at 4 °C to hold the sample in position, avoiding it moving in the solution during imaging.

2.4 Calculations and image processing

After obtaining images of phantom or prostate tissue at different modes, the standard deviation of the energy-normalized histograms of raw data were calculated as the image contrast [22]. The contrast values were then normalized by the contrast of SPIM mode alone to show the contrast enhancement after adding eCSD, SI or both.

For axial resolution measurement, a sequence of 500 nm fluorescent beads images was obtained with stage translation step size of 1 μm. The actual distance between two sequential x'y' slices is the translation step size divided by $\sqrt{2}$ considering the 45° angle. After image shifting in Fiji [34], the z' profile of a bead was plotted and fitted with Gaussian function. The full-width-at-half-maximum (FWHM) of the profile was then calculated based on the fitting parameter. Twenty (20) beads were calculated for each data set.

For 3D imaging and visualization, a stack of oblique images was acquired which represents a parallelepiped-shaped part of the tissue. To visualize the correct shape of the data, the stack was also shifted in Fiji. The shifted stack can then be loaded into 3D visualization tools such as Paraview or Amira (FEI, Thermo Fisher Scientific). A faster way of image shifting is to use the shear function in Amira. For example, to shift a 4GB data in our desktop with Xeon CPU E5-2699 v3 and 128 GB RAM, shifting in Fiji took about 8 minutes while Amira took less than 1 minute.

3. Results

Figure 3 shows the validation results of the eCSD look-up table. A stack of x'y' slices of 500 nm fluorescent beads in gelatin phantom were acquired with either SPIM mode or eCSD. After shifting and maximum intensity projection (MIP) along the z' direction, we compared the two volumes in the form of MIPs. To better verify our results, three random subsets across the FOV were chosen to compare individual beads (Fig. 3(C)-3(E)). The same distribution of beads was observed in confocal mode (B) and in SPIM mode (A), which along with the detailed comparison in C-E shows good synchronization of electronic confocal slit rolling and the beam scanning.

Confocal, SI, and the combined (confocal + SI) mode all exhibited superior contrast enhancement over SPIM mode alone in gelatin phantom (Fig. 4), especially SI SPIM and
confocal-SI SPIM, which were about 2.8 times better compared to SPIM alone. Note that SI SPIM still had excellent SNR at a normalized frequency of 0.16 for the low scattering gelatin phantom. Detailed comparison of contrast enhancement (average ± standard deviation, SD) among different modes and parameters is shown in Table 1.

![Fig. 4. MIPs of 81 shifted x'y' slices along z' in different imaging modes. For better comparison, a nonlinear gamma transformation (γ = 0.4) was applied to each image and 0.3% of pixels were saturated in the lookup table. Quantitative measurement of the 81 raw x'y' images shows that compared to SPIM mode alone, confocal SPIM with 9.75 μm slit size was 1.11 ± 0.02 (SD) times better on image contrast, SI SPIM at frequency of 0.16 was 2.76 ± 0.15 (SD) times better, and the combined mode with 9.75 μm slit size and frequency of 0.16 was 2.80 ± 0.1 times better. The intensity range of the four images is 29665 ± 1196 (SD). Scale bar: 10 μm.](image)

**Table 1. Contrast enhancement over SPIM mode among different modes and parameters**

| ν' | Slit Size | Global | 39 μm | 29.25 μm | 19.5 μm | 9.75 μm |
|----|-----------|--------|--------|-----------|---------|---------|
| 0  | 1.00 ± 0.02 | 1.06 ± 0.01 | 1.09 ± 0.02 | 1.11 ± 0.02 | 1.11 ± 0.02 |
| 0.01 | 2.42 ± 0.06 | 1.74 ± 0.05 | 1.76 ± 0.04 | 1.78 ± 0.05 | 1.70 ± 0.11 |
| 0.04 | 2.64 ± 0.07 | 2.00 ± 0.06 | 1.95 ± 0.05 | 1.98 ± 0.04 | 1.93 ± 0.07 |
| 0.08 | 2.81 ± 0.08 | 2.26 ± 0.07 | 2.27 ± 0.06 | 2.35 ± 0.06 | 2.14 ± 0.08 |
| 0.16 | 2.76 ± 0.15 | 2.67 ± 0.09 | 2.68 ± 0.07 | 2.71 ± 0.09 | 2.80 ± 0.10 |

This contrast enhancement was also observed in the highly-scattering PI stained human prostate tissue (Fig. 5). The scattered light as seen in the left part of the SPIM image (Fig. 5(A)) was largely rejected in confocal, SI, and the combined modes. Figure 5(J) quantitatively compares the contrast increase compared to SPIM alone. The best contrast improvement achieved by confocal SPIM was 1.72 when the slit size was 9.75 μm, which is about 80% of the illumination beam waist width. SI SPIM and confocal-SI SPIM achieved even higher contrast, which were about 2.2 times better than SPIM mode alone. Unlike the low scattering gelatin phantom results, as the SI pattern frequency was increased to 0.16, the contrast was decreased due to the lower SNR (Fig. 5(F)). However, combining eCSD with SI can help maintain the SNR and the contrast enhancement (Fig. 5(I)). Figure 5(K) shows the depth (in the x' direction) response of image contrast with SPIM mode and the optimized mode. From the peaks in the contrast plot, it can be shown that the optimized mode enhanced contrast along depth without losing signal information.
Fig. 5. Contrast comparison among different imaging modes. Same position from a PI stained prostate tissue was imaged with SPIM (global exposure mode) (A), confocal SPIM (B, C), SI SPIM (D-F), and confocal-SI SPIM(G-I) respectively. The parameters used in different modes were labeled in the lower part of each image. (D) and (H) had the best contrast. However, the image contrast at SI SPIM mode with frequency of 0.16 was decreased to even lower than SPIM mode (F, J) due to the decreased SNR. By adding confocal detection to SI (I), the contrast can be maintained by improving SNR. For better visualization and comparison, 0.5% of pixels were saturated in the lookup table. The yellow line in (A) separates the in-focus right part and the scattering left part which can be rejected in other modes. The red line in (J) indicates the size of the beam waist. (K) Contrast comparison between SPIM mode and Confocal-SI mode versus depth. The yellow stars indicate the top surface of the sample, which is where the illumination light originates. The green and blue dash lines indicate two different depths along x’, with distance of about 175 μm. Scalebar, 50 μm.

Theoretically, the optical sectioning ability of SI increases (optical section thickness decreases) when the pattern frequency increases - the optimal achievable section thickness of SIM with our particular setup is 10.1 μm when the normalized frequency goes to 0.86 based on the Stokseth approximation to the microscope OTF [35]. However, as shown in the highly scattering case, the image contrast is not necessarily increased when the frequency is higher, and sometimes can be even lower than SPIM mode alone (Fig. 5). The possible reason could be the deteriorated modulation depth of the structured patterns at high frequencies lowers the
signal recovered. Figure 6 compares the modulation depth of SI patterns in fluorescent dye solution (2.5 μL/mL acridine orange solution) between SI SPIM and confocal-SI SPIM. We found that the combination of eCSD and SI dramatically improved illumination pattern modulation depth at high pattern frequencies compared to SI SPIM alone, thus helping to recover more signal at higher frequencies. As shown in Fig. 6(A), at a normalized grid frequency of 0.16, compared to global shutter mode (SPIM mode), modulation depth in eCSD mode has been improved by 6.6 times, 7.6 times, 7.8 times, 9.0 times and 15.3 times, respectively, using slit size of 39 μm, 29.25 μm, 19.5 μm, 9.75 μm and 3.25 μm. The enhancement of pattern modulation depth can be directly visualized in Fig. 6(B), which shows the pattern images at normalized grid frequency of 0.16 with different electronic confocal slit sizes. The increased modulation depth by adding eCSD to SI could be one of the reasons that the images in confocal-SI mode have much higher SNR and contrast than SI SPIM at the same high frequencies. Also, the amplification of background shot noise by the square-law detection algorithm can decrease the SNR of SI SPIM, while the reduction of initial background signal (and its associated shot noise) prior to collection achieved when combined with eCSD resulted in higher SNR for confocal-SI SPIM than SI SPIM at the same frequency.

![Fig. 6. Comparison of illumination pattern modulation depth versus normalized grid frequency at global mode and confocal mode with different slit sizes (A). Corresponding pattern images at grid frequency of 0.16 at global mode and confocal mode with different slit sizes (B). For better visualization and comparison, images in B were normalized to show the min-max intensity.](image)

Even though eCSD improved modulation depth dramatically and reduced initial background signal, confocal-SI SPIM did not necessarily yield better image contrast than SI SPIM at low pattern frequencies, and the optimal contrast achieved by confocal-SI SPIM was similar to that of SI SPIM. In fact, the influence of confocal-SI SPIM on imaging performance can be complex, due to its side effect of an overall reduction in collected signal. A longer integration time or higher power can improve signal levels, but at the same time it will increase the background. We have tested the contrast differences on the prostate sample by increasing laser power to get comparable signal levels across the different modes, and found that the trends of contrast when adjusting laser power (data not shown) were similar to the ones shown in Fig. 5. As the result, in the prostate tissue imaging, confocal-SI SPIM can only be more effective than SI SPIM in contrast enhancement, when the pattern frequency is too high to enable good performance in SI SPIM.

One possible reason to adopt confocal-SI SPIM with high pattern frequencies instead of SI SPIM could be to improve axial resolution. As measured on gelatin phantom with 500 nm fluorescent beads, the axial resolution can be improved when the pattern frequency is increased, especially when the beam waist is thick, or at the two ends of a Gaussian beam, where the beam is much thicker than at the waist. The axial resolution was increased by
10.3% with SI SPIM at a normalized frequency of 0.16 compared to SPIM mode only, when the beam thickness was 27 μm (at the two ends of the Gaussian beam); whereas, it increased to 19.4% improvement with the addition of confocal detection (confocal-SI) at a normalized frequency of 0.16 and a confocal slit width of 9.75 μm (Fig. 7).

Finally, Fig. 8 shows xy and xz planes of the reconstructed 3D volumes of a prostate tissue using SPIM mode, confocal mode and confocal-SI mode. The contrast has been improved significantly using confocal-SI mode compared to SPIM alone. The averaged contrast enhancements of the volume for the optimized modes have been calculated: compared to the SPIM mode, confocal SPIM achieved 2.31 ± 0.01 (standard error, SE) better contrast, and confocal-SI was 4.57 ± 0.02 (SE) better. Excellent SNR was still maintained after reducing background, which enabled better appreciation of tissue features and details without further adopting imaging processing methods for background subtraction.
4. Discussion

In this paper, we have compared the imaging performance of SPIM, confocal SPIM, SI SPIM and confocal-SI SPIM on low-scattering phantoms and highly-scattering human prostate tissue. We found that the best contrast was achieved when using SI or confocal-SI mode. If not considering the longer acquisition time, SI SPIM and confocal-SI both are more suitable methods for thick, scattering tissue imaging to achieve better contrast, compared to SPIM alone. Moreover, adding confocal detection to the SI mode helps to recover SNR and contrast at higher pattern frequencies, which can be useful to achieve better axial resolution, especially when the beam thickness is large and the contribution of out-of-focus background is larger. The enhancement of contrast and SNR for SI, when combined with confocal slit detection, is due to removal of part of the scattered background, and its associated shot noise, which would otherwise be amplified in SI-SPIM without confocal detection. On the other hand, although its performance enhancements are more modest, confocal SPIM is still useful for fast 3D imaging when moderate contrast enhancement is sufficient.

These methods are useful where 3D thick tissue imaging is needed but optical clearing is not practical, such as intra-operative pathology samples that need to be processed and imaged quickly (i.e. within minutes of removal). By leveraging pseudo-H&E fluorescent staining [36] and creating 3D digital histology data sets without the sampling issue resulting from physically cutting slices as in standard histopathology, we can better understand and diagnose diseased tissue.

One of the limitations of single-sided imaging with the iSPIM system is the anisotropic resolution. The axial resolution of iSPIM is mostly dependent on the beam thickness, which
in our case, is almost 10 μm at best. In our current work we are extending these methods to dual-sided imaging, using double-view capture and fusion [37], to achieve z-resolution that is equivalent to the lateral resolution (here, about 1.5 μm, but ultimately depending on the objective lens used). Using Bessel beams can also help improve axial resolution [38], but at the cost of extra background from side lobes, which however can be mitigated by combining with eCSD and SI.

Finally, data processing, 3D visualization and sharing are common challenges when dealing with large data sets. The size of the data can be several times larger after shifting because of the need for zero-padding, which further aggravates the situation. On the other hand, current readily available 3D visualization tools are either limited to small data sets [39], or are limited in their utility in open data publishing. New developments of readily-available web-based 3D visualization tools are always welcome additions to this burgeoning field.

5. Conclusions

A versatile iSPIM system has been optimized by combining with eCSD and SI. The imaging performance in highly scattering tissue (i.e. intact human prostate biopsy) has been assessed and compared. We found that although eCSD could achieve better contrast than SPIM alone and is the most rapid method to improve optical sectioning in SPIM, SI and confocal-SI could achieve even higher contrast. The combination of confocal + SI mode reduces background signal and its associated shot noise prior to detection with the SI algorithm, and enables us to achieve higher pattern modulation depths with less dependence on illumination pattern frequency, both of which results in better SNR for SI at high illumination pattern frequencies which can be used to improve axial resolution. Current work is aimed at extending to dual-sided imaging and demonstrating the utility of dual confocal-SI SPIM for rapid multicolor diagnostic imaging.

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