Volumetric fluorescence retinal imaging in vivo over a 30-degree field of view by oblique scanning laser ophthalmoscopy (oSLO)

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Abstract: While fluorescent contrast is widely used in ophthalmology, three-dimensional (3D) fluorescence retinal imaging over a large field of view (FOV) has been challenging. In this paper, we describe a novel oblique scanning laser ophthalmoscopy (oSLO) technique that provides 3D volumetric fluorescence retinal imaging with only one raster scan. The technique utilizes scanned oblique illumination and angled detection to obtain fluorescent cross-sectional images, analogous to optical coherence tomography (OCT) line scans (or B-scans). By breaking the coaxial optical alignment used in conventional retinal imaging modalities, depth resolution is drastically improved. To demonstrate the capability of oSLO, we have performed in vivo volumetric fluorescein angiography (FA) of the rat retina with ~25 μm depth resolution and over a 30° FOV. Using depth segmentation, oSLO can obtain high contrast images of the microvasculature down to single capillaries in 3D. The multi-modal nature of oSLO also allows for seamless combination with simultaneous OCT angiography.

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1. Introduction

Fluorescence retinal imaging is an essential tool in vision science, and has important clinical applications in ophthalmology. Currently, four major forms of fluorescent contrasts are utilized. Fluorescein angiography (FA) has been a gold-standard imaging technique for evaluation of the retinal vasculature for more than 50 years [1]. Intravenous injection of fluorescein allows for dynamic identification of areas of chorioretinal vascular leakage or nonperfusion. Indocyanine green angiography (ICGA) is utilized in cases where retinal hemorrhage may block FA signal or when the choroidal vasculature is the suspected area of pathology. Blue light and NIR fundus autofluorescence (FAF), techniques not requiring exogenous contrast agents, take advantage of the intrinsic autofluorescence (AF) of retinoids in photoreceptors [2] and lipofuscin and melanin in the RPE [3, 4] to image areas of pathology in non-exudative age-related macular degeneration [5] and detect photoreceptor damage indicative of retinal dystrophies and degenerations [6].

Despite the extensive use of fluorescence retinal imaging, prevalent fundus photography techniques have significant limitations due to the lack of 3D imaging capability. As a result, signals from different depths are superimposed to each other which confounds the image interpretation. For example, the significant AF background signal from the outer retina and RPE can overwhelm the weak fluorescein signal from small vessels and capillaries, limiting FA’s resolution and sensitivity.

While advanced retinal imaging techniques have attempted to address the issues of fluorescence imaging, each new method has its own limitations. Scanning laser ophthalmoscopy (SLO) and confocal SLO (cSLO) use confocal gating to remove diffused light, resulting in crisper image quality [7, 8]. The depth-resolved fluorescence imaging has been successfully demonstrated in rodents thanks to the high numerical aperture (NA) of the dilated pupil (NA~0.5) [9], and using two photon excitation [10–13]. However, the volumetric imaging of SLO requires to compile z stacks, which can be challenging and time-consuming. In human, the dilated pupil has smaller NA of about 0.2; and thus the depth of focus of SLO on human retina is typically in the order of several hundreds of microns, which is insufficient to provide depth discrimination. Improving upon SLO, adaptive optics SLO (AOSLO) corrects for ocular aberrations [14–16], allowing diffraction-limited resolution in both axial and lateral resolution, in human [17] and in small animals [18–21]. This technique is limited, however, by its small field of view (FOV) and also the necessity of z stacks for volumetric imaging. Optical coherence tomography (OCT) permits wide-field volumetric imaging and is capable of 3D label-free angiography by OCTA [22–26]. However, its inability to image leakage limits its capacity to diagnose chorioretinal vascular dysfunction [17]. In addition, OCTA requires comparison of multiple raster scans to detect blood flow and thus is vulnerable to motion artifact.

To address the limitations of current retinal imaging technology, we have developed a novel technique termed oblique scanning laser ophthalmoscopy (oSLO) for in vivo volumetric fluorescence retinal imaging. The technique allows “OCT-like” cross-sectional images contributed solely by the fluorescent contrast, without the need for z stacking. We
demonstrated high contrast depth-resolved FA imaging of retinal vascular structure at the single capillary level. The 30° FOV is comparable to that of other commonly used clinical imaging modalities, and the seamless integration of OCT with oSLO allows for simultaneous multimodal volumetric retinal imaging.

2. Principle

The principle of oSLO for volumetric imaging is illustrated in Fig. 1. The existing retinal imaging modalities all employ coaxial alignment for excitation and emission/reflection detection (Fig. 1(a)). For laser scanning methods such as SLO/cSLO and AOSLO, this alignment relies on the focusing power of the ocular optics and confocal gating to achieve depth resolution, similar to the working principle of fluorescence confocal microscopy. One dilemma posed by coaxial alignment is the tradeoff between FOV and depth resolution. SLO in human uses a small beam size on the pupil, i.e. small numerical aperture (NA) to achieve a large FOV at the expense of poor depth resolution. AOSLO fully uses the dilated pupil size, i.e. the highest possible NA, to achieve high depth resolution but with limited FOV. In oSLO, we break the coaxial alignment of excitation and emission detection. The laser illumination is offset from the ocular axis to create an obliquely focusing beam on the retina (Fig. 1(a)). At the same time, a separate fluorescence detection system is aligned in such a way to image the retina sideways and refocus the oblique laser incidence onto a planar camera. By crossing the illumination and detection beam paths, depth resolution is decoupled from the focusing power of the ocular optics and drastically improved, while still maintaining a wide FOV. We simulated the point spread functions (PSFs) for excitation and emission detection under a
coaxial alignment, and their multiplication as the combined PSF in Fig. 1(b)-1(d). Because of the coaxial alignment, the axial extent of the combined PSF is in the same order of the Rayleigh range determined by NA. In contrast, under the oSLO setup, the excitation beam is focused obliquely, intersecting the detection beam path to significantly improve the depth resolution (Fig. 1(e)-1(g)). It is important to note that each acquisition of the camera records a 2D cross sectional image of the scanned oblique illumination plane. The oSLO essentially implements a scanned light sheet microscopy [27] using the natural ocular optics for volumetric imaging, without the need of compiling z-stacks.

3. Experimental setup and methods

We now describe the methods and materials that were used in this paper. All animal procedures were approved by the Institutional Animal Care and Use Committee at Boston Medical Center and conformed to the guidelines on the Use of Animals from the National Institutes of Health (NIH).

3.1 Oblique scanning laser ophthalmoscopy

The system setup is modified from our previous publication [28], and the schematic is shown in Fig. 2(a). The light source is a supercontinuum laser (SuperK, NKT, Denmark) providing a broad band illumination. Visible light under 650nm was filtered out by a dichroic mirror (DM1), polarized, and dispersed by a pair of identical prisms (P1, P2). A beam block (B) passed the blue light from ~420nm to 490nm. The blue light was reflected by the mirror M1, redirected by a D-shaped mirror (DSM), and then coupled into a 50/50 fiber coupler (OFC1) to further combine a NIR band. In the illumination optical path, the light was first collimated by an $f = 6$ mm lens (L2). Two galvanometer scanning mirrors (GM1, GM2) and two relay lenses (L3: $f = 50$ mm, L4: $f = 50$ mm) were mounted to steer the laser. A telescope system
(L5: $f = 75$ mm, L6: $f = 25$ mm) with 0.33x magnification relayed the beam to the pupil of the eye. A dichroic mirror (DM3) was used to deflect the fluorescence emission to the detection optical path. The telescope and the dichroic mirror were installed on a custom-made dove tail mount, which can adjust the offset of the optical axis of the telescope system to create an oblique focusing beam onto the retina (see appendix Fig. 6 for details). An offset of 5 mm resulted in a ~1.7 mm shift of the laser spot from the apical center of the cornea, and a corresponding ~15° angle of the oblique illumination assuming the diameter of the rat eyes is ~6 mm. The offset on the dove tail mount was measured precisely by using a digital caliper. The magnification in the illumination optical path was around 3, approximating a 6mm focal length of rat eye. The effective illumination NA is estimated to be around 0.04 given the fiber NA = 0.12-0.14.

In the fluorescence detection optical path, a fluorescence band pass filter (F2) was installed in the optical path to further filter the emission. Another lens (L7: $f = 60$ mm) together with L6 relayed the beam from the pupil plane to the third galvanometer mirror (GM3), which de-scanned GM1 and created a stationary image during the slow scanning. Another 1:1 telescope system (L8: $f = 50$ mm, and L9: $f = 50$ mm) relayed the beam again to the objective lens (OL3, Olympus UplanSApo 20 × /0.75), after which, a tilted image of the oblique laser incidence was formed. The magnification from retina to the image space of OL3 is estimated to be $M = -0.4$. Therefore, the angle of the tilted image of the oblique laser incidence $\phi$ is ~34°, following the Scheimpflug condition:

$$
\tan(\phi) = \frac{\tan(\theta)}{M},
$$

where $\theta = -15°$ is the oblique angle of the incidence with respect to the optical axis [28].

A final imaging system (OL4, UplanFL N 10 × /0.3, and L10) was mounted on a 2D translational stage, and aligned in an angle of ~30° to the optical axis of OL3, to refocus the tilted image of the oblique laser incidence onto a planar CCD camera (Pixelfly). The choice of the angle is to balance the depth of view and the fluorescence collection efficiency. The iris before L10 was closed down to ~1mm to avoid the resolution deterioration due to the spherical aberration of the rat eye.

The 3D model of the eye in Fig. 2(b) illustrated the operation of the oSLO system and denoted the imaging dimension. The fast scanning galvanometer scanned the $x'$ direction and formed a scanned oblique light sheet illumination in $x'$, $z'$ plane. The laser incident direction is denoted by $z'$. This oblique plane was refocused by the tilted imaging system (OL4, L10) and a tomographic fluorescence image was captured by the planar CCD camera (Fig. 2(c)). The resulting image is similar to the B-scan of the OCT images except that oSLO here relies on the fluorescent contrast.

### 3.2 Optical coherence tomography

We combined an additional OCT system in parallel to obtain complementary structural imaging and OCTA. The illumination from 800 to 900nm was filtered by a dichroic mirror (DM2) and a high pass filter (F1), and coupled into the 50/50 fiber coupler (OFC1) to combine with the blue light excitation. A second 50/50 fiber coupler (OFC2) served as the interferometry. The wavelength multiplexing scheme for both oSLO and OCT is illustrated in Fig. 2(d), where the dichroic mirror (DM3) allowed transmission of both blue and NIR incidence and reflection of fluorescence emission at 500-520nm. The sample arm of the OCT shared an identical optical path with oSLO. The backscattering light returned into the interferometer and interfered with the light from the reference arm. The reference arm consisted of a variable neutral density filter (VNDF), dispersion compensation glass plates (DC), and a mirror. A custom-made spectrometer took the spectrum from 780 to 900nm as in a conventional Fourier domain OCT configuration.
3.3 Data acquisition

The system synchronization was modified from an OCTA scanning protocol [29–31], and has been explained in detail in our previous publications [28]. Briefly, a saw tooth pattern with 80% duty cycle was fed to $x'$ fast scanning mirror (GM2) for OCT B-scans. The OCT acquisition took 400 A-lines in the forward scanning direction, and halted in the backward direction. The round trip was repeated for 5 times at each B-scan location for OCTA. This sequence was then carried out at 512 steps along $y'$, which was controlled by a ramping voltage pattern to the slow scanning mirror (GM1). The A-line rate was 50 kHz, and the frame rate was 100 fps for OCTA. At each location along $y'$, a single trigger was sent to the CCD camera to capture one 2D cross sectional fluorescence image $I_{\text{oSLO}}(x', z')$ with 300 × 400 pixels. As the raster scan completed, a volumetric fluorescence retinal imaging data set was obtained as $I_{\text{oSLO}}(x', z', y')$ with 300 × 400 × 512 pixels. Due to the slow data transfer speed, only one fluorescent cross sectional frame was captured while OCTA completed 5 B-scans at each $y'$ location. The CCD camera was operated with 40% duty cycle (i.e. 20ms exposure and 30ms data transfer), and the frame rate was 20 fps. The incident power on the cornea is 200μW for oSLO, and 0.8mW for OCT. The total power is ~1mW for simultaneous oSLO/OCTA. The total acquisition time for simultaneous oSLO/OCT was 25.6s.

3.4 Image processing

The raw data for oSLO is three-dimensional denoted by $I_{\text{oSLO}}(x', z', y')$. In order to perform depth segmentation, we manually selected the capillaries at the outer plexiform layer (OPL) from the cross sectional images about every 10 frames in ImageJ, and output the coordinates $(x''_n, z''_n, y''_n)$, where $n$ represents individual sampling points. Then 2nd polynomial fitting was subsequently performed in $x'$ and $y'$ directions. The fitted plane $z'(x', y')$ denoted the depth of OPL, based on which the volumetric oSLO image was flattened to the retinal surface. The depth-resolved images were then obtained by segmenting the signal from a desired depth range.

The method to generate OCT images followed the standard procedure, including DC spectral background removal, spectral normalization and resampling, digital dispersion compensation, and fast Fourier transformation (FFT). The OCTA algorithm followed our previous methods [29]. Briefly, after compensating for the axial global phase shift, we took the subtractive difference between two subsequent complex OCT B-scans. The absolute value of the difference yielded OCTA contrast.

3.5 Animals

Adult Long-Evans pigmented rats were used in this study. We initially anesthetized adult Long Evans rats with 4.5% isoflurane for 10 mins followed by 2.5% isoflurane during the imaging session. The animal was placed on a custom-made 5-axis ($x$, $y$, $z$ translations, yaw and pitch) holder to allow adjustments of eye position and angle. We used a bite bar to stabilize the rat head, and used strips to gently restrain the body onto the holder. We applied 0.5% Tetracaine HCl ophthalamic solution to the rat’s eye for local anesthesia and applied 1% Tropicamide ophthalamic solution to dilate the pupil. Commercial artificial tears were applied to the rat’s eye every other minute to prevent corneal dehydration. Prior to imaging, 10% w/w of fluorescein salt (30 mg) was diluted in 0.3 ml saline and injected intravenously through the tail vein. During imaging, the eye was positioned roughly coaxial with the telescope system, so that the laser was offset to the apical center. Fine adjustments were made based on the real-time display of the oSLO image. After the experiment, the animal was released from the holder and placed into a warm ventilated box for recovery.
Fig. 3. Feasibility and resolution characterization of oSLO system using fluorescent microspheres under a 6mm ball lens simulating the rat eye. (a) Simultaneously captured OCT and oSLO fluorescence images and their overlay. The fluorescence images were pseudo-colored green. Bar = 100 μm. (b-d) The maximum intensity projection of the volumetric fluorescence microsphere image on the planes of x’-z’, y’-z’, and x’-y’. (e) Magnified images of three representative microspheres in the center of the field of view in (b-d). The locations of three microspheres are labeled in (b-d). (f) The intensity profile across the center of three beads in x’, y’, and z’ directions. Bar = 5 μm. A cross sectional fly-through video is included in Visualization 1.

4. Results

In order to characterize the system resolution of oSLO in 3D, we first performed a phantom experiment with a 6mm diameter ball lens mimicking the rat eye (See appendix Fig. 7 for experimental setup). Fluorescent microspheres with 1 μm diameter were sealed within 1% agarose gel and placed underneath the ball lens, with water filling the space in between. Figure 3(a) shows the cross-sectional images of the fluorescent microspheres taken by OCT, oSLO, and their overlay. We first compressed the axial scale of the OCT image assuming a 1.33 refractive index in the gel and warped the oSLO image to match OCT using the method
in our previous publication [28]. As the refractive index is a constant within the gel, OCT provides an absolute scale for characterization of the axial resolution of oSLO. The scale of the lateral extent has been calibrated with images of a caliper jaw. After applying the same warping process to the entire 3D data set, the maximum intensity projections in three directions were made as shown in Fig. 3(b)-3(d). The side projections in Fig. 3(b) and 3(c), show that the image sharpness was largely maintained within a depth of view of ~250μm. Given that the thickness of rat retina is about 200 μm, oSLO is capable of providing focused images throughout the entire retina.

Because of the small size of the microspheres, the images of the individual beads approximate the PSF and can be used to estimate the oSLO system resolution. We selected three representative beads from the center of the field of view, and plotted their magnified images in three directions in Fig. 3(e). The intensity profile plotting through their centers reveals resolutions of about 7 μm, 8 μm, and 25 μm in x’, y’ and z’ respectively, by the full-width-half-maximum of the curves (Fig. 3(f)). We used the prime superscripts to distinguish from Cartesian coordinate system due to the oblique illumination.

After testing the system and characterizing its resolution, we proceeded with in vivo FA imaging of rat retina. Fluorescein solution was injected intravenously to highlight the vasculature. Figures 4(a)-4(c) show the simultaneously acquired cross-sectional OCT, OCTA, and oSLO FA images. The OCT structural image displays the typical stratified structures of retina, with the inner (IR) and outer retina (OR) separated by the outer plexiform layer (OPL). The outer retina is composed of photoreceptors with a monolayer of RPE separating the retina and choroid. A major blood vessel is visible with a shadow underneath it. The OCTA image enhances the blood motion contrast from the active circulation as shown in Fig. 4(b). The signals from capillaries within OPL as well as the major blood vessel in the inner retinal circulation are apparent in OCTA. The outer retina is avascular and thus has no OCTA contrast, except for the shadowgraphic projection artifacts on IS/OS and RPE form the superficial vasculature [30, 32]. The OCTA contrast from choroidal circulation reappears beyond RPE. In comparison, the cross-sectional oSLO FA image (Fig. 4(c)) clearly identifies the corresponding vessels in NFL/GCL, and capillaries in OPL. In addition, we observed background fluorescence signals in the avascular outer retina, which may be contributed by AF originated from the biretinoids in photoreceptors and RPE [2]. The existence of the AF signal was verified by imaging without fluorescein injection (Appendix Fig. 9). The signal diminishes gradually beyond outer retina. A cross sectional flythrough movie of a representative oSLO volumetric data is shown in Visualization 2.

The system’s volumetric imaging capability allows us to generate depth-resolved FA through image segmentation. Figure 4(d)-4(f) show the oSLO FA images of the superficial, intermediate, and deep capillary plexuses. Corresponding OCTA images from the same depth ranges were acquired simultaneously (Fig. 4(g)-4(i)). The two angiography methods show excellent agreement in their vascular structural imaging, but there are several distinct differences. In the OCTA image in Fig. 4(g), motion artifact from the cardiac cycle is manifested as vertical stripes in NFL/GCL. Since oSLO utilizes fluorescence emission contrast and is free from motion artifact, the stripes are not present in the corresponding oSLO FA image (Fig. 4(d)). In the intermediate vasculature, the oSLO FA image (Fig. 4(e)) clearly shows the vertically diving vessels that extend into the deep capillary plexus. However, the same structures are not apparent in OCTA, presumably due to fringe washout from the vertical blood flow (i.e. smaller Doppler angle causing higher projective blood velocity). In the deep capillary plexus, OCTA generally has a crisper image quality than oSLO FA. However, the vasculature appears more continuous with better contrast in oSLO FA than OCTA (blue arrows in Fig. 4(f), 4(i)). This difference is probably due to the fact that dye-based FA is independent of blood flow velocity and vessel orientation, both of which would impact OCTA. Interestingly, the size of the venule in oSLO (Fig. 4(f)) appears larger than the corresponding vessel in OCTA (Fig. 4(i)). Slow blood flow velocity along the venous
vessel wall may result in this decreased OCTA signal. In this regard, oSLO FA image resembles the actual vascular morphology more accurately than OCTA, since it is not dependent upon blood flow speed.

**Fig. 4.** *In vivo* volumetric oSLO FA and OCTA acquired simultaneously from the rat retina. (a-c) Examples of cross sectional OCT B-scan, and the corresponding OCTA and oSLO FA. (d-f) Depth-resolved oSLO fluorescence images at varying depth ranges. (g-i) The corresponding depth-dependent OCTA images. Red arrows point to OCTA motion artifacts, appearing as vertical stripes. Yellow arrows point to locations where retinal vessels dive down into the deep capillary plexus, seen more clearly on oSLO FA than OCTA. White arrows point to a venule that appears larger in oSLO than OCTA. Blue arrows point to a region where oSLO FA shows better contrast and capillary resolution than OCTA. Bar = 200 μm. An *en face* fly-through video from both oSLO and OCTA is included in Visualization 3.
Fig. 5. Wide-field volumetric fluorescence retinal imaging by oSLO over 30° angle of view. (a-b) Depth-encoded volumetric oSLO FA image and the corresponding conventional SLO image from a rat retina in vivo. (c-d) The magnified images of an ROI between a major retinal arteriole and venule, as outlined in panel (a) and (b). (e-h) The vascular sketch showing the detailed vessel branching and derivation in upper layer in NFL and GCL (e), intermediate layer in OPL (f), deep layer in OPL (g), and their overlay (h). The connections between adjacent layers were labeled by end dots. An enface fly-through video is included in the Visualization 4.

Finally, we tested the capability of oSLO for large FOV volumetric fluorescence retinal imaging. Figure 5(a) presents a volumetric oSLO FA over a 30° viewing angle with the superficial and deep vasculatures displayed in red and green, respectively. The viewing angle is calculated at the pupil. The scanning angle range of the galvanometer mirrors was controlled to be ±2.5° by ±2° driving voltage range. The angle of the scanning laser was then ±5° off the galvanometer mirrors, and further amplified to ±15° at the pupil by the 3:1 telescope system (L5, L6). The signal beyond inner retina was excluded by depth segmentation. For a side-by-side comparison, we generated the en face mean value projection of the entire volumetric data set as in Fig. 5(b), equivalent to the conventional SLO. Without depth discrimination, the significant fluorescence background in conventional SLO blurs the image, rendering vessels from different layers indistinguishable.

The clarity and high contrast of the volumetric oSLO FA allow to closely study the vascular organization. It has been previously shown that the retinal vasculature is distributed in three layers, with precapillary arterioles in superficial layer and a capillary plexus in the deep layer [33, 34]. We zoomed in on a region (Fig. 5(c), 5(d)) and manually traced the vascular branching from the retinal arterioles into the capillary plexus and finally the collecting venules (Fig. 5(e)-5(g)). We confirmed that arterial branching occurs in NFL/GCL (Fig. 5(e)), further distributes within IPL/INL (Fig. 5(f)), and finally forms a planar capillary network at OPL (Fig. 5(g)). The majority of the collecting venules are in the same deep capillary layer and directly drain to the major venous circulation as shown in Fig. 5(g). The locations where the vessels dive down through the three layers are labeled with ending dots. Altogether, the evolution of the retinal vasculature can be traced down to the single capillary level in 3D, as exemplified in Fig. 5(h).

5. Discussion and conclusion

We have presented oSLO, a novel technique for volumetric fluorescence retinal imaging in vivo over a large FOV. It only requires one raster scan to obtain volumetric imaging without the need for depth sectioning. We demonstrated the elegant combination of oSLO with OCT.
and OCTA. This multimodal technique allows simultaneous structural and functional imaging \textit{in vivo}, another valuable feature, as demonstrated in Fig. 4. Although we only demonstrated the fluorescence imaging in this paper, the scattering contrast is also feasible using oSLO without the fluorescence filtering. We have demonstrated the volumetric FA over a 30° FOV using oSLO. Despite the angle change of both excitation and the detection beams as the laser scans through retina, the combined PSFs by intersecting two beam paths do not change significantly (Appendix Fig. 8). The ultimate FOV will be determined by the fluorescence optical system.

The concept of oblique illumination using a single lens has been earlier demonstrated in oblique plane illumination microscopy and SCAPE microscopy [35–37]. In these works, a microscopic objective lens with high NA ~1.0 was used to produce a large crossing angle between the excitation and fluorescence detection PSFs to obtain a higher depth resolution. In oSLO, the natural ocular optics was used as the objective lens leading to two distinct differences and challenges. First, with a fully dilated pupil, the effective NA is only about 0.5 for rodents and 0.2 for humans, which fundamentally limits the depth resolution of oSLO. Our previous simulations suggested that the diffraction-limited depth resolution can reach <10μm for dilated rodent eyes, and ~30μm for human eyes [28]. Given a retinal thickness of about 200 μm in rodents and 300 μm in the perifoveal region in humans, oSLO still has sufficient resolving power to separate at least the retinal and choroidal circulations, also inner and outer retina. Another major difference resulting from reliance on ocular optics is the eye’s spherical aberration, which is the primary reason for the reduced depth resolution in our current oSLO setup (~25 μm). Future designs correcting for spherical aberration would allow for even higher depth resolution.

There are some limitations in the current oSLO system. First, the imaging speed was only 20 fps and the total imaging time took 25.6s, which is impractical for clinical use. For a single oSLO frame, the CCD camera took ~30ms to complete the data transfer permitting only 40% duty cycle. The speed will be dramatically improved by replacing the CCD camera with a scientific CMOS camera that offers high speed and sensitivity, as demonstrated in SCAPE microscopy [37]. Second, the alignment of the eye position can be challenging in oSLO due to the requirement of the offset illumination. The current method used a rough positioning of the eye and a fine adjustment based on the real-time feed from the camera. Future improvement using a pupil camera to monitor the laser offset would facilitate the imaging. The setup of the oSLO system is also more complicated than SLO and OCT making it more difficult to align, because the fluorescence detection is currently separated from the illumination optical path. Future design using the same optical path for both illumination and detection would simplify the system. Third, the principle of oSLO requires pupil dilation in order to permit oblique scanning and detection. However, pupil dilation is a standard-of-care practice in current clinical ophthalmology associated with minimal risk. Finally, the angled imaging system reduces the efficiency of fluorescence detection. We anticipate that the use of the high sensitivity scientific CMOS cameras would offset the reduced fluorescence detection.

The simultaneous acquisition of OCT/OCTA and oSLO cross sectional images in Fig. 4(a)-4(c) shows considerable fluorescent signal from avascular photoreceptor layer. Although the current oSLO resolution is not sufficient to definitively separate photoreceptor from RPE, the depth range of the fluorescence band appears much wider than monolayer RPE. The presence of the similar AF band was confirmed by imaging rat retina without fluorescein injection (Appendix Fig. 9). These results suggest that there may be some contribution of photoreceptor AF, corroborating with a recent study showing contribution of biretinoids in photoreceptors in blue light FAF [2]. Previously it was believed that blue light FAF stems primarily from accumulation of lipofuscin in RPE, at least in human eyes [38]. This discrepancy can be addressed by future use of oSLO combined with OCT in human to specifically locate the anatomical AF signal origin.

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To conclude, a novel oSLO technique is presented for the first time being capable of volumetric fluorescence retinal imaging \textit{in vivo} with only one raster scan. Volumetric FA and AF capabilities were demonstrated in rat retina, and over a 30° FOV was achieved.

**Appendix**

We provide the design and the photography of the dove tail mount that controlled oblique illumination, the components lists, and the phantom experimental setup using a ball lens. We provide illustration on the angle changes of the excitation and detection PSFs. We also provide supplemental data on intrinsic AF on retina without dye injection.

\textit{Mechanical design for oblique incidence}

![Dove Tail Mount Diagram](image)

Fig. 6. (a) The solid work model for the dove tail mount installed in the optical setup. (b-c) Zoomed in view of the dove tail mount. (d) The photography of the actual setup in oSLO system.

We designed and custom-made a dove tail mechanical mount compatible to the off-the-shelf cage mechanical system from Thorlabs, to adjust the oblique laser incidence. Figure 6 shows the solid work model and the actual photography of the mechanical mounting. The upper part of the dove tail mount is attached to the galvanometer cage, and the bottom part is connected with L5, DM3 and L6 through the cage system. The adjustable sliding of the dove tail mount offsets the laser beam to the optical axis for the oblique illumination into the eye.

\textit{Component table}

We included the detail part numbers and manufacturers for all the optical components in the current oSLO setup, as in Table 1.
Table 1. The information of the system components.

| Component | Model               | Manufacturer        |
|-----------|---------------------|---------------------|
| SL        | SuperK EXTREME EXU-6| NKT Photonics       |
| DM1       | DMLP650R            | Thorlabs            |
| DM2       | DMLP900R            | Thorlabs            |
| DM3       | ZT514/1064rpc       | Chroma              |
| BT1/BT2   | BTC30               | Thorlabs            |
| PBS       | CM1-PBS251          | Thorlabs            |
| P1/P2     | PS853               | Thorlabs            |
| OL1/OL2   | DIN 20x 0.4         | Edmund Optics       |
| OL3       | UplanSapo 20 × 0.75 | Olympus             |
| OL4       | UplanFL N 10 × 0.3  | Olympus             |
| F1        | FEL0800             | Thorlabs            |
| F2        | ET512/20            | Thorlabs            |
| OFC1/ OFC2| TW850R5A2           | Thorlabs            |
| VNDF      | NDC-50C-4M-A        | Thorlabs            |
| DC        | WG11010             | Thorlabs            |
| L1        | HPUCO-23A-400/700-S-10AC | OZ Optics |
| L2        | HPUCO-23A-400/700-PSM-6AC | OZ Optics |
| L3*       | AC254-100-A × 2     | Thorlabs            |
| L4*       | AC254-100-A × 2     | Thorlabs            |
| L5*       | AC254-150-A × 2     | Thorlabs            |
| L6*       | AC254-50-A × 2      | Thorlabs            |
| L7        | AC254-60-A          | Thorlabs            |
| L10       | 12-36mm/1:2.8       | Computar            |
| GM1/GM2   | GVS201              | Thorlabs            |
| GM3       | GVS011              | Thorlabs            |
| Iris      | CP20S               | Thorlabs            |
| Camera    | Pco.pixelfly usb    | PCO                 |

* Two identical doublets back to back were used to reduce aberration. The effective focal length is ~half of each doublets.

**Experimental setup using a ball lens**

A 6mm diameter ball lens was used to mimic the rat’s eye and characterize the oSLO resolution. The lens was attached to a rod and mounted on a three-dimensional translational stage (not shown here). The agarose gel phantom was placed underneath the lens with water
filling between the spaces, mimicking the aqueous vitreous. Figure 7(a) shows the photography of the setup. The fast scanning creates a scanned oblique light sheet illumination, as shown in Fig. 7(b). From a side view in Fig. 7(c), we can see the oblique illumination with respect to the ball lens.

![Image](image1.png)

Fig. 7. The photography of the phantom experiment using a ball lens mimicking the rat eye. (a) The photograph of the system setup. (b, c) The photography of the in-action oblique scanning laser illumination from two side views.

**Angle change of PSFs as the laser scans through retina**

We simulated the angle change of the excitation, emission and combined PSFs as the laser scans through the retina along y' direction over a 50° FOV, as in Fig. 8. The depth resolution of the combined PSFs does not change significantly.

![Image](image2.png)

Fig. 8. Illustration of the angle changes of excitation and detection PSFs at various scanning angles over 50° FOV, and the resulting combined PSFs. Bar = 20μm.

**In vivo oSLO AF retinal imaging**

Figure 9 shows the oSLO AF images taken without injection of fluorescein contrast agent from a rat eye *in vivo*. Without the fluorescein, the major blood vessels appear dark in the *en face* projection due to the blood absorption (Fig. 8(a)). The cross sectional image in Fig. 8(b) showed the similar fluorescent band as we observed in the main Figure 4(c) in the avascular outer retina.
Fig. 9. In vivo oSLO AF retinal fluorescence imaging from a rat eye. (a) En face maximum value projection of the volumetric oSLO AF data set. (b) The cross sectional image along the dash line in panel (a). Bar = 0.2 mm.

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**Disclosures**

Ji Yi holds a pending patent for oSLO. The other author(s) declare no competing financial interests.