Gabor domain optical coherence microscopy combined with laser scanning confocal fluorescence microscopy

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Abstract: We report on the development of fluorescence Gabor domain optical coherence microscopy (Fluo GD-OCM), a combination of GD-OCM with laser scanning confocal fluorescence microscopy (LSCFM) for synchronous micro-structural and fluorescence imaging. The dynamic focusing capability of GD-OCM provided the adaptive illumination environment for both modalities without any mechanical movement. Using Fluo GD-OCM, we imaged \textit{ex vivo} DsRed-expressing cells in the brain of a transgenic mouse, as well as Cy3-labeled ganglion cells and Cy3-labeled astrocytes from a mouse retina. The self-registration of images taken by the two different imaging modalities showed the potential for a correlative study of subjects and double identification of the target.

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1. Introduction

Since its introduction in 1991 as a noninvasive biomedical imaging modality based on low-coherence interferometry [1], optical coherence tomography (OCT) underwent numerous technical innovations aimed at improving the imaging performance in terms of sensitivity, speed, resolution and field of view. Fourier domain OCT (FD-OCT), which eliminates the need for scanning the reference mirror in time domain OCT (TD-OCT) was proposed by Fercher et al. [2]. FD-OCT is typically preferred to TD-OCT for its fast speed, high signal-to-noise ratio (SNR), and simplicity [3–5]. Implementations of FD-OCT include spectral domain OCT (SD-OCT) [2], which employs a broadband source and a spectrometer, and swept source OCT (SS-OCT) [6,7], which employs a frequency-swept laser and a photodetector. OCT systems utilize the light backscattered from a sample to visualize its three-dimensional morphology but lack the capability of intrinsically discriminating between different tissues and organelles, which is a characteristic of fluorescence imaging, where the fluorescence mainly derives from a selectively stained target.

Software and hardware approaches have been proposed to extract spectral and fluorescence properties of the sample from OCT data. The frequency-dependent spectral response, instead of reflectivity, can be obtained by post-processing through wavelet transformation [8–10]. Hardware approaches to directly combine fluorescence imaging and OCT have been proposed. Fluorescence spectra of tumors and healthy tissues from uterine cervixes and vulvas were compared with OCT structural visualization [11], and this attempt first cast light upon the complementary

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relationship between the two imaging techniques: their combination has the potential to decrease the false-positivity of fluorescence detection and improve the specificity of OCT-based diagnosis. Simultaneous but parallel operation of OCT and fluorescence imaging was achieved by attaching a fluoroscope to an OCT endoscope [12–15]. In a study of early bladder cancer in rats, a multi-modal endoscopic OCT system improved the sensitivity and the specificity of 5-ALA fluorescence detection by 21% and 28%, respectively [16]. In a study of the mouse adenomatous colon, the dual imaging technique demonstrated transverse correlation between the colon structures and the autofluorescence-emitting area [17]. Approaches to merge the OCT and fluorescence optical systems using either a dual-cladding fiber in fiber-based interferometry [18–22] or a dichroic mirror in free-space interferometry [23,24] were proposed. These methods enabled synchronous - not parallel - dual imaging. A high-definition synchronous dual imaging system was demonstrated in the form of combining either TD-OCT [25], SD-OCT [26], or SS-OCT [27] with two-photon fluorescence microscopy. A different implementation combined full field OCT (FF-OCT) and structured illumination fluorescence microscopy in single-source [28] or dual-source [29] illumination. SD-OCT was combined with confocal fluorescence [30–32] or phosphorescence [33] microscopy in dual-source illumination, or with confocal fluorescence microscopy using a single supercontinuum light source [34,35]. Dual-source illumination has two benefits: firstly, various fluorophores, i.e., in the visible (VIS) spectrum, can be selected for fluorescence imaging, while the near-infrared (NIR) spectrum remains available for conventional OCT imaging; secondly, the optical signal of each imaging modality can be detected without any interference.

In this paper, we report on the first combination of Gabor domain OCM (GD-OCM) [36] and laser scanning confocal fluorescence microscopy (LSCFM) with dual-source illumination ($\lambda_{\text{GD-OCM}}=790-890 \text{ nm}$ and $\lambda_{\text{LSCFM}}=556 \pm 1 \text{ nm}$), which we named Fluo GD-OCM. The GD-OCM microscope served as the illumination path for both imaging modalities. The GD-OCM microscope provided its unique dynamic refocusing capability, leveraging an embedded liquid lens to vary the effective focal length with no moving parts [37,38]. The combination of GD-OCM and LSCFM can enhance the individual capabilities of each instrument used on its own, in particular, by adding fluorescence labeling to the structural imaging of GD-OCM.

2. Methods

2.1. Merging LSCFM into GD-OCM

The underlying architecture of GD-OCM is a fiber-based SD-OCT system, shown in Fig. 1. The light source is a superluminescent diode with the central wavelength of 840 nm and 100-nm-bandwidth. The dispersion mismatch between the two interference arms is corrected using a custom single-pass frequency domain optical delay line [39–41] located in the reference arm. The beam at the sample arm is collimated and steered by an XY-galvanometer scanner and is focused by the dynamic focusing probe with numerical aperture of 0.2. The GD-OCM spectral interference signal is recorded by a custom astigmatism-corrected Czerny-Turner spectrometer [42]. This spectrometer is equipped with a 4096-pixel line-scan camera (spL4096-140km, Basler) with a line period of 70 kHz. The spectral resolution is 0.041 nm, and the corresponding maximum imaging depth is 4.29 mm in the air (1 pixel = 2.09 $\mu$m depth in the air). The scanning period of the galvanometer mirror is synchronized to the line-period for the spectrometer data acquisition by using the same trigger signal from a custom electronics board.

A modular form of LSCFM, also shown in Fig. 1, was designed and combined with the GD-OCM system. The light source of the LSCFM was chosen for exciting red fluorescent proteins with a modeling fluorophore of tdTomato ($\lambda_{\text{EX}}=554 \text{ nm}$ and $\lambda_{\text{EM}}=581 \text{ nm}$) [43]. The wavelength of the excitation source was 556 $\pm$ 1 nm (MGL-FN-556, UltraLasers). After collimation, the excitation beam was deflected by 45° at the dichroic mirror #1 ($\lambda_{\text{edge}}=560 \text{ nm}$, FF560-FDi01, Semrock) then combined with the optical path at the sample arm of the GD-OCM.
in free-space through the dichroic mirror #2 ($\lambda_{\text{edge}}=735$ nm, FF735-Di02, Semrock). We refer to this configuration in which a dichroic mirror is introduced in the sample and reference arms as modified GD-OCM. The optical axis of the excitation beam was micro-adjusted to be precisely overlaid with that of the GD-OCM beam. The GD-OCM microscope objective was positioned such that its front focal plane could be located between the two scanning mirrors for equalizing the optical path lengths of all the scanned beams. The emission filter consisted of two band-pass filters–a long-pass filter (LP01-561RE, Semrock) and a short-pass filter (FF01-715/SP, Semrock). Figure 2 shows the efficiencies of the dichroic mirrors and emission filter. The spectra of excitation of the LSCFM, the fluorescence of tdTomato, and the source of the GD-OCM are overlaid in the figure to show the filter design. The dichroic mirrors #1 and #2 were chosen to accept as much fluorescence as possible from the sample. Theoretically, $\sim 88\%$ of tdTomato-fluorescence will be detected as the output, $\sim 2.6 \times 10^{-6}\%$ of the excitation beam will creep into the detection of the fluorescence, and $\sim 3\%$ of the backscattered light from the sample will be lost in the GD-OCM imaging due to the dichroic mirror #2. The fluorescence emission was focused with an aspheric achromat (#49-657, Edmund Optics) then passed through the two-Airy-disk pinhole (P30S, Thorlabs) for improving the sensitivity of fluorescence detection. The location of the cathode of the current-typed photomultiplier tube (PMT; H7422-20, Hamamatsu) was designed to be underfilled with the fluorescence by 72%. The resulting current was trans-impedance amplified (HCA-1M-1M, FEMTO) to the voltage with a gain of $1 \times 10^6$ V/A. The voltage was then digitized with a 12-bit analog-to-digital converter (ATS9350, AlazarTech).

2.2. Data acquisition and image processing

The three-dimensional (3D) GD-OCM images were acquired with GD-OCM 4D™ software (LighTopTech) in PC #1, and the two-dimensional (2D) LSCFM images were generated with a custom LabView algorithm in PC #2, as illustrated in Fig. 1.
The need for synchronization of the data acquisition between the two imaging modalities causes the maximum imaging speed of Fluo GD-OCM to be determined by that of the slower system, which was typically GD-OCM. Limited by the line period of the GD-OCM spectrometer, a sampling rate of 2 Msamples/sec was chosen to meet the Nyquist condition when the bandwidth of the amplifier was 1 MHz. The number of records (i.e., the number of vertical pixels assigned to a 2D LSCFM image) was 1000, to match the number of vertical pixels in a 2D GD-OCM en face image. The mega-sampling rate of the LSCFM allocated more pixels in the horizontal dimension (i.e., along the fast-scanning axis) than the vertical dimension (i.e., along the slow-scanning axis). The horizontal dimension of the raw 2D LSCFM image was therefore rescaled using a custom MATLAB algorithm to match the vertical dimension; thereby creating a 2D LSCFM image of $1000 \times 1000$ pixels, which corresponds to the dimension of a 2D GD-OCM en face image.

2.3. Sample preparation

Three mouse specimens were prepared to test the performance of the Fluo GD-OCM system: brains from NG2-DsRed-expressing mice, Cy3-labeled RGCs, and Cy3-labeled retinal astrocytes. All experiments were approved by the University Committee on Animal Resources (UCAR). An adult female NG2-DsRed mouse (Stock No. 008241, Jackson Laboratory) was anesthetized with ketamine-xylazine (ketamine: 100 mg/kg; xylazine: 10 mg/kg; intraperitoneal injection). Once the mouse had a negative pedal reflex, the mouse was transcardially perfusion-fixed with ice-cold phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). The brain was

![Fig. 2. Efficiencies of the dichroic mirrors and emission filter used in Fluo GD-OCM. (a) The dichroic mirror #1 for combining/splitting the optical paths of the tdTomato-fluorescence (red) and excitation (green), (b) the emission filter for blocking the excitation beam and passing the fluorescence, and (c) the dichroic mirror #2 for combining/splitting the optical paths of the GD-OCM and LSCFM. The light source of the GD-OCM (blue) was D-840-HP-I, Superlum.](image-url)
extracted and allowed to post-fix in 4% PFA overnight at 4°C. The following day, the brain was sectioned into 100-µm-coronal sections using a vibratome (VT1200S, Leica), and slices were kept in PBS and mounted on microscope slides (Superfrost Plus, Fisherbrand).

All retinal tissues were obtained from 2-5-month-old-adult C57BL/6J mice, which were fed chow and water ad libitum and housed on a 12-hour light to dark cycle. Retinal experiments were conducted in adherence with the Association for Research in Vision and Ophthalmology’s statement on the use of animals in ophthalmic and vision research and were approved by UCAR. Eyes were harvested and fixed in 4% PFA in 1 × PBS for 90 minutes. The retinas were dissected free from the optic cup and blocked in 10% horse serum and 0.4% TritonX in 1 × PBS overnight at 4°C. For staining RGCs, the retina was then incubated at 4°C for 3 days in the primary antibody of 1:250 rabbit anti-RBPMS (GTX118619, GeneTex) diluted in 10% horse serum and 0.4% TritonX in 1 × PBS. The retina was then washed and incubated for 24 hours at 4°C in donkey anti-rabbit 555 (Cy3) secondary antibodies (A31572, Invitrogen) diluted at 1:1000 in 1 × PBS. For staining retinal astrocytes, the retina was incubated at 4°C for 3 days in the primary antibody of 1:1000 mouse anti-GFAP (MAB360, Millipore) diluted in 10% horse serum and 0.4% TritonX in 1 × PBS. The retina was washed and incubated for 24 hours at 4°C in donkey anti-mouse 555 (Cy3) secondary antibodies (A32727, Invitrogen) diluted at 1:1000 in 1 × PBS. Both retinas were washed, mounted on a 1-mm-thick glass slide ganglion using Fluoro-Gel with Tris buffer (Electron Microscopy Sciences), and covered with a 160-190-µm-coverslip for preserving the moisture and, more importantly, fixing the curvature of the tissues. The ganglion cell layer faced up.

2.4. Protocol of imaging

When imaging samples on a glass slide with a coverslip, significant portions of the illumination beam were reflected at the glass interfaces, and the reflected beams interfered with the backscattered light from the tissue in GD-OCM imaging. Consequently, multiple duplicate images of the tissue appeared in every GD-OCM B-scan. These specular reflections from glass interfaces can be suppressed by using oblique illumination and detecting the signal mostly deriving from tissue scattering on axis, even though this technique was not implemented in this particular work [44–49]. Instead, we used the dynamic focusing capability of the GD-OCM probe to identify the focal plane location in which the scattering from the tissue can be maximized. Optimal focal length and working distance settings for GD-OCM imaging were investigated, and are reported in Appendix A.

In all cases, the tissue in contact with the glass was imaged with GD-OCM in a common-path configuration. We refer to this configuration as common-path modified GD-OCM. The reference arm of GD-OCM, as shown in Fig. 1, was disconnected. The working distance was chosen to avoid overlap of duplicate images in the B-scan. In experiments done without a coverslip (i.e., for the 100-µm-thick brain slice from NG2-DsRed mouse), the beam was focused at the top surface of the tissue, while the specular reflection from the top surface of the glass slide acted as the reference, as shown in Fig. 3(a). In experiments done with a coverslip (i.e., the <100-µm-thick Cy3-labelled mouse retinas), the beam was focused on the top surface of the tissue, while the specular reflection at the same surface, interfacing with the bottom surface of the coverslip, was the reference, as shown in Fig. 3(b).
Fig. 3. A schematic of the imaging protocol: (a) when a 100-µm-thick brain slice is mounted on a glass slide and (b) when a <100-µm-thick retina is mounted on a glass slide and covered by a cover-slip.

3. Results

3.1. Transverse imaging performance

The transverse imaging resolutions of the common-path modified GD-OCM and LSCFM modules were assessed imaging a USAF resolution target (R1L1S1P, Thorlabs), as shown in Fig. 4(a). The minimum resolvable separation between the two mirror-bars edge-to-edge in common-path modified GD-OCM was 2.2 µm (=228 line pairs; lp/mm). For assessing the LSCFM resolution, the image shown in Fig. 4(b) was taken after removing the emission filter and using the excitation beam (λ<sub>EX</sub>=556 ± 1 nm) to reflect off the resolution target. The minimum separation was 3.5 µm (=144 lp/mm). Despite having a shorter wavelength than GD-OCM, the resolution of the LSCFM was worse. Spherical aberration and coma caused a reduction in resolution since the GD-OCM probe was originally designed for NIR (λ<sub>design</sub>=800 ± 60 nm [36]). In this first integration of fluorescence imaging with GD-OCM, it is natural to plan custom optics at a later stage of the research, which will help resolve these limitations.

Fig. 4. Transverse imaging resolutions of the Fluo GD-OCM system. The USAF resolution target was imaged with (a) common-path modified GD-OCM and (b) LSCFM. The smallest resolvable group is marked in a red box. (c) The overlay of (a) and (b) demonstrate synchronization and transverse registration.
3.2. Longitudinal imaging performance

The axial resolution of the modified GD-OCM system was estimated as the full-width at half-maximum (FWHM) of the axial point spread function (PSF). In the case of LSCFM, since the optical sectioning capability derived from confocal gating, the width was estimated by the FWHM of the through-focus intensity profile. Additionally, the effective focal lengths for GD-OCM ($\lambda_{\text{GD-OCM}} = 790-890$ nm) and LSCFM ($\lambda_{\text{LSCFM}} = 556 \pm 1$ nm) were measured to assess the effect of the axial color between the GD-OCM focus and the LSCFM focus, which results in a difference in the viewing depths for the two imaging systems.

Thouvenin et al. [29] reported a reduction in the interference visibility in FF-OCM when a dichroic mirror was added to the sample arm. To assess the impact of the dichroic mirror #2 (Fig. 1) on the imaging performance, the FWHM of the axial PSF was recorded for varying optical delays for common-path modified GD-OCM and modified GD-OCM (i.e., with the reference arm). For common-path modified GD-OCM, the specular reflection at the GD-OCM probe window acted as the reference, and the optical delay was adjusted by axially translating a mirror acting as the sample. The beam was focused on the probe window. On the other hand, for modified GD-OCM with the separate reference arm, the optical group delay was added with the dispersion compensator. Figure 5(a) shows the FWHMs of the axial PSFs vs the optical delay for the two cases, in comparison with regular GD-OCM (without the dichroic mirror at the sample arm) [36], which has experimental depth of focus (DOF) at any focus position of $\sim 80 \mu$m [38]. For the common-path modified GD-OCM, $<5$-µm-FWHM axial PSFs were maintained up to a depth of $\sim 100$-µm. The broadening of the PSF was primarily caused by the translation of the mirror over the limit of the DOF, and not by the dichroic mirror, since reference and sample beams both traverse the dichroic mirror in the common path configuration. For the modified GD-OCM with the reference arm, $<5$-µm-FWHM axial PSFs were achieved up to a depth of $\sim 400$ µm. This result was obtained after compensating the mismatch in group delay dispersion of the two interfering beams with the dispersion compensator, which included the same dichroic mirror (#2). We speculate that the dichroic mirror caused a ripple-like high order group delay dispersion in the sample arm that was not fully compensated by the same dichroic mirror placed in the reference arm due to manufacturing variabilities and differences in the beam’s angle of incidence.

![Fig. 5. Longitudinal imaging resolution of Fluo GD-OCM. (a) The FWHMs of the axial PSFs over depth for the modified GD-OCM in common-path configuration (solid blue curve), the modified GD-OCM with the reference arm (solid black curve), and the original GD-OCM (solid red curve). The error bar is the standard deviation of the FWHM over ten measurements. (b) The optical sectioning capability of LSCFM was characterized by the FWHM of the through-focus intensity profile when a mirror was axially translated around the focus of the excitation beam ($\lambda_{\text{EX}} = 555-557$ nm).](image-url)
The through-focus intensity of $\lambda_{\text{EX}} (=556 \pm 1 \text{ nm})$ was measured by translating a mirror near the focus of the excitation beam ($\lambda_{\text{EX}}=556 \pm 1 \text{ nm}$). The intensity was averaged over ten measurements, and the result is shown in Fig. 5(b). The FWHM of the intensity profile was $\sim 70 \mu\text{m}$.

Due to the wavelength dependence of the refractive optics, the GD-OCM probe performs differently at different operating wavelengths. For each working wavelength of GD-OCM and LSCFM, the effective optical power was characterized by the working distance between the GD-OCM probe window to the focal plane in the air. The focal plane location was defined to show the maximum intensity when a mirror was axially translated around each focus. Figure 6 shows the result for varying electric voltages to refocus the liquid lens. Since the liquid lens displays hysteresis [50], the voltage was applied in a decreasing manner for each measurement from 54V to 40V with a step size of 1V, and the measurement was repeated ten times. The error bar represents the standard deviation of the working distance. The depth difference between the focal plane locations of GD-OCM and LSCFM ranged from 29 $\mu\text{m}$ to 71 $\mu\text{m}$, and the working distance of LSCFM was always shorter than that of GD-OCM due to the shorter working wavelength of LSCFM.

The LSCFM and GD-OCM en face images of the tissue are shown in Fig. 7(a) and 7(b-c), respectively. The two GD-OCM en face sections were taken at different depths, 30 $\mu\text{m}$ apart. Both GD-OCM images showed hyper-reflective strands that corresponded to white matter - myelinated axon tracts [49,53–56] projected from the thalamus to the cortex - surrounded by neighboring thalamic grey matter, and hypo-reflective spots that connected and spanned the entire tissue volume. The spots may correspond to air/fluid inside blood vessels that could be

3.3. Imaging DsRed-expressing cells in the mouse brain

The mouse brain slice containing NG2-DsRed-positive cells was imaged without a cover-slip, as shown in Fig. 10(a) in Appendix A. Both GD-OCM and LSCFM images were taken at the same region of the posterior thalamus that expressed the red fluorescent protein, DsRed, after the NG2 promoter. The NG2 promoter is expressed in several cell types in the brain but mostly in pericytes and smooth muscle cells [51,52].

The LSCFM and GD-OCM en face images of the tissue are shown in Fig. 7(a) and 7(b-c), respectively. The two GD-OCM en face images were taken at different depths, 30 $\mu\text{m}$ apart. Both GD-OCM images showed hyper-reflective strands that corresponded to white matter - myelinated axon tracts [49,53–56] projected from the thalamus to the cortex - surrounded by neighboring thalamic grey matter, and hypo-reflective spots that connected and spanned the entire tissue volume. The spots may correspond to air/fluid inside blood vessels that could be
found throughout the entire brain. Although it was not clear if the hypo-reflective spots were arteries, arterioles, capillaries, venules, or veins, imaging the same area with the LSCFM system, as shown in Fig. 7(a), showed clear co-localization between the hypo-reflective trajectories and the NG2-positive smooth muscle cells that are commonly seen sheathed around arterioles (yellow arrows in Fig. 7(a)) [51]. Cell bodies of pericytes with fine processes, radiating along the vessel, could also be seen and could be used to identify pre-capillary arterioles (green arrow in Fig. 7(a)) [57]. Smaller pericycle somas, seen as equidistant red puncta, were normally found along capillaries (blue arrow in Fig. 7(a)). Thus, combining these cellular markers from the LSCFM complemented the GD-OCM structural imaging by enabling the identification of specific vascular segments in the rodent brain.

**Fig. 7.** Fluo GD-OCM images of a transgenic NG2-DsRed mouse brain slice. (a) An LSCFM image of the tissue showed several NG2-positive cells, confirmed by the DsRed fluorescence emission. NG2 was expressed by pericytes and vascular smooth muscle cells in the brain, and their cellular morphology allowed to differentiate segments of the vascular network: arterioles (yellow arrows), pre-capillary arterioles (green arrow), and capillaries (blue arrow) [56]. (b-c) Two GD-OCM *en face* images, taken at different depths separated by 30 μm, showing hyper-reflective strands of white matter surrounded by hypo-reflective gray matter. The hyper-reflective white matter corresponded to myelinated axon tracts that projected from the thalamus to cortex. (d-e) Overlays of the GD-OCM images in (b) and (c) with the LSCFM image in (a) showed that hypo-reflective spots corresponded to blood vessels that were drained during the perfusion-fixation process. The artifacts (e.g., the dark spots contained within the white oval dotted line in Fig. 7(b) and 7(c)) were caused by the difference in reflectivity of the reference beam deriving from non-uniform contact between the sample and the glass slide.

### 3.4. Imaging mouse Cy3-labelled RGCs

The retina was stained with anti-RBPMS antibodies for labeling the somas of RGCs that are the output neurons of the retina [58,59]. RGCs have axons, not labeled by the anti-RBPMS
antibodies, which coalesce at the back of the optic disc and exit to form the optic nerve. A coverslip covered the sample, as shown in Fig. 10(b) in Appendix A.

The LSCFM and GD-OCM images of the tissue were taken near the retinal optic disc, and are shown in Fig. 8(a) and 8(b-c), respectively. The two GD-OCM en face sections were taken at different depths separated by 78 µm. As shown in Fig. 8(a), no RGC somas were present at the optic disc (green arrow in Fig. 8(a)), and the optic disc was commonly seen at the two GD-OCM images (green arrow in Fig. 8(b-c)). The retinal vasculature (yellow arrows in Fig. 8(a)) radiated from the optic disc, and the RGC somas were not visible where the blood vessels ran because the layer of the retinal vasculature lay above the RGC layer. The GD-OCM image in Fig. 8(b) showed hyper-reflective granular features that mostly corresponded to the rods [60,61] and seemed less developed in the optic disc (area A) than the surrounding area (B). The small size (diameter: 1.4 µm [62]) of mouse rods was not clearly resolved with the 2.2 µm transverse imaging resolution of the GD-OCM. The hyper-reflective string-like structures shown as yellow arrows in Fig. 8(c) corresponded to retinal vasculature [32,34] and their locations and alignments were correlated with those seen in the LSCFM image. The labeled RGCs were only visible in the LSCFM image.

3.5. Imaging mouse Cy3-labelled retinal astrocytes

The retina was stained with anti-GFAP antibodies for labeling retinal astrocytes in the RGC layer. Astrocytes are the resident “star-shaped” support cells of the central nervous system. Among their many supportive roles, the astrocyte end-feet wrap around the retinal vasculature and function to regulate a blood flow and maintain blood-retinal-barrier [63]. Thus, the retinal vessels are apparent by locating the abundant astrocyte end-feet. A coverslip covered the sample, as shown in Fig. 10(b) in Appendix A.

The LSCFM and GD-OCM images of the tissue were taken near the retinal optic disc and are shown in Fig. 9(a) and 9(b-c), respectively. The two GD-OCM en face sections were taken at different depths, 84 µm apart. The retinal astrocytes [64,65] were only visible in the LSCFM image, as shown in Fig. 9(a), while the rods were only seen in the GD-OCM image, as shown in Fig. 9(b). All LSCFM and GD-OCM images commonly showed the optic disc (green arrow),
and the alignment between the retinal vasculature found in the LSCFM image (yellow arrows in Fig. 9(a)) and the hyper-reflective strand seen in the GD-OCM image (yellow arrows in Fig. 9(c)).

Fig. 9. Fluo GD-OCM images of the mouse GFAP-Cy3 labeled retina. (a) An LSCFM image of the tissue showing several star-shaped GFAP-positive astrocytes whose end-feet wrap around the vasculature, confirmed by the Cy3-fluorescence emission. (b-c) Two GD-OCM en face images taken at different depths, separated by 84 µm. All images displayed co-localization of the optic disc (green arrow), and alignment between the hyper-reflective strands, shown as yellow arrows in (c), and the retinal vasculature in (a). The granular features shown in (b) mostly corresponded to the rods.

4. Discussion

Fluo GD-OCM demonstrated the capability to simultaneously image structural features with GD-OCM and fluorescence labels with LSCFM. The registration of GD-OCM and LSCFM images was demonstrated in the transverse plane. The dynamic refocusing capability of the GD-OCM probe was leveraged when acquiring either a GD-OCM or an LSCFM image. For example, the working distance for the GD-OCM image was chosen to acquire an image with good contrast while minimizing specular reflections at the glass interfaces (i.e., the coverslip and the glass slide). For LSCFM, the working distance was chosen in such a way that the corresponding image showed the best contrast for the feature. Typically, two different liquid lens voltages were required to meet the objectives due to the axial color, as reported in Fig. 6. An attempt to correlate the depths viewed in each imaging modality was not successful, despite the freedom of independently accessing each module’s focal plane. This may be attributed to the mismatch between the optical sectioning performances of GD-OCM and LSCFM (Fig. 5(a) and 5(b)). In the future, a new GD-OCM custom probe designed for both working wavelengths, i.e., VIS and NIR, will be able to achieve equivalent imaging performances (transverse imaging resolutions and optical sectioning) between the two modules. Thereby, 3D co-registration will be achieved over the entire imaging depth range of GD-OCM. The axial color that resulted from using the GD-OCM probe beyond its designed spectrum (i.e., 800 ± 60 nm) to include the visible fluorescence (i.e., 554/581 nm) is best corrected redesigning custom optics across the full spectrum. A potential temporary solution could be to explore decollimating the excitation beam; however, the impact on the induced aberrations and imaging quality would need to be carefully considered.

In this first demonstration, a common-path configuration for the GD-OCM acquisition was used to avoid the effects of multiple reflections from glass interfaces. This resulted in artifacts (e.g., the dark spots contained within the white oval dotted line in Fig. 7(b) and 7(c)) due to the difference in reflectivity of the reference beam. The latter derived from non-uniform contact
between the sample and the glass slide. Furthermore, the common-path configuration results in dimmer images due to a lower intensity of the reference beam. Finally, the maximum imaging depth of GD-OCM was also limited by the extent of the depth of focus in the common-path configuration. In the future, strategies to perform GD-OCM imaging in the traditional Michelson configuration with an external reference arm will be investigated using a non-contact probe. To avoid astigmatism introduced by the two XY-galvanometer mirrors, a two-axis-scanner, i.e., a micro-electro-mechanical-system (MEMS) mirror should be implemented [66].

5. Conclusion

In this study, we presented a technical fusion between GD-OCM and LSCFM, which we named Fluo GD-OCM, and demonstrated ex vivo imaging of three rodent tissues: NG2-DsRed-expressing cerebral pericytes and smooth muscle cells, Cy3-labeled RGCs, and Cy3-labeled retinal astrocytes. Fluo GD-OCM achieved self-registration of images taken by GD-OCM and LSCFM over a 1.0 × 1.0 mm² field of view. The GD-OCM module provided morphological visualization of the tissue microscopic structures based on detection of backscattered light, i.e., the myelinated axon tracts and vasculature in the brain sample, and the rods and the vasculature in the retinal samples. On the other hand, the LSCFM module could visualize the stained targets that were not visible by GD-OCM like pericytes and the type of vessels in the brain samples, and RGCs and astrocytes in the retinal samples. The features that were commonly seen by the two modules, such as blood vessels and the optic disc, were correlated in their locations. Thanks to the dynamic refocusing of GD-OCM, both structural and functional images with good contrast could be acquired under dual-source illumination, especially when glass plates surrounded the tissues. We expect this technical merger will have the potential for a correlative study for features that can only be visible by each modality individually, and for intensifying the identification of targets that are jointly visualized by the two modalities.

Appendix. A

In order to define an optimal setup for imaging a glass-covered sample with GD-OCM and to aid selection of the best image out of duplicates, optimal illumination conditions with and without a coverslip were investigated by varying the focal plane locations. Figure 10(a) and 10(d) illustrate two imaging configurations that are described by the following parameters: the thicknesses of the GD-OCM probe window $t_W$, sample $t_S$, glass slide $t_G$, and air gap $\phi_1$ (or $\phi_2$ with a coverslip); the working distance $D_G$; and the longitudinal location of the $i$-th glass interface, where $i$ is 1 (or 2) for interior (or exterior) surface of the window, 3 (or 4) for top (or bottom) surface of the glass slide, t (or b) for top (or bottom) surface of the coverslip, respectively. The specular reflectance associated with the interfaces is denoted as $R_i$, while $R_S$ denotes the scattering intensity from the sample.

In common-path interferometry, imaging a glass-covered sample with OCT creates as many duplicate images as the number of glass interfaces. Figure 10(b) and 10(e) show theoretical GD-OCM B-scans corresponding to configurations in Fig. 10(a) and 10(d), respectively, which contain images of the glass interfaces (red line) and the sample (thickened white line). $I_{Si}$ denotes an OCT cross-sectional image of the sample, created by the interference between the backscattered light from the sample and the specular reflection as a reference at the interface $i$. All images of the glass interfaces and the sample are vertically aligned based on the optical path length difference of the pair-to-pair interferences. For example, in Fig. 10(b), $I_{S1}$ is an image based on the interference between the two beams with each reflectance of $R_1$ and scattering intensity $R_S$. Varying the focal plane locations ($D_G$), two 100-µm- and 25-µm-thick mouse brain slices were imaged in each setup, as shown in Fig. 10(a) and Fig. 10(d), respectively, where $t_W=1$ mm, $t_G=1$ mm, and $t_C=160-190$ µm.
Fig. 10. Optimization of the optical focus in GD-OCM for imaging either covered- or uncovered-sample fixed on a glass slide with two imaging configurations: (a) without or (d) with a coverslip. The corresponding theoretical GD-OCM B-scans are illustrated in (b) and (e), respectively, which contain images of the glass interfaces (red line) and the sample (thickened white line). Varying the focal plane locations, two 100-µm- and 25-µm-thick mouse brain slices were imaged in the setup of (a) and (d), respectively, where $t_W=1$ mm, $t_G=1$ mm, and $t_c=160-190$ µm. For the setups as shown in (a) and (d), the corresponding experimental A- and B-scans are shown in (c) and (f), respectively, within each region of interest (ROI).

Figure 10(c) shows the experimental GD-OCM B-scans of the uncovered 100-µm-thick sample, corresponding to the two $D_G$s, with $\phi_1\approx100$ µm. Without the coverslip, $I_{S2}$ became the most visible when the exterior surface of the probe window was focused by the light ($D_G=0$). On the other hand, when the beam was focused on the tissue ($D_G\approx\phi_1$), $I_{S3}$ was the brightest. Given the backscattered light from the sample whose intensity was weaker than the specular reflection from the glass interface, the glass interface where the beam was focused decided which image became the most noticeable out of the duplicates. In order to use the sensitivity gain at a short optical path length difference [67,68] and increase the backscattering from the tissue, $D_G$ was set equal to $\phi_1$ (at the top of the sample), then $I_{S3}$ was selected as the optimal image.

Figure 10(f) shows the B-scans of the covered 25-µm-thick sample, corresponding to the two $D_G$s with $\phi_2\approx190$ µm. For the same reason above, $D_G$ was set equal to $\phi_2+t_c$ (at the top of the sample) when the sample was covered with the coverslip. $I_{Sb}$ was then selected as the optimal image. By principle of OCT, when a covered-sample fixed on a glass slide is illuminated, two images of $I_{gb}$ (associated with the specular reflection from the bottom surface of the coverslip) and $I_{S3}$ (associated with the specular reflection from the top surface of the glass slide) can be located in the B-scan coordinate with an opposite order in the optical path length, as shown in Fig. 10(e). Typically, the image $I_{Sb}$ was not seen in the experiment regardless of the choice of
D_G, perhaps, due to the lower specular reflection from the top of the glass slide than that from the bottom surface of the coverslip.

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