Hyperspectral scanning laser optical tomography

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In order to study physical relationships within tissue volumes or even organism-level systems, the spatial distribution of multiple fluorescent markers needs to be resolved efficiently in three-dimensions. Here, rather than acquiring discrete spectral images sequentially using multiple emission filters, a hyperspectral scanning laser optical tomography (SLOT) system is developed to obtain hyperspectral volumetric data sets with 2 nm spectral resolution of optically transparent mesoscopic (mm-cm) specimens. This is achieved by acquiring a series...
of point-scanning hyperspectral extended depth of field images at different angles and subsequently tomographically reconstructing the 3D intensity distribution for each wavelength. This technique is demonstrated to provide robust measurements via the comparison of spectral and intensity profiles of fluorescent bead phantoms. Due to its enhanced spectral resolving ability, this technique is also demonstrated to resolve largely overlapping fluorophores, as demonstrated by the 3D fluorescence hyperspectral reconstruction of a dual-labelled mouse thymus gland sample and the ability to distinguish tumorous and normal tissues of an unlabeled mouse intestine sample.

1. Introduction
Fluorescence metrology encompasses a range of powerful techniques employed in physics, life science, biomedical research, and has seen a number of innovative advances recently, including novel labeling [1-3] and imaging strategies [4-8]. In recent decades studies of organism-level systems are rapidly proliferating and have led to increasing interest in the development of three-dimensional (3D) whole-body imaging techniques to extract structural and functional information throughout “mesoscopic” (mm-cm) samples, e.g. small animals, embryos and engineered tissues. To date, a number of mesoscopic 3D imaging techniques have been developed including optical projection tomography (OPT) [5], scanning laser optical tomography (SLOT) [6] and light sheet microscopy (LSM) [7,8], providing high-resolution 3D images of mesoscopic samples with fluorescence and/or absorption contrast. They are relatively cost-effective and have been widely applied to facilitate system-level studies, e.g. mapping 3D neural circuit networks in brain [9-11].

OPT and SLOT, also known as optical computed tomography, reconstruct the 3D structure of a specimen (i.e. a stack of cross-sectional images) from a series of 2D projections acquired at different angles. The use of visible light affords rich spectroscopic contrast, but also restricts OPT and SLOT to optically transparent samples that are either inherently
transparent (e.g. *D. rerio* and *C. elegans*) or have been made transparent by optical clearing methods [5, 9-13]. The key differences between OPT and SLOT are the use of wide-field illumination and direct 2D imaging in the former while the latter employs scanning of focused illumination and point detection. SLOT decouples image formation and light collection from each other allowing a higher collection efficiency compared to OPT. Both techniques provide the advantage of isotropic spatial resolution, easy scaling to larger samples (>mm), low cost of implementation; and have been successfully applied to a variety of mesoscopic samples (e.g. *C. elegans, D. melanogaster, D. rerio, mouse organs, human tissue, etc.*) [14-19]. While there have been demonstrations of quantitative fluorescence optical computed tomography, for example fluorescence lifetime OPT and SLOT [20,21], they have been largely restricted to intensity-based imaging providing mainly structural rather than functional information (e.g. molecular dynamics, signaling).

To study the spatial organization and relation of tissue constituents, different fluorophores are used to selectively label different structures and it is desirable to distinguish each label efficiently. Multispectral imaging is commonly used in such measurements by employing different filter sets to image each label sequentially or to split the fluorescence emission into discrete spectral channels simultaneously measured on one or more detectors [5, 22]. These filter-based approaches are limited by many factors, including fluorophore spectral overlap, filter bandwidth and cross-talk. In practice, it is difficult to resolve more than 4 colors within the visible spectrum or to efficiently isolate one signal from others when emission spectra are significantly overlapped. Alternatively, hyperspectral techniques in which a diffraction grating or prism permits the acquisition of the full spectrum have been developed to significantly enhance spectral resolution and are widely used in confocal microscopy [4]. However, confocal microscopy of mm scale sample volumes is prohibitively slow, so it is more practically applied to regions of interest within such samples. Recently, hyperspectral imaging has been introduced in LSM for samples of the few hundred µm to mm
scale (e.g. zebrafish or fruit fly embryos) [23]. To date, there has been no report of hyperspectral volumetric acquisition for larger mesoscopic samples (i.e. mm-cm scale) using optical computed tomography to the best of our knowledge.

Here we propose an approach to mesoscopic fluorescence tomography that extends SLOT to include hyperspectral imaging. We demonstrate that this system provides hyperspectral volume data sets with 2 nm spectral resolution for large mesoscopic samples up to ~5 mm in diameter, and unlike hyperspectral imaging with confocal/LSM, the system provides isotropic spatial resolution. This is achieved by acquiring a series of hyperspectral projection images, each produced from a 2D scan, as a function of the sample angle and extracting the spectral information of each voxel by subsequently reconstructing the 3D intensity distribution at each wavelength. We characterize its application by imaging fluorescent phantoms comprised of both single and mixed species of labelled beads. We also demonstrate its application to image dual-labelled mouse thymus gland tissue and to distinguish tumorous and normal tissue components of an unlabeled mouse intestine sample.

2. Materials and Methods

2.1. Sample preparation

Two cylindrical phantoms of ~2.8 mm diameter consisting of a low concentration suspension of fluorescent beads in 1.5% agarose were used as model samples for measurements. The first phantom, referred to as BP1, consisted of a single type of fluorescent bead (F8827, Thermo Fisher Scientific Ltd) of 2 µm diameter and excitation/emission maxima at 505/515 nm. The second phantom, referred to as BP2, consisted of two types of fluorescent bead. In addition to the previous bead type, a second type (T8880, Thermo Fisher Scientific Ltd) of 1 µm diameter and excitation/emission maxima at 488/560 nm was also present.

Excised mouse thymus gland samples from wild-type mice (BALB/c, 12-week-old, female) were fixed with 4% paraformaldehyde (PFA, 16005, Sigma-Aldrich) in phosphate-buffered saline (PBS) at 4 °C overnight. The immunostaining and clearing procedures...
followed the UbasM [10] and uDISCO [11] protocols respectively. UbasM1 was prepared as a mixture of 25 wt% Urea, 25 wt% Meglumine, 20 wt% 1,3-Dimethyl-2-imidazolidinone, and 0.2 wt% Triton X-100. Each fixed sample was immersed in 5 ml diluted UbasM1 (50%) at room temperature for 2-4 hours and subsequently treated with 5-10 ml UbasM1 (100%) for 12 hours. The treated sample was then washed with PBS several times and then incubated with the primary antibody for 48 hours at 37°C, washed with 0.2% Triton/PBS for 12 hours and then subsequently incubated with the secondary antibody for 48 hours at 37°C. The following primary antibodies were used: anti-alpha smooth muscle actin primary antibody to smooth muscle cells (Abcam, ab5694, 1:100) and anti-CD24 primary antibody to epithelial cells (Abcam, ab64064, 1:100). The following secondary antibodies were used: goat polyclonal secondary antibody to rabbit IgG conjugated to Alexa-488 (excitation/emission maxima 495/519 nm, Abcam, ab150077) and goat polyclonal secondary antibody to rat IgG conjugated to Cy2 (excitation/emission maxima 489/505 nm, Abcam, ab6952). After immunostaining, the samples were cleared as follows: samples were dehydrated through a series of incubations in 20 ml of 30 vol%, 50 vol%, 70 vol%, 80 vol%, 90 vol%, 96 vol% and 100% tert-butanol at room temperature (6 hours for each step), followed by incubation in BABB-D4 (i.e. prepared by mixing BABB (benzyl alcohol + benzyl benzoate 1:2) with diphenyl ether (DPE) at a ratio of 4:1) at room temperature for at least 2 h until samples became transparent.

An unlabeled mouse intestine sample using the 4T1 tumor model (a murine breast cancer cell line) was generated by an orthotopic injection of \( \sim 5 \times 10^4 \) cells into the mammary fat pads of the mouse. After 4 weeks, the tissue was carefully excised and subjected to fixation in 4% PFA/PBS(-) at 4 °C overnight and then cleared in uDISCO in preparation for autofluorescence imaging. All the procedures in the experiments were carried out in accordance to the Institutional Animal Care and Use Committee of Guangdong Province.
2.2. Imaging system and acquisition procedure

An experimental schematic of the hyperspectral SLOT system, arranged in a transmission geometry, is depicted in Figure 1. In brief, the sample was mounted under a rotation stage (T-NM08AS-T4, Zaber Technologies Inc.) and suspended in a cuvette filled with clearing solution to provide an index matched environment. A 488 nm CW laser diode (Cobolt 06-MLD, Cobolt Inc.) was used for excitation. The beam passed through an excitation filter (475/23 nm) and a neutral density filter and then was directed onto the sample via a two-axis scanning mirror system (ProSeries, Cambridge Technology Inc.) and an F-theta-lens with a focal length of 100 mm (LINOS F-Theta-Ronar Lenses). The excitation power at the sample was ~3.5 mW. The scanning laser focus was located a quarter of the way into the sample and the depth of field was arranged to extend through at least half of the sample, which is a standard arrangement in OPT [5,24]. The emitted fluorescence was reflected by a dichroic mirror (wavelength cut-off at 490 nm), transmitted through a long-pass filter (>500 nm) and directed to a spectrograph (ISO-Plane 160, Princeton Instruments), with a maximum collection of NA ~0.13, via an image relay comprised of two 100 mm focal length doublets and a second two-axis scanning mirror system. This second scanning system was used to descan to the signal onto the same point of the spectrograph’s entrance slit. The spectrograph entrance slit width was set to 75 µm and a grating of 150 lines/mm was used. The output from the spectrograph was projected on to a CCD camera (PIXIS 1024, Princeton Instruments) cooled to -70 °C.

The hardware and acquisition settings were controlled using a LabVIEW 2013 program developed in-house. To realize synchronization of the scanning mirrors and camera integration, a DAQ board (USB-6361, National Instruments Corp) was used to generate appropriate trigger signals. The CCD was set to readout a region of interest (ROI), providing a hyperspectral projection for each position of the excitation beam (X, Z) where X is the lateral coordinate and Z a common vertical coordinate (parallel to the axis of rotation) in each
projection image. Once a complete 2D hyperspectral projection image was acquired, the rotation stage was switched to the next angular position and the process repeated until all the projection images were measured.

For the data presented, 128 hyperspectral projection images, scanning an area of 3 × 3 mm (i.e. 300 × 300 pixels with a pixel size of 10 µm) with Δλ = 2 nm spectral resolution over 60 -160 nm bandwidth at the desired wavelength, were acquired over a full rotation (360º). The recorded spectral bandwidth was determined by the CCD ROI and the corresponding total acquisition time was ~17-35 minutes, limited by the corresponding read-out rate of the CCD ROI (i.e. ~11 – 5.4 KHz). For example, to acquire the raw data of a cylindrical phantom of ~2.8 mm diameter over a 60 nm spectral range, the total pixel number was 300 × 300 × 128, and for each “pixel” here, there was a ROI read out to obtain a 60 nm spectral range. The acquisition time was ~17 minutes, which was limited by the ~11 kHz per “line” read-out rate of the CCD (the ROI here used as a “line” detector for each pixel). Since the beads used in the phantoms were much smaller than the expected spatial resolution of the system, the reconstructed bead images specify an achieved spatial resolution of ~20 µm determined by line-sections through bead centers (as shown in Figure S1), which was in agreement with the calculated optical resolution with respect to the spot size at the sample of 20 µm.

2.3. Reconstruction
The most common method for intensity reconstruction of 3D projection data (X, Z, θ) in OPT and SLOT is standard filtered back-projection (FBP) [25], although iterative algorithms have also been developed in the case of fast acquisition with a reduced number of projection images [26]. In addition, reconstruction techniques incorporating point spread function information [24, 27, 28] to improve the image quality have also been demonstrated.
To reconstruct our hyperspectral data \((X, Z, \lambda, \theta)\), where \(\lambda\) is the fluorescence wavelength and \(\theta\) the projection angle, each individual spectral channel was reconstructed independently, i.e. \((X, Z, \lambda_i, \theta)\), using FBP. Given the multiple reconstructions required per 4D data set, a FBP algorithm was implemented using graphics processing unit acceleration in MATLAB.

**Figure 2** (a, b) shows example raw projection images \((X, Z)\) at \(\lambda = 515\) nm and 560 nm respectively and (c, d) shows the sinograms \((X, \theta)\) for a central row of pixels at each wavelength respectively. These sinograms were reconstructed by FBP to produce the cross-sectional \((X, Y)\) images at their respective wavelengths, as shown in **Figure 2** (e, f). The intensity profile for different wavelengths in each voxel was obtained and then restored as a spectrally resolved 4D spatial-spectral data set, \((X, Y, Z, \lambda)\).

After reconstruction, a variety of characteristics can be extracted, e.g. peak emission wavelength and spectral width. As an example of characterization in this publication, the peak emission wavelength was determined from the spectral profile of every voxel in the hyperspectral reconstruction. For visualization, this peak wavelength was represented on a false color scale over an appropriate range (e.g. 500-580 nm) and weighted by the integrated fluorescence intensity summed over the wavelength range (see **Figure 3**). A conventional intensity reconstruction, equivalent to a single broadband fluorescence acquisition, is provided by the integrated fluorescence intensity data.

### 2.4. Software used

All algorithms, including OPT intensity reconstruction, maxima wavelength calculation and image merging, were implemented in MATLAB. 3D rendering of the volumetric reconstruction data were produced using Imaris (see **Figure 3-5**).

### 3. Results and Discussion

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Figure 3 (a) shows the 3D rendered integrated fluorescence intensity reconstruction while (b) (also see Video S1) shows the corresponding intensity merged peak wavelength reconstruction of BP1 respectively. Assuming the beads comprise a single fluorescent species with uniform properties (i.e. size, fluorophore concentration, etc), the fluorescence intensity of each reconstructed bead in this sample should ideally be constant within measurement noise across the whole phantom. In practice, however, the measured fluorescence intensity can be affected by potential variations in illumination intensity, light collection and detection efficiency and labelling between beads. In addition, the distribution of the beads and shadowing in the acquisition can also affect the reconstructed intensity. Therefore, a clear variation in the mean bead intensity per cross-sectional slice from the reconstructed intensity profile is observed (solid line in Figure 3 (c)). As indicated by the green and orange circles in Figure 3 (a) and (b), these extremely bright beads significantly affect the mean intensity value of their respective planes flagged by the arrows in (c). These large bead-to-bead intensity variations make it difficult to determine whether these beads are the same kind. In comparison, the measured peak emission wavelength is robust against bead-to-bead variation since this is an inherently ratiometric measurement. Therefore, the mean peak emission per bead (dashed line in Figure 3 (c)) does not vary significantly. This robust measurement provides consistent information across the whole sample and thus supports the inference that this phantom consists of a single type of bead. Indeed, the data can be analyzed more extensively if required (e.g. spectral width, shape, etc.).

Figure 3 (d) and (e) (also see Video S2) show the 3D rendered integrated fluorescence intensity and the corresponding intensity merged peak wavelength reconstruction of the BP2. Although the intensity varies significantly between beads, the peak wavelength reconstruction illustrates that hyperspectral SLOT can clearly distinguish the two fluorescent labels. The mean peak emission wavelengths for the two kinds of beads were calculated as $515.3 \pm 0.9$ nm and $559.9 \pm 1.4$ nm, which are in good agreement with their fluorescence properties [29, This article is protected by copyright. All rights reserved.]
The separation and small variation of the determined peak emission wavelength is such that the F8827 (515 nm) and T8880 (560 nm) bead signals appear binary (blue and orange respectively) despite the continuous color scale used. Figure 3 (f) shows the average fluorescence spectra for the two kinds of bead in the BP2 extracted from the reconstruction. After precise determination of the spectra, each population (i.e. bead type) can be identified and segmented in the reconstruction. It is noted that having additional spectral information can significantly decrease any ambiguity when closely located beads exhibit either similar intensity or significantly different intensity (white boxes and circles respectively in (d) and (e)), which is also shown in line plots through the white boxes and circles (Figure S2).

To further illustrate the potential of hyperspectral SLOT, a dual-labelled mouse thymus gland sample and an unlabeled mouse intestine sample were prepared and imaged. Figure 4 (a) shows the 3D rendered integrated fluorescence intensity of the dual-labelled mouse thymus gland sample, obtained by summing over the wavelength range 510-540 nm (i.e. similar to a typical bandpass filter used for Alexa-488, 525/30 nm). Based on this intensity reconstruction, it is not possible to distinguish between the Alexa-488 and Cy2 labelling (i.e. epithelial and smooth muscle cells) due to the large spectral overlap of the two fluorophores. Figure 4 (b) (also see Video S3) shows the corresponding intensity merged peak emission wavelength reconstruction, clearly illustrating the labelled structures with different colors. This result demonstrates that hyperspectral SLOT can resolve significantly overlapped fluorophores and has the potential to separate and quantify many labels within a sample. Figure 5 (a) shows the 3D rendered integrated autofluorescence intensity reconstruction of the unlabeled mouse intestine sample and (b) and (c) show the corresponding intensity merged peak emission wavelength reconstruction on a continuous and discrete color scale respectively. The results show the slight spectral difference between tumor and normal tissue. Considering this slight difference together with a large spectral overlap, we believe it may be possible but would be challenging to measure using discrete multispectral fluorescence
projection tomography. This demonstrates the potential of hyperspectral SLOT to distinguish between signals from diseased and normal tissue without prior knowledge.

For fluorophores with little spectral overlap that are well separated, multiple discrete emission filters could be used in SLOT to distinguish between them, but would require several sequential acquisitions or multiple detectors. This could result in extended exposure time and therefore increased light dose or require additional detectors. Here, we demonstrate that hyperspectral SLOT provides much higher spectral resolution in a single acquisition. In multispectral SLOT, signal intensity would be severely reduced for fluorophores with a large spectral overlap, due to the necessity of ensuring a sufficient separation of the emission signals, which also limits the ability to discriminate between them. Moreover, in applications where even more fluorescent labels are required to explore the relationship between tissue structures or their components (e.g. protein expression), it becomes impossible to sufficiently distinguish multiple colors with multiple discrete filters due to the increasing spectral overlap, leading to significant spectral cross-talk. However, while we have demonstrated that hyperspectral SLOT can separate two fluorophores with a large spectral overlap, we believe it could be further used to separate and quantify many fluorescent species since it provides access to a large spectral range with high spectral resolution. This can also be applied to the quantitative analysis of more complex interactions and readouts, e.g. Förster resonance energy transfer [31].

In the demonstrations presented here, the acquisition time was up to ~17 minutes for a 60 nm spectral range, limited by the read-out rate of the CCD (equivalently ~11 kHz per “line”, the CCD ROI used as a “line” detector). Considering that the long exposure times may have phototoxic side effects in live samples, this system is more practically suitable for ex vivo experiments. The acquisition time could be reduced by using a fast (>100 kHz) line detector (e.g. linear CCD or photomultiplier tube linear array) to approach those of standard single-color SLOT. The spectral resolution (i.e. 2 nm) was determined by the 75 µm entrance
slit and 150 lines/mm grating. This resolution could be improved by changing the width of the slit (e.g. 37.5 μm for 1 nm resolution) and/or the grating used (e.g. 300 lines/mm for 1 nm resolution). However, such fine sampling over a large spectral range may not be needed for most applications. Nevertheless, the optimal spectral channels need to be determined based on the aim and practical experimental conditions for a given application and therefore it is useful to employ hyperspectral imaging to explore this in initial experiments. Once the optimal spectral windows are determined, the spectral resolution and/or binning of spectral channels can be set accordingly.

To characterize the system, the signal-to-noise (S/N) was approximated by

\[
\frac{S}{N} = \frac{N_p E}{\sqrt{N_p E^2 + \sigma_r^2}}
\]

where \(N_p\) is the number of fluorescence photons incident on the spectrometer, \(E\) is the efficiency of detection, which is the product of the diffraction grating efficiency (varies between 60 - 80% in the range 500 – 700 nm), the CCD quantum efficiency (>90% in the range 500 – 700 nm) and the CCD readout conversion efficiency (~0.99 in high gain mode), and \(\sigma_r\) is the CCD read noise (12.4 e\(^-\) at maximum readout rate mode). The CCD dark noise was considered negligible at the operating temperature of -70 °C. For a typical acquisition the measured peak wavelength was ~5-32% of the full dynamic range of the camera, resulting in a typical signal-to-noise of ~63-170. A maximum signal-to-noise of ~300 could be achieved by integrating for longer or increasing the excitation power, and therefore the fluorescence signal flux, dependent on the sample characteristics. The sensitivity of the system could also be improved by using an EMCCD camera or photomultiplier tube linear array.

4. Conclusion

In conclusion, we have demonstrated a technique for mesoscopic multidimensional fluorescence imaging that utilizes hyperspectral SLOT to realize spectrally-resolved 3D
fluorescence imaging of volumetric samples up to several mm or even a cm in scale. To the best of our knowledge this is the first demonstration of 3D hyperspectral reconstruction using optical computed tomography for such large samples. The spectral resolving capabilities were demonstrated using fluorescent bead phantoms, a multi-immuno-labelled mouse thymus gland tissue sample and an unlabeled mouse intestine sample, showing clear fluorescence spectral discrimination. We have shown that this approach provides improved resistance to intensity-based artefacts compared to integrated intensity imaging. It demonstrates the potential of hyperspectral SLOT to facilitate the mapping and quantitative analysis of functional information in 3D mesoscopic biomedical samples (e.g. whole organs, embryos, etc.). We believe that as the demand to image samples containing multiple fluorophores increases, our mesoscopic 3D fluorescence hyperspectral imaging technique will provide a convenient and broadly applicable platform for system-level identification and quantitative analysis, and will be useful for achieving a more profound understanding of organism-level biological systems.

**Supporting Information**

Additional supporting information may be found in the online version of this article at the publisher’s website.

**Figure S1** Reconstructed (a) X–Y and (b)X–Z image slices of a bead which is much smaller than the expected spatial resolution of the system. (c, d) Line plots and Gaussian fits through the Y and Z axis of the reconstructed bead in (a, b). A number of beads at different distances from the axis of rotation were analyzed and their measured average full width half maximum (FWHM) in the system was 20.6±0.9 μm. Scale bar, 30 μm.

**Figure S2** Line plots through (a) white circles in Fig. 3 in which a dim T8880 bead close to a bright F8827 bead that are impossible to identify in the intensity-only reconstruction and (b) white boxes in which two beads exhibit similar intensity but different emission spectra.

**Video S1** 3D intensity merged peak emission wavelength rendering of bead phantom BP1, consisting of a low concentration suspension of fluorescent beads (F8827).

**Video S2** 3D intensity merged peak emission wavelength rendering of bead phantom BP2, consisting of a low concentration suspension of two kinds of fluorescent bead (F8827 and T8880).

**Video S3** 3D intensity merged peak emission wavelength rendering of the epithelial and smooth muscle cells of a thymus gland of a wild-type (BALB/c, 12-week-old, female) mouse
immunostained as follows: rat anti-mouse CD24 directly conjugated to Cy2 used to label epithelial cells and mouse anti-smooth muscle actin antibody and Alexa-488 secondary antibodies used to label smooth muscle cells.

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References


Figure 1. Schematic of hyperspectral SLOT setup. The sample is illuminated with a laser point that is quickly scanned by 2-axis scanning mirror system. The fluorescence signal is directed into a spectrograph where a diffractive unit (e.g. grating) is used to separate the spectral components spatially. After \((X, Z, \lambda)\) data was obtained, the rotation stage switches to the next angular position, resulting in a 4D raw data cube \((X, Z, \lambda, \theta)\). A conventional intensity (i.e. single colour) acquisition is achieved by summing over all or selected wavelength bands.
Figure 2. (a,b) Intensity projection images of BP1 acquired at 0° rotation angle at 515 and 560 nm; (c,d) corresponding raw sinograms for the central plane of pixels at 515 and 560 nm; (e,f) corresponding reconstructed cross-sectional (X, Y) images of central plane of pixels at 515 and 560 nm. It is noted that the images acquired of opposing views of the sample are actually focused on different regions and therefore, they do not contain the exact same information.
Figure 3. 3D (a) intensity and (b) intensity merged peak emission wavelength (see Supporting Information Video S1) rendering of bead phantom BP1. (c) The variation of the reconstructed peak fluorescence wavelength (dashed line) and intensity (solid line) with respect to their global mean values plotted as a function of vertical position (i.e. Z). The green and yellow circles in (a) and (b) indicate extremely bright beads which significantly affect the mean intensity value of the respective planes as shown in (c). 3D (d) intensity and (e) intensity merged with peak emission wavelength (also see Supporting Information Video S2) rendering of bead phantom BP2. White boxes show that some beads exhibit similar intensity but different emission spectra. White circles show a dim T8880 bead close to a bright F8827 bead that are impossible to identify in the intensity-only reconstruction. (f) The average reconstructed fluorescence spectra of the two kinds of beads: F8827 (Green, solid) and T8880 (Orange, dashed). Scale bar, 1 mm.
Figure 4. 3D (a) intensity and (b) intensity merged peak emission wavelength (also see Supporting Information Video S3) rendering of the epithelial and smooth muscle cells of a thymus gland of a wild-type (BALB/c, 12-week-old, female) mouse immunostained as follows: rat anti-mouse CD24 directly conjugated to Cy2 used to label epithelial cells and mouse anti-smooth muscle actin antibody and Alexa-488 secondary antibodies used to label smooth muscle cells. It should be noted that the spectra of Cy2 (excitation/emission maxima at 489/505 nm) and Alexa-488 (excitation/emission maxima at 495/519 nm) are quite similar. Scale bar, 1 mm.
Figure 5. (a) 3D intensity rendering of the autofluorescence of an unlabeled mouse intestine sample. (b) Intensity merged peak emission wavelength using a continuous color scale and (c) a discrete color scale. The tumor tissue shows a slightly longer peak wavelength compared to the normal regions displaying a shorter peak wavelength. Scale bar, 1 mm.
Graphical Abstract

Hyperspectral scanning laser optical tomography is developed to provide spectrally-resolved volume data sets with high spectral resolution for large mesoscopic samples. It can be used to distinguish between signals from autofluorescence of diseased and normal tissue without prior knowledge.

ToC figure