Abstract: We report replacement of one side of a static illumination, dual-sided, thin-sheet laser imaging microscope (TSLIM) with an intensity modulated laser scanner in order to implement structured illumination (SI) and HiLo image demodulation techniques for background rejection. The new system is equipped with one static and one scanned light-sheet and is called a scanning thin-sheet laser imaging microscope (sTSLIM). It is an optimized version of a light-sheet fluorescent microscope that is designed to image large specimens (<15 mm in diameter). In this paper we describe the hardware and software modifications to TSLIM that allow for static and uniform light-sheet illumination with SI and HiLo image demodulation. The static light-sheet has a thickness of 3.2 µm; whereas, the scanned side has a light-sheet thickness of 4.2 µm. The scanned side images specimens with subcellular resolution (<1 µm lateral and <4 µm axial resolution) with a size up to 15 mm. SI and HiLo produce superior contrast compared to both the uniform static and scanned light-sheets. HiLo contrast was greater than SI and is faster and more robust than SI because it produces images in two-thirds of the time and exhibits fewer intensity streaking artifacts.

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References and links

1. Introduction

The thin-sheet laser imaging microscope (TSLIM) is a light-sheet microscope that was developed for nondestructive optical sectioning of organisms and thick tissues such as the mouse cochlea, zebrafish brain/inner ear and rat and mouse brain [1]. TSLIM was designed as a dual beam, light-sheet microscope with air mounted illuminating and observing objectives so that large specimens can be imaged. Although it can image transparent live specimens, it was optimized for observing fixed tissue that has been fluorophore labeled and chemically cleared to transparency. TSLIM is similar to other light-sheet based microscopes in which specimens are optically sectioned using a light-sheet, and the fluorescent tissue planes are recorded by a camera mounted perpendicular to the light-sheet. By moving the specimen in the z-axis through the light-sheet, serial optical sections are collected and three-dimensional (3D) reconstructions of structures can be generated for visualization and morphometry. However, since the light-sheet is thinnest only in a narrow region (i.e., the beam waist) an x-axis scanning procedure was used in TSLIM to produce a well-focused composite image across the full width of the specimen.

In 3D microscopy, resolution is determined in lateral and axial planes. While the lateral resolution is mainly governed by the Abbe diffraction limit and therefore the numerical aperture (NA) of the detection objective, the axial resolution is mainly determined by the thickness of the light-sheet. In TSLIM, the axial resolution was more than three times greater than the lateral resolution. Because of physical limitations and the fact, that the confocal parameter is proportional to the beam waist, the beam waist can’t be focused as an arbitrarily thin light-sheet. That is the reason why investigators like Neil [2] and Mertz [3] developed techniques using pattern illumination for enhancing image contrast and axial resolution independent of light-sheet thickness. In this paper we describe hardware and software modifications to TSLIM for a scanned light-sheet and patterned illumination.

TSLIM’s scanned laser side is similar to a digital scanned laser light-sheet fluorescence microscope [4], or DSLM with SI [5] but exhibits important differences such as air mounted objectives, the use of a single galvanometer mirror and the addition of HiLo modulation.

2. Methods and results

The illumination light sources of the original TSLIM [1] uses two ND:YAG solid state Lasers (Optotronics VA150532/473) with green 532nm and blue 473nm wavelengths. The use of the specific wavelength depends on the choice of the fluorophore used to stain the specimen. A beam splitter for a dual sided illumination splits both lasers. For the static light-sheet side, the laser is guided by a mirror assembly into either a 5X or 10X Galilean beam expander (Edmund Optics NT 55-577/8) where the beam is expanded and collimated. After shaping the beam, it is focused by an f = 50.8 mm plano-convex cylindrical lens (Newport CKX525-C) in order to focus the light in the y-dimension. The light-sheet then travels through an infinity-corrected, long working distance plan apochromat microscope objective (Edmund Optics, NT59-876/7) of either 5X (NA = 0.14) or 10X (NA = 0.28) magnification which focuses the illuminating light-sheet. With different combinations of the illumination setup, light-sheet thickness can be selected between 3.2 µm to 4 µm (e⁻²).

These data were measured by reflecting the beam onto the camera sensor via a pentaprism positioned in the specimen chamber and calculating a Gaussian best fit function based on the measured intensity profile.

While the beam is compressed by the cylindrical lens in one dimension the illuminating microscope objective produces a thin light-sheet which is projected through the specimen. The focused light-sheet does not have a constant thickness, rather a hyperbolic profile. The light-sheet is thin only in its narrow region called the confocal parameter which, by definition, is the region on both sides of the beam waist where the beam thickness is less than \(2^{1/2}\) times larger than the beam waist. Image quality is optimal only in this region. By
using a stitching technique described by Schacht et al. [6], and Buytaert and Dirckx [7] a composite image of the full width of the specimen is produced which contains columns of image data from the confocal region. After recording one image plane, the specimen is moved an increment in the z-direction to produce a stack of serial images. The detection system is an Olympus MacroView MVX10 microscope, with a 1X (MVPLAPO1X, NA = 0.25 WD = 65mm) and a 2X (MVPLAPO2XC, NA = 0.50, WD = 20mm) objective lens. Attached to the microscope is either a full-frame CCD digital camera (Retiga 2000R) or a TDI line scan camera (Dalsa, Piranha HS-40-04K40). The specimen is mounted on a xyz-translater and a y-rotation stage to position the specimen and to enable the necessary movement for image stitching and stack creation. The specimen is placed in an optical glass or quartz specimen chamber (Starna, 3G20; DLC300Q20) filled with clearing solution. The chamber material has a refractive index that is similar to the clearing solution and the tissue to minimize light scattering. While imaging, the chamber and the illumination and detection objectives are stationary so the optical path length remains constant as the specimen is translated in the chamber.

As can be seen in Fig. 1, the device has a static and a scanned illumination side for comparison of two types of generated light-sheets and the SI and HiLo image demodulation techniques. The left side of the illumination system was modified for intensity modulated scanning. The laser light travels through an acousto optic modulator (AOM) (Noah Industries, AOMOD-HPFS-100), which modulates the intensity of the first order diffracted beam. An aperture blocks all other beam orders before the central beam is expanded and collimated by a variable Gaussian beam expander (Edmund Optics, NT68-479). A mirror assembly guides the beam into a single axis scanning galvanometer mirror system (galvano) (Thorlabs, GVS001). The galvano (GM) is positioned at the focal point of the telecentric F-Theta lens (Sill Optics, S4LFT0061) and is used to scan the beam and focus it in an image plane. Because the spot diameter of the F-Theta lens is still too large (~6 µm), it is projected to a smaller size by an afocal system consisting of an achromatic tube lens duplet (Thorlabs, AC508-100-A-ML) and a microscope objective (Edmund Optics, NT59-877). Tests with a plano-convex lens singlet used as tube lens produced a larger beam waist. The beam expander is variable from 1 to 8X magnification. The F-Theta lens limits the maximal entrance aperture to 5 mm diameter. With an original beam diameter of 1.2 mm before the beam expander, the entrance beam diameter in the F-Theta lens can be selected between 1.2
mm to 5 mm and therefore a measured illumination light-sheet thickness between 6.1 µm to 3.7 µm (e^2, measured as previously described).

The main differences between the scanned beam of sTSLIM and DSLM-SI [5] are the field of view, resolution, and the method for changing the optical sectioning plane. In DSLM-SI [5] a second galvano mirror is used for scanning the light-sheet in z-direction. The specimen is fixed and the focal plane of the detection system is moved to the same position of the light-sheet with another translation stage. In sTSLIM the z-position of the detection objective and the light-sheet is fixed and only the specimen is moved with a translation stage. Also, in sTSLIM the illuminating and observing objectives are mounted in air to accommodate large specimens and interchangeability of lenses. In DSLM-SI the illumination and observing objectives are fixed and mounted within the specimen chamber. This allows for higher NA lenses and better resolution, but drastically reduces the working distance and hence the size of the specimen that the system can image. In sTSLIM the chamber is fixed and the specimen moves within the clearing solution, thus the optical path length stays constant and it is not necessary to correct the focal position of the illumination or the detection system. The advantage is that the focal plane of the illumination and detection system remain well aligned and the overall costs of the system is reduced because it only requires a single-axis galvano mirror and no focusing translation stage for the detection system.

2.1. Programming the control of sTSLIM

Image demodulation techniques require a grid illumination with flexible grid period, duty cycle and grid phase. Rapid shifting between uniform and grid illumination and the grid phases is necessary for time efficient imaging. sTSLIM is controlled by a PC running Windows XP and custom National Instruments (NI) LabVIEW (ver. 2011) software. For data acquisition a NI PCIe-6321 X card is used. It is coupled via −10V to 10V analog output to the GM control and via TTL to the AOM driver. The GM is controlled by an analog signal with a high sampling rate generated by the NI PCIe 6321 card and the AOM by a digital signal synchronized to the sampling rate of the GM control signal. The frequency of the AOM digital signal is an integer multiple of the GM frequency which results in a static pattern. Depending upon the ratio between the AOM and the GM frequency, the number of gridlines over the field of view and therefore the grid frequency can be adjusted.

Since the F-Theta lens has a linear ratio between scan angle and scan position, a triangle wave is used for movement control of the GM in order to produce an even distributed illumination. Because of the moment of inertia, the GM could not follow the signal exactly on the edges. That is why the triangle amplitude is set 20% greater than necessary for illuminating the whole field of view. To couple the AOM frequency with the GM frequency, the AOM is driven by a hardware counter clock output on the PCI card. The same counter clock output is used for triggering the frequency of the GM. This prevented the drifting of the frequencies. The phase shift of the grid is selected by an offset added to

![Fig. 2. Optical model of the illumination pathway. The galvano mirror is positioned at the focal point of the F-Theta lens in order to produce a scan angle. The tube lens is mounted afocally to the F-Theta lens and the microscope objective to decrease the spot diameter of the F-Theta lens and illuminate the specimen.](image-url)
the GM control signal. Figure 2 shows the optical diagram of the focusing optic for the scanning setup.

To calculate the offset required to shift the grid illumination it is essential to understand the relationship between the scanning angle and the beam position. Equation (1) describes the spot position \( y' \) depending on the scan angle \( \Theta \) in the first image plane after the F-Theta lens:

\[
y' = f_1 \times \Theta
\]

(1)

An F-Theta lens has a linear ratio between scanning angle and scanning position. Because the chosen F-Theta lens has a telecentric behavior, the focused beam is always parallel to the optical axis and the beam angle \( \Theta' \) equals zero. The angle after the tube lens \( \Theta'' \) is described by the trigonometric Eq. (2):

\[
\Theta'' = \arctan \left( \frac{y'}{f_2} \right)
\]

(2)

The spot position after the microscope objective \( y'' \) compiles with Eq. (3):

\[
y'' = f_3 \times \tan(\Theta'')
\]

(3)

Equation (4) is the result of inserting Eq. (2) in Eq. (3):

\[
y'' = f_3 \times f_1 \times \Theta
\]

(4)

The required GM angle is calculated by the given scanning position with the help of Eq. (5):

\[
\Theta = \frac{y'' \times f_2}{f_3 \times f_1}
\]

(5)

2.2. Image demodulation techniques

For optimizing the resolution of sTSLIM the two image demodulation techniques SI and HiLo are compared in Table 1.

<table>
<thead>
<tr>
<th>Method</th>
<th>Structured Illumination</th>
<th>HiLo Background Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td>• minimum three phase shifts grid illumination required</td>
<td>• two images, one with a pattern illumination and one with a uniform illumination</td>
</tr>
<tr>
<td></td>
<td>• phase control of the grid pattern (0°, 120° and 240° phase), more phase shifts are possible</td>
<td>• low pattern quality necessary</td>
</tr>
<tr>
<td></td>
<td>• high accuracy for phase shift required</td>
<td>• grid, or speckled for pattern illumination possible</td>
</tr>
<tr>
<td></td>
<td>• high grid quality required</td>
<td>• robust algorithm against pattern failure</td>
</tr>
<tr>
<td></td>
<td>• simple algorithm for image post processing</td>
<td>• complicated algorithm required</td>
</tr>
<tr>
<td>Speed</td>
<td>minimum three images</td>
<td>two images</td>
</tr>
<tr>
<td>Energy Load</td>
<td>minimum three images with 50% intensity = 150% intensity</td>
<td>one image with 100% = 1 image with 50% = 150% intensity</td>
</tr>
<tr>
<td>(photobehaving/phototoxicity)</td>
<td>[2,5,8]</td>
<td>[3,7,8]</td>
</tr>
</tbody>
</table>

Table 1. Comparison between structured illumination and HiLo background rejection
Fig. 3. Comparison images of the scala media from a mouse cochlea. The arrows indicate the illumination direction. The images are recorded and processed as follows: (a) static, uniform illumination, (b) scanned, uniform illumination, (c) structured illumination, and (d) HiLo background rejection. Scale bar = 100 µm.

Since the photobleaching in the three SI images was noticeable (horizontal striped lines in Fig. 3c), image intensity was normalized before they were post processed with the SI algorithm. The post processing for SI was done by a custom LabVIEW program. There are still lines of different intensity visible in the SI image, which are caused by the sinusoidal intensity of the grid pattern (Fig. 3c). This is discussed by Schaefer [8]. Also SI images with six and twelve phase shifts were recorded, but the images had no noticeable quality enhancement at this grid frequency. However, photobleaching and camera gain induced noise were worsened by using more phase shifts. For producing the HiLo image modulation we used the HiLo Fiji Plugin (http://biomicroscopy.bu.edu/r_hiloplugin.htm) written by Daryl Lim in Dr. Jerome Mertz’s laboratory. Contrast was quantified by calculating the energy normalized standard deviation of each image in Fig. 3 according to Eq. (6) in Ref.
The standard deviation measurements, summarized in Fig. 4, reflect the observed qualitative differences in the images.

HiLo processing was more resilient to interruptions in the grid pattern than SI (Fig. 5). Since SI processing includes a subtraction step, interruptions in the grid pattern can lead to common information between component images and thus darkened areas in the final image (Figs. 5b, 5c). HiLo uses scanned grid as well as scanned uniform illumination, making it possible to recover information from the uniform illumination image that would have been otherwise lost during SI processing.

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3. Conclusions

We have demonstrated implementation of an intensity modulated laser scanner into an existing static illumination light-sheet fluorescence microscope system and we described the control programming using LabVIEW. Using sTSLIM the image quality of whole, inner ear optical sections using static and scanned light-sheets as well as SI and HiLo were compared. The scanned light-sheet reduced out-of-focus “haze” and streaking artifacts in the specimen while retaining a resolution comparable to the static light-sheet. SI was performed with
three phase shifts (0°, 120°, 240°) rather than six or twelve as these appeared to introduce noise without a noticeable increase in image quality. SI and HiLo produced superior contrast compared to both the static and the unmodulated, scanned light-sheets. HiLo contrast was greater than SI. Out-of-plane objects and structures that blurred images tended to cause darkened areas after SI but not after HiLo. This is likely due to the subtraction step in SI image processing. HiLo appears to be faster and more robust than SI because it produces images in two-thirds of the time and exhibits fewer intensity artifacts.

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