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<th><strong>Title</strong></th>
<th>Dual spectrometer system with spectral compounding for 1-m optical coherence tomography in vivo</th>
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One of the major advantages of optical coherence tomography over other diagnostic imaging tools is the ability to resolve clinically relevant tissue microstructures at 10-µm level [1, 2]. Over the past 15 years, research has been focused on high resolution technologies with resolution from several micrometers to one micrometer in an attempt to achieve cellular and subcellular level imaging [5-8]. It has been demonstrated that many cellular and subcellular structures of biological tissues can be reliably visualized using a class of imaging techniques characterized by ~1-µm axial resolution [4, 6, 7, 9, 11]. In order to achieve such a high axial resolution, repetitive pulsed laser sources had been used to obtain a large spectral bandwidth and visible light was included at the blue end of the spectrum in the improved resolution [4, 6, 8, 12]. As near infrared (NIR) radiation from superluminescent diodes (SLD) is less likely to cause tissue damage than visible, repetitive pulsed radiation, a 1-µm axial resolution OCT technology which operates with NIR, SLD light sources is desirable especially for ocular and skin imaging applications in vivo.

In this Letter, we report a novel OCT imaging system that achieves an axial resolution of ~1.27 µm in air which, to the best of our knowledge, is the highest axial resolution OCT using NIR SLD sources up to date. Based on its merits in spatial resolution, safety and cost aspects, the proposed technology provides a viable solution for cellular and subcellular resolution imaging.

The imaging system presented in this Letter uses two SLD arrays to provide NIR illumination from 755–1105 nm (3-dB spectral range) (Fig. 1). In order to detect spectral interference signal across the entire illumination bandwidth, we employed two spectrometers based on an InGaAs camera (Sensors Unlimited GL2048L) and a Si camera (E2V, AViVA EM4) respectively. There is a ~100-nm overlap, which allows us to combine spectral interference signals acquired from the two cameras with high accuracy in spite of differences in optical arrangements between the two spectrometers (Fig. 2 (a)).
directed to the reference arm and the annular wavefront goes to the sample arm. The optical power on the sample is 690 μW. An objective lens (Mitutoyo Plan Apo NIR, 20X, NA = 0.4, ~70% transmission) is used to focus the light beam into a focal spot of 1.7 μm (full-width at half maximum, FWHM). Light backreflected from the reference arm and backscattered from the sample arm is recombined through the rod mirror and guided by the single mode fiber back to a 10 μm pinhole via the 50:50 beam splitter (BS2). The interference signal is split by another 50:50 non-polarizing beam splitter (BS3) to the two spectrometers. The spectrometer 1 is composed of an 830 lines/mm ruled grating (NT43-850, Edmund Optics Inc.), a camera lens (Nikon AF Nikkor 85mm f/1.8D), and the Si based linear sensor. We use 1050 camera pixels to detect a total spectral range of 850 ± 100 nm. The spectrometer 2 is composed of a 1,200 lines/mm transmission grating (830 nm, Wasatch Photonics Inc.), a camera lens (Nikon AF Nikkor 50mm f/1.8D), and the InGaAs based linear sensor. We use 1,350 camera pixels to detect a total spectral range of 1,020 ± 80 nm. For each linear sensor, we digitized the detected signals at 12-bit resolution and transferred them to the personal computer through camera link cables and an image acquisition board (KBN-PCE-CL4-F, Bitflow). Both cameras and the galvo scanner are synchronized by a triggering signal generated by the computer.

In order to combine the two spectra acquired from the two spectrometers, we developed a semi-automatic spectral mapping algorithm. Firstly, a set of calibration interference fringes was acquired from both cameras when a partial reflector is located at the focal plane of the sample arm focusing optics. By changing the reference delay, this step was repeated at two optical pathlength differences between the two arms. After background subtraction, we secondly obtained the indices of the zero crossing points of the calibration spectra using linear interpolation. In the third step, we manually selected a subset of a spectrum in the overlap region acquired from the Si camera which started from the $n$ th ($n = 1, 2, \ldots, N$, $N$ is the total number of zero crossing points) zero crossing point and ended with the $(n+9)$ th zero crossing point. We then conducted an exhaustive search for the best match between the selected spectral subset and subsets of 10 consecutive zero crossing points in the corresponding spectrum from the InGaAs camera acquired at the same optical pathlength difference. Finally, index mapping data that provided the best match was verified with the other two sets of calibration interference fringes acquired from the other two pathlength differences. By compounding two spectrometers using the index mapping data, an extended FWHM spectral range of 293 nm centered at 927.5 nm was achieved (Fig. 2 (b)).

The axial resolution resulted from the combined spectrum was tested to be ~1.27 μm in air which corresponds to 0.93 μm in tissue (n = 1.36) (Fig. 3 (a), red dashed profile), which matches well with a theoretical resolution of 1.16 μm in air. The axial sidelobes of ~5 dB are due to spectral fluctuations of both SLD arrays and the decayed spectral responses from 850 – 980 nm of both cameras. A spectral shaping method was implemented to reduce the spectral intensity fluctuation [13]. In Figs. 2 (a) and (b), the red line outlines the envelope of original signal and the blue curve is the compounded spectrum after sidelobe suppression. The improved axial profile (purple solid line in Fig 3 (a)) shows that the sidelobes are suppressed by ~2 dB without significant degradation of axial resolution. We also measured axial point-spread functions (PSF) at various depths of a mirror sample by changing the delay in the reference arm. The results show that the axial resolution decreased to ~1.7 μm at a depth of 0.5 mm where signal dropped by 6 dB (Fig. 3 (b)). To test the system stability, we monitored the axial profile over 10,000 sec and observed little change in the axial resolution.

![Figure 2](image1.png)  
**Figure 2:** Spectral compounding. Interference spectra acquired using a reflective interface by (a) the Si camera (green) and the InGaAs camera (cyan). (b) Combined interference spectrum demonstrating an extended spectral range. Red curves: envelopes of spectrometer output.

![Figure 3](image2.png)  
**Figure 3** (a) Measured axial point-spread function using an attenuated mirror; (b) Measured axial PSFs on a linear scale for different delays relative to the reference arm length; (c) Axial resolution as a function of distance to focal plane.
chromatic focal shift in the working wavelength range. Theoretical axial resolution was estimated based on the simulated center wavelength and spectral bandwidth (Fig. 3 (c), blue stars). The measured values (Fig. 3 (c), red circles) matches well with the simulation results.

In order to test the phase stability of this dual-spectrometer scheme with respect to a single spectrometer system, we measured phase variations of all the pixels over 512 A-lines with an optical pathlength difference of 0.09 mm. The phase stability is defined as the standard deviation of the phase differences between sequential A-lines according to reference [14]. The results demonstrate that the mean phase stability within the overlap region is around 9% higher than that outside of the overlap region (Fig. 4), which may be partially attributed to the spectral intensity variation.

To characterize the sensitivity of the system, we measured the signal to noise ratio (SNR) using a partially-reflecting mirror sample (~40.7 dB reflectivity) when the pathlength difference between the mirror sample and reference arms was maintained at 0.1 mm. The sensitivity for the system (combined spectrum), Si camera alone, and InGaAs camera alone was measured to be 95.8 dB, 95.4 dB, and 91.1 dB respectively. In order to understand the measured sensitivity, we analyzed the SNR in the case of individual camera using the following formulas [15]:

\[
S[\text{dB}] = 10 \times \log \left( \frac{N_{\text{ref}} \times \sum N_{s}}{N_{\text{el}}^2 + N_{\text{sh}}^2 + N_{\text{RIN}}^2} \right) \tag{1}
\]

\[
N_{\text{RIN}} = (f \Delta v)^{1/2} N_{\text{ref}} \tag{2}
\]

\[
N_{\text{sh}} = (N_{\text{ref}})^{1/2} \tag{3}
\]

where \(N_{\text{ref}}\) is the number of electrons per pixel generated by the reference arm light, \(N_{\text{el}}\) is the electrical noise of the photo detector, \(N_{\text{sh}}\) is the number of shot noise electrons, \(f\) is the reciprocal of twice the exposure time of the CCD, \(\Delta v\) denotes the FWHM spectral bandwidth of the reference light received by single pixel, \(\sum N_{s}\) denotes the sum of electrons over the entire array generated by sample arm light returning from a 100% reflector. Since the two cameras are synchronized, the line rate and the exposure time of both cameras were 10,000 Hz and 90 μs respectively. The parameters mentioned in Eqs. 1-3 for SNR analysis are listed in Table 1 for the spectrometer 1 and 2 respectively. Assuming a uniform spectral density, the sensitivity was predicted to be 98.44 dB and 93.46 dB for the spectrometers 1 and 2 respectively. We think the difference between the measured sensitivity and the theoretical prediction is mainly due to non-uniformity of the spectral response of both cameras.

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<td>Nel (e⁻)</td>
</tr>
<tr>
<td>NRIN (e⁻)</td>
</tr>
<tr>
<td>Nsh (e⁻)</td>
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<tr>
<td>Δv (GHz)</td>
</tr>
<tr>
<td>Spectrometer efficiency</td>
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<tr>
<td>Full well depth (e⁻)</td>
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<td>N_ref (e⁻)</td>
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<td>(\sum N_s) (e⁻)</td>
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It is expected that the measured sensitivity of combined spectrum is higher than the measured sensitivity of individual camera. This is because the two spectra has a ~100 nm overlap where the noise was suppressed relative to the signal based on the SNR model [15]. Because the full well depth of the Si camera is 6.72 times smaller than that of the InGaAs camera, under the condition of a uniform input spectral density and a uniform spectral reflectivity of the reference arm, each InGaAs camera pixel was only 7.54% filled when the Si camera was saturated. The InGaAs camera should have had ~5.5 dB higher sensitivity given enough reference light power in the range of 930-1105 nm. This problem can be solved by using a light source with higher power or more efficient system design. In addition, the sensitivity can be further improved by increasing the sample arm light power at the cameras using an objective lens with higher transmission rate and/or replacing BS3 with a dichroic filter.

In order to validate the proposed system for in vivo cellular resolution applications, imaging was conducted using three-day-old zebrafish larvae from the dorsal side near the tail. Images were recorded over a field view of 873 μm × 436 μm (transverse × longitudinal) at a frame rate of 20 Hz with 512 lines per frame. Numeric dispersion compensate was used to balance the water dispersion. In a representative image (Fig. 5 (a)), the blood flow (Green box and the inset) in the artery can be clearly visualized near the notochord. We identified a few similar reflective/scattering patterns in each frame, and each pattern was composed of two bright and distinct interfaces with ~3.3 μm (n = 1.36) spacing in between (Figs. 5 (a-d), orange arrows). Based on the width and height of individual signal pair, we think that each of them represents the top and bottom surfaces of individual disk-like [16] red blood cell as illustrated in Fig. 5 (e). We further identified flat, spindle shaped structures lining along the luminal surface of the blood vessel wall (Figs. 5 (a-d), 0, green arrows). Based on the shape, dimension, and anatomical location (Fig. 5 (e)), these structures are possibly endothelial cells.

A dual spectrometer design is more powerful than the single spectrometer design in high resolution applications. For a single spectrometer based OCT system to achieve axial resolution better than 1 μm in
Figure 5 in vivo one-micrometer resolution images of zebra fish larvae. (a) A representative imaging showing blood flow in the tail artery (green box and inset) near the notochord (N) (see Media 1). Scale bars in the main figure: 50 μm; Scale bars of inset: 25 μm: (b)-(d) Each RBC appears as individual signal pair (orange arrows) originating from the top and bottom surfaces of the disk-like cell body. (e) Illustration of endothelium and RBCs in an artery. (f) Structures at the surface of the vessel wall suggest endothelial cells (green arrows) in a 10-frame averaged image. Scale bars: 25 μm.

tissue, the practical option is to shift the spectrum to the blue end which will unavoidably decrease the penetration depth and include visible radiation where much stricter safety regulations than NIR radiation apply for in vivo applications. The dual spectrometer design makes it possible to improve axial resolution using NIR radiation and gain improved penetration by use of longer center wavelength.

In conclusion, we developed a one-micrometer axial resolution OCT system using NIR SLD light sources, which gives the highest resolving power comparing to other systems with same classes of light sources. We have validated the axial resolving power of the proposed system in zebra fish larvae by visualizing cellular structures in vivo. Our system employs NIR radiation from SLD sources which is safer compared with the repetitive-pulsed source with visible radiation [4, 6-8, 12, 17]. In addition, the proposed imaging system is also superior in cost and sensitivity compared with existing high resolution system employing high brightness broadband light sources [7, 8, 12, 17]. Based on these facts, the proposed system provides a viable solution for one-micrometer resolution cellular level OCT imaging in vivo.

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