Cell Motility as Contrast Agent in Retinal Explant Imaging With Full-Field Optical Coherence Tomography

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We previously showed full-field optical coherence tomography (FFOCT) imaging of retina1,2 and cornea,3,4 and used multimodal fluorescence-FFOCT to identify cells in the retinal ganglion cell layer (GCL)2 and stem cells in the corneal limbus.2 FFOCT revealed micrometric morphologic detail in these tissues, and allowed visualization of fibers, vessels, collagen, and cellular details. However, some cells were sometimes masked by the stronger scattering signals from overlying fibrous structures, and cell contours were often hard to accurately define. Additionally, tissue health could only be assessed if substantial structural disorganization had occurred.

Here we apply a new method, named dynamic FFOCT,6,7 to the imaging of retinal explants in order to reveal more retinal cells. This technique relies on cell motility to create intrinsic contrast in the image, and tends therefore to highlight cells and intracellular details. This cellular contrast is observed in the ganglion cell, inner and outer nuclear, and photoreceptor layers. Thanks to the high transverse resolution offered by FFOCT in retinal explants, subcellular details are revealed including the nucleus position and size, which permits more precise identification of cell types.

We then explore quantification of the motility signals, including questions such as whether the signal can be a biomarker of cell viability, and whether it can be used to help differentiate specific cell types.

METHODS

Tissues

All animal manipulation was approved by the Quinze Vingts National Ophthalmology Hospital and regional review board (CPP Ile-de-France V), and was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Macaque ocular globes were obtained from a partner research facility and transported to the Vision Institute in CO₂-free neurobasal medium (Thermo Fisher Scientific, Wal-tham, MA, USA) inside a device that maintained oxygenation, for transport to the laboratory for dissection. Mouse ocular globes were obtained from the Vision Institute animal facility, and euthanasia and dissection took place immediately prior to imaging. Mice were C57Bl6J wild type from Janvier Laboratories (Le Genest Saint Isle, France).

We imaged 11 macaque and 6 mouse retinas. An incision was made in the sclera to remove the anterior segment. For macaque retinas, the retina was gently removed from the choroid, with separation occurring at the RPE, and flattened into petals by making four incisions (nasal, temporal, superior, inferior). Pieces of an area ~1 mm², approximately 3 mm from the fovea toward the median raphe, were selected for imaging. Inevitably, the FFOCT signal is reduced in depth due to aberrations accumulated on passage through the tissue, and less photons return from lower depths due to multiple scattering.11
If therefore we consider that we have a “fixed” total penetration depth in any given tissue (bearing in mind that the “fixed” amount does vary depending on parameters such as transparency, freshness, flatness, contact with the imaging window, etc.), we wanted to cover as many retinal layers as possible within this fixed depth, hence our choice of position toward the median raphe where the nerve fiber layer (NFL) is proportionally thinner. For mouse retinas, the whole eye cup, with retina still attached to the choroid, was placed in the sample holder after flattening into petalas by making four incisions. Retinal samples were placed in transwells, and immersed in CO₂ free neurobasal and HEPES (Thermo Fisher Scientific, Waltham, MA, USA) medium for imaging.

**Fluorescent Labeling**

For fluorescence-FFOCT, live macaque retinal cells were labeled in vitro by particle mediated acute gene transfer of green fluorescent protein (GFP). A Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA) was used for particle-mediated acute gene transfer in macaque retinal explants, similarly to what has been previously described. Briefly, 10-mg gold microcarriers (1.6 μm; Bio-Rad Laboratories) were coated with 20 μg of a plasmid encoding CMV:CatCh-GFP in an adeno-associated virus (AAV) backbone in 3.2 mL ethanol solution and loaded into Tefzel tubing (Saint Gobain, Forge, PA, USA) using a gene gun (Tubing Prep Station; Bio-Rad Laboratories). The gene gun barrel was held 5 mm above the retinal explant (RGC side facing the barrel) and the plasmid bullets were propelled at a pressure of 110 psi. The explants were incubated at 37°C 5% CO₂ for 3 to 4 days post gene transfer.

Bipolar cells in a mouse retina were labeled in vivo with a previously described method. This retinal sample was recuperated from a mouse that had undergone AAV gene therapy, and whose ON-bipolar cells had been labeled with GFP. Briefly, a humanized version of channel rhodopsin-2 with H134R mutation (ChR2/H134R) was fused to GFP to facilitate cellular localization. Gene expression was restricted to the ON bipolar cells by the use of a 200-base pair enhancer sequence of the mouse Grm6 gene, which encodes the ON bipolar cell-specific metabotropic glutamate receptor, mGluR6.7m8 (a genetic variant of AAV2), was used to deliver ChR2 across the retinal layers. ChR2 was delivered to the retinas of C57BL/6J wild-type mice. Four to six weeks after intravitreal delivery of AAV2-7m8, vector encoding hChR2/H134R-GFP led to strong panretinal expression in bipolar cells, as confirmed by two-photon microscopy.

**Imaging Setups**

Full-field OCT is a variant of regular OCT in which two-dimensional (2D) en face images are captured on a camera and three-dimensional (3D) data sets may be obtained by scanning in the depth direction. This configuration, together with the use of a white light source, allow for higher axial and en face resolution than conventional OCT, on the order of 1 μm. FFOCT can perform micrometer resolution 3D imaging noninvasively in fresh or fixed ex vivo biologic tissue samples.

We used two FFOCT setups in this study. The first was a commercial system (LITech Management, Paris, France), similar to that described previously, but equipped with a high speed (300 Hz), high sensitivity complementary metal-oxide semiconductor (CMOS) camera (Adimec, Netherlands) and full field illumination provided by either a high power LED (623 nm) giving an optical section of 5-μm thickness, or a halogen source, giving an optical section of 1 μm thickness. The interferometer arms held a matched pair of microscope objectives in the Linnik configuration. Water immersion objectives (×10 with 0.3 numerical aperture (NA) lead to a lateral resolution of 1.0 μm and field size of 1.3 × 1.3 mm. Penetration depth depends on tissue content and transparency and is approximately 200 μm in retina. Wide field images can be obtained by automated mounting to image samples up to 25 mm in diameter, or stacks of images in depth can be captured. A 2 × 2 cm² montaged 2D field is captured in ~1 minute, and a 200 μm × 1.3 × 1.3 mm 3D stack in less than 3 minutes.

The second FFOCT system was a laboratory setup that offers higher magnification (×40, 0.8 NA) and multimodal imaging with pixel-to-pixel colocalized FFOCT and fluorescence channels. Compared to previous fluorescence-FFOCT setups, in the setup used here, simultaneous measurements of fluorescence, static and dynamic FFOCT can be acquired. Briefly, this setup operated in a similar fashion to conventional FFOCT but with the addition of a blue light-emitting diode (LED, M470L2, 650 mW; Thorlabs, Newton, NJ, USA) centered at 470 nm blocked by an excitation filter (FF01500, λc = 500 μm; Thorlabs) to excite fluorophores. The two illumination beams are combined and focused onto the sample, and in the detection path, FFOCT and fluorescence signals are separated by a single edge dichroic beam splitter (BrightLine, λ = 593 nm, D025R594-22x27; Semrock, Rochester, NY, USA), and captured by two cameras, a CMOS (Adimec) for the FFOCT image and a scientific CMOS (5.5, PCO.edge; Kelheim, Germany) to capture the fluorescence image, with filters placed in front of the detection cameras to ensure independence of the two paths. As the optical path into the tissue is parallel for the FFOCT and fluorescence channels, an identical region of the sample is captured on the FFOCT and fluorescence cameras. Water immersion objectives (×40, 0.8 NA; Nikon, Paris, France) lead to a lateral resolution of 0.5 μm and field size of 220 × 220 μm. FFOCT image capture time is 3 ms, at 100 Hz. The fluorescence channel displays only one image, exposed for 500 ms. High NA objectives offer better photon collection in both fluorescence and FFOCT paths which increases the available signal-to-noise ratio (SNR) for small scatterers.

Figure 1 presents the principle of dynamic FFOCT image extraction. In static FFOCT, the amplitude image at a given depth is computed from 4 direct images, phase shifted by π/2. In dynamic FFOCT, contrast arises from the temporal phase fluctuations of the tissue. These phase fluctuations can be captured either from direct or phase images to similar effect, but the impact of minor vibrations from the piezo was avoided by using direct images. Therefore, we stopped the modulation of the piezo (i.e., of the optical path). Due to the low coherence of the setup and the NA of the objectives, we are only sensitive to phase changes that happen at a given depth inside the coherence gate of the microscope. We acquired sequences of 1000 consecutive direct images, and computed the standard deviation over groups of 100 images, then averaged the 10 resulting images (Supplementary Movie S1). Computing the standard deviation on the direct images cancels the incoherent light that does not produce interference. The dynamic FFOCT image obtained therefore corresponds to phase fluctuations occurring at a given depth, just as the static FFOCT image also corresponds to phase changes, though in the static case these occur in defined increments and are imposed via piezo modulation, while in the dynamic case these occur naturally within the tissue itself. Even though the final image is obtained in 1 second, we are probing phase fluctuations, caused by intracellular displacements that are occurring in the 10-100 Hz range.

Computing the standard deviation over groups of 100 images, then averaging over the 10 resulting images produced...
the highest contrast in retinal tissues compared to various other signal analysis procedures. This approach actually probes the amplitude of fluctuations, which depend on both the fluctuation dynamics and the signal strength. The dependency on the signal strength is important to lower the contribution of regions without meaningful signal, where the shot noise still creates a significant fluctuation. Several other techniques to analyze interferometric signal fluctuations are discussed in a previous publication.17

**Imaging Protocols**

Fluorescence experiments were performed on the laboratory setup. En face fluorescence, static and dynamic FFOCT images of the sample were captured at planes of interest.

Dynamic and static FFOCT depth stacks were captured on both commercial and laboratory setups, with an en face image captured either every 1 μm in depth, or in larger depth steps in order to reduce data volume. Some stacks were also acquired every hour for 24-hour periods to monitor the tissue over time. The depth stacks with high NA are performed by correcting the defocus and adjusting the coherence volume and focal volume position according to a procedure previously described.16

Image acquisition was performed via custom-developed software in (LABVIEW; National Instruments, Austin, TX, USA) and laboratory system (MATLAB; MathWorks, Natick, MA, USA). Acquisition time for dynamic images took 5 seconds per plane, 10 minutes per stack on the commercial setup, and 1 second per plane, 2 minutes per stack on the laboratory setup.

**Image Processing**

Images are processed using Fiji (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health [NIH], Bethesda, MD, USA). Multimodal images are displayed in two different color channels and merged (e.g., Fig 2: fluorescence in green, OCT in gray; Fig 3: static in red, dynamic in green).

In color-coded dynamic FFOCT analysis (Figs. 4–8), the Fourier transform of the temporal fluctuations of each single pixel is calculated and divided into three normalized frequency bands.17 The red channel corresponds to the low frequency spectrum intensity (fluctuations between 0.1 and 0.5 Hz), green is associated with intermediate dynamics (fluctuations between 0.5 and 5 Hz) and blue with the fastest fluctuations (above 5 Hz). All channels are normalized to the maximal value of each channel of each image. The faster the fluctuations are, the faster the signal decorrelates, so that calculating frequency bands gives similar results in dynamic FFOCT to computing a decorrelation time or a fractal dimension.17,18 Here, we used frequency bands as they are much faster to compute for the large data sets involved (each plane corresponds to 4 GB of data). Identical color scale was used for color-coded images in Figures 4 through 8. This causes the images taken with the commercial setup to look “redder” (i.e., slower) than those with the laboratory setup which are “greener” (i.e., faster), as the high NA of the laboratory setup allows detection of more rapid movements.

Cell counting was performed manually using the “Cell Counter” plugin in Fiji (NIH) on en face slices by two authors. Cell density per optical slice was calculated, then multiplied by...
the number of cell layers detected per retinal layer, in order to obtain cell density per retinal layer in cells/mm² (Table). As FFOCT can produce volumetric data, in theory each cell in each layer could have been counted manually to directly count the number of cells in the entire retina. However, manual counting within a volume requires complex tracking of cells from one slice to the next. In addition, complete data stacks were not available for every sample due to data volume constraints, as each optical slice contains 4 GB of data. Our approach of counting cell density per optical slice and multiplying by the number of cell layers per retinal layer was devised as a compromise that combined both efficient counting practice and efficient data management.

For display purposes, time series were presented as movies showing an hour in each frame that passed, or with one time point displayed in magenta and a later time point in blue, as in Figure 9, in order to appreciate which structures are ceasing to be mobile over the imaging period. For analysis of signal decrease over time series, cells needed to be manually identified and tracked through the time stacks as tissue tended to move slightly during the acquisition period (e.g., due to swelling from edema and/or shrinkage as immersion liquid evaporated). The plot shown in Figure 9 shows the analysis of mean grayscale level on cells measured by manual plotting (i.e., by selecting the cell in each layer and measuring its mean grayscale level. In this plot, we show the mean over 4 macaque retinas, with 3 to 10 cells tracked per retina.

**RESULTS**

**Fluorescence-FFOCT Comparison**

Figure 2 gives an example of live GFP-labeled cells in the GCL, which precisely match in location to cells seen in the FFOCT channel, though not all cells visible in FFOCT are labeled. In particular the two large cells with a large eccentric nucleus, indicated by arrows in Figure 2, are unlabeled, suggesting that they are more likely to be displaced amacrine cells than ganglion cells, as the labeling is intended to be specific to ganglion cells. In addition their morphology fits with that of starburst amacrine cells. Figure 2 also gives an example of live GFP-labeled bipolar cells in the mouse inner nuclear layer (INL), where the distribution and morphology of the labeled cells is mirrored by cells of similar morphology and density in the dynamic FFOCT image, along with additional cells of different morphology and density which we infer are not bipolars.

**Static-Dynamic FFOCT Comparison and Color Coding**

Figure 3 and Supplementary Movie S2 show a depth stack in inner retinal layers acquired with the commercial system where merging the static and dynamic information in two color channels highlights the combination of static FFOCT (red) signal on fibers, vessels, and collagen of plexiform layers and dynamic FFOCT (green) signal on cells. The contrasts revealed with static and dynamic FFOCT are complementary as dynamic FFOCT reveals cells in the nuclear layers that produce little static FFOCT contrast compared to the plexiform layers.

Figure 4 shows selected slices from a color coded analysis of a dynamic image stack in the macaque retina where stationary (fibrous, red), slow moving (cells with large nuclei, green) and fast moving (smaller structures, blue) are revealed. Most retinal cell populations in the different layers of the retina can be recognized with dynamic FFOCT, by considering aspects such as cell morphology and stratification, along with particular motility dynamics revealed by color coding, in order to infer cell classifications. We note that in addition to retinal neurons, dynamic FFOCT is particularly efficient (Fig. 5) in detecting red blood cell contours since their membranes rapidly fluctuate with large amplitude. In retinal explants, red blood cells are trapped inside the capillaries, offering the possibility to study their dynamics in conditions close to physiology.

**Ganglion Cell Layer**

The Table contains all quantitative data on cell densities and sizes.

The GCL contains ganglion cells of many types, sizes and functions, as well as displaced amacrine cells. The GCL in Figures 3 through 5 appears to contain cell somas of varying size, some with a large dark eccentric nucleus surrounded by bright cytoplasm, while others have no visible nucleus. Others cells are smaller, with large nuclei but little cytoplasm. The sizes of these cells appear consistent with the literature on
Figure 3. Merged static (red) and dynamic (green) FFOCT images in macaque retina identify stationary (fibers, vessels, collagen) and dynamic (cell) structures. En face images, left to right, top to bottom, and associated cross-section (center), descending through the inner retinal layers: NFL, GCL, IPL, INL, OPL, and ONL. Supplementary Movie S2 shows the associated full image stack. Note that the color representation in this figure (static red and dynamic green) is different from that used in subsequent figures (color coding analysis within the dynamic FFOCT image, according to signal decorrelation time).

Figure 4. Color coding the dynamic signal for speed of signal decorrelation time allows discrimination of different structures in all retinal layers. Color-coded, three-channel stack of dynamic FFOCT images in macaque retina at 2 hours postmortem; color coded for speed of movement/signal decorrelation: the red channel shows low temporal frequencies (<0.5 Hz); green shows the intermediate frequencies (between 0.5 and 5 Hz); and blue shows the fastest pixels (>5 Hz). Fibrous structures are slowest (red); cells are intermediate (green); and subcellular details are fastest (blue).
RGC and amacrine cell diameter range (we find an average 16-μm GCL cell diameter in macaque and 14 μm in mouse, while the literature states a range of RGC diameter 8–23 μm, amacrine cell diameter 7–17 μm). As nuclear to cytoplasmic ratio in amacrine cells is greater than for RGCs,22,23 it may be that the small cells with large nuclei correspond to amacrine cells. On average, dynamic FFOCT measures cell densities in the ganglion cell layer between 3,000 to 6,000 (macaque at the center) cells/mm², which, for the macaque is 4 to 20 times lower than the value reported for macaque fovea24 and in mouse, includes the range reported.25 If we then scale the reported foveal data for macaque by the distribution of ganglion cells in human from near fovea to periphery, where 10 times fewer ganglion cells are found at the periphery,23 then our GCL cell density for macaque is also within the expected range.

**Inner Nuclear Layer**

The INL contains amacrine, Müller, bipolar, and horizontal cells, with somas of overlapping diameter ranges.25,26 Along with their relative proportions, we can use the stratification of these cells to help to deduce their identity. Within the INL, amacrine cells are known to occupy the first cell layer after the inner plexiform layer (IPL), followed by 3 to 4 layers of bipolar cells, with the (rare) horizontal cells lying at the INL/outer plexiform layer (OPL) junction.24,26 Amorphous, elongated Müller cells fit into the space between bipolar and amacrine somas.27,28 Dynamic FFOCT can identify in the central INL at least two different cellular morphologies, as illustrated in Figure 6. One type of neurons exhibit a small size and a nucleus that occupies almost the entire cellular space with a tiny nucleolus (possibly bipolar cells) and a second type are larger cells with higher cytoplasm-to-nucleus ratio (possibly amacrine cells). We could not identify Müller cells, possibly due to their unusual form. We found an average INL soma diameter of 7 μm in both macaque and mouse, in accordance with the literature.25,26 The cell density in the macaque INL was counted to be 100,000 to 130,000 cells/mm², and in mouse 120,000 to 160,000 cells/mm², which is within the ranges reported for macaque fovea,24 and for mouse.25 At the bottom of the INL in mouse, a few large cells with intermediate nucleus size were detected almost at the frontier with the OPL (Supplementary Movie S3). We infer that these cells are horizontal cells due to their stratification and size, and counted a density below 2,000 cells per mm² which is close to agreement with the literature.25

**Outer Retina**

At the frontier between the OPL and the outer nuclear layer ONL (Fig. 7), static FFOCT shows the fibrous layer of the OPL to the left of the image and many small spheres in the ONL that we identify as the photoreceptor pedicles and spherules (i.e., the synaptic endings of the photoreceptors that can be as large as 8 μm for the cones pedicules and 4 μm for the rod spherules).29 Dynamic FFOCT additionally detects photoreceptor cell bodies in the ONL on the right-hand side of Figure 6, due to slight tilting of the sample. Color-coded dynamic FFOCT shows a uniform green (average color) in this layer, indicating that dynamics are similar in all structures.

Finally, dynamic FFOCT can reveal photoreceptor inner segments (IS; Fig. 8), probably due to their high concentration of mitochondria. The larger cone IS can be easily distinguished from the smaller rod IS. Both static and dynamic FFOCT can detect the photoreceptor outer segments (OS), but dynamic FFOCT emphasizes their contours. The origin of the dynamic
signal in OS could include several possibilities: membrane fluctuations\(^30\); recycling of rhodopsin\(^31\); intrinsic phase change (elongation)\(^32\); swelling due to edema; or a combination of these and other factors. In the macaque retina presented in Figure 8, we counted a cone density of 2000 cells/mm\(^2\) and rod density 50,000 cells/mm\(^2\), which are reasonable values for the periphery of the primate retinas.\(^33\) Mouse photoreceptors were at the resolution limit of our systems and were therefore not quantified.

**Time Series**

Time series on retinal explants showed the death of cells over time (Fig. 9) and the associated decrease in the dynamic signal. After several hours imaging in an uncontrolled environment (immersed in Neurobasal and HEPES medium, but not oxygenated and not maintained at 37°C), most of the cells can no longer be detected with dynamic FFOCT, indicating that their activity has ceased. Mean dynamic signal of cell nuclei decreases over time (e.g., Fig. 9 shows 25% intensity reduction over 18 hours). Signal in background tissue (e.g., collagen) remained relatively constant compared to signal in cells.

**DISCUSSION**

FFOCT is capable of imaging cells, and in conjunction with fluorescence, cells can be identified for validation purposes. However, the addition of dynamic FFOCT reveals a far greater quantity of cells throughout the retina, without the need for invasive labeling. Dynamic FFOCT complements rather than...
replaces static FFOCT, as static FFOCT gives the highest contrast on fibers, vessels, and collagen, while dynamic FFOCT gives the highest contrast on cells. The dynamic component therefore acts as a contrast agent for the cells. Static and dynamic FFOCT should therefore be used together to produce the best possible image, purely in the interests of maximizing contrast, particularly considering that dynamic FFOCT requires virtually no hardware modification other than holding the piezo mirror stationary, and acquisition of the static and dynamic parts can be performed sequentially by the software in an automated fashion. Additionally, high transverse resolution dynamic FFOCT can reveal subcellular contrast including nuclei size and position, and frequency-dependent color coding serves to highlight subtle differences in these structures that can aid in identification of the imaged cell type. Due to the high level of complexity of the retina, structural information is however not always enough to infer the cell type. Functional information contained in the decorrelation time, or fractal dimension, or any other frequency-dependent parameter characterizing the dynamic signal, exhibits small differences between cells. We were able to successfully separate the fractions of slow and fast dynamics in spheroids using such an analysis, and we hope to further improve analysis in order to refine label free cell classification in other cell cultures or tissues.

### Table

<table>
<thead>
<tr>
<th>Species</th>
<th>Layer</th>
<th>Measured Cells/mm² in En Face Optical Slice</th>
<th>Detected Number of Cell Layers</th>
<th>Calculated Cells/mm² per Retinal Layer</th>
<th>Cells/mm² in Retinal Layer in Literature</th>
<th>Measured Cell Soma Diameter</th>
<th>Cell Soma Diameter in Literature</th>
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<tbody>
<tr>
<td>Macaque</td>
<td>GCL</td>
<td>1,500</td>
<td>2–4</td>
<td>5,000–6,000 (measured at periphery)</td>
<td>26,000–65,000/mm² (measured at fovea)</td>
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<td>8–25 µm</td>
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<td></td>
<td>INL</td>
<td>16,000</td>
<td>6–8</td>
<td>100,000–130,000 (measured at periphery)</td>
<td>75,000–180,000 (measured at fovea)</td>
<td>7 µm</td>
<td>7–17 µm (amacrine); 6–9 µm (bipolar)</td>
</tr>
<tr>
<td>Macaque</td>
<td>PR</td>
<td>2,000 (cones); 50,000 (rods)</td>
<td>1</td>
<td>2,000 (cones); 60,000 (rods) at periphery</td>
<td>2,000 (cones); 12 µm (cones); 4 µm (rods)</td>
<td>4 µm (rods) in far periphery</td>
<td>7–17 µm (amacrine); 6–9 µm (bipolar)</td>
</tr>
<tr>
<td>Mouse</td>
<td>GCL</td>
<td>4,000</td>
<td>1–3</td>
<td>4,000–12,000</td>
<td>5,000–10,000²⁵</td>
<td>9–22 µm</td>
<td>10–20 µm</td>
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<tr>
<td>Mouse</td>
<td>INL</td>
<td>20,000</td>
<td>6–8</td>
<td>120,000–160,000</td>
<td>100,000²⁵</td>
<td>7 µm</td>
<td>6–9 µm²³</td>
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</table>

**Figure 9.** Dynamic FFOCT contrast decays over time in macaque retinal GCL. The top left panel shows a typical GCL image immediately after tissue dissection (time point, T = 0). The top-right panel shows the same region 6 hours later and illustrates the decay of the dynamic FFOCT contrast over time. The top center panel displays the overlay of left (in magenta) and right (in blue) panels to illustrate which features have decayed. The graph shows an analysis of the decay of the dynamic FFOCT signal in manually tracked cells in four different macaque retinal explants (3 to 10 cells tracked per time stack) over an 18-hour period. Manual cell tracking through the stacks was necessary for analysis due to slight tissue motion over the imaging period, due to tissue swelling or shrinkage. The signal reduces by 25% over this time.

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Although we cannot be certain that dynamic FFOCT is revealing all of the retinal cells, or indeed all retinal cell types, the cell densities we measured are reasonable values when compared to the literature, where figures exist at these eccentricities. The packing of the cells seen in the images seems to be at its maximum (i.e., with no empty space) in several layers (Figs. 4, 5), meaning the cell density we are measuring should be valid for the samples we have imaged. Departures from cell densities reported in the literature, by a factor of 4 to 20 for macaque\textsuperscript{24} could be explained by differences in retinal location between our study (peripheral, situated along median raphe where axon density is lower and cells are larger, rather than at the fovea where density is maximal), and differences from animal to animal (i.e., the literature is not all based on the type of macaque (Macaca fascicularis) and mouse (C57) we measured here). Finally, dynamic images may not reveal all cells, as motility within the explanted retinas may be reduced compared to the in vivo case, i.e. we do not see cells that are completely inactive. Interestingly, a recent demonstration of in vivo dynamic adaptive optics OCT (AO-OCT) imaging (Liu Z, et al. IOVS 2017;ARVO E-Abstract 3430) found fewer cells than predicted due to their high motility (Thouvenin O, unpublished observations, 2017).

Another future direction for dynamic FFOCT could include functional imaging in retinal explants, by modifying the setup to move to longer imaging wavelengths to approach scotopic conditions, and adding a visible light stimulation channel, to record possible changes in the dynamic signal due to activation, in a similar manner to intrinsic optical signal imaging demonstrated with optical coherence or confocal microscopy.\textsuperscript{39,40} With the additional option of using the fluorescence channel, this type of approach to functional imaging may have potential in the field of optogenetics research,\textsuperscript{41} and calcium imaging.\textsuperscript{42} Interestingly, as dynamic FFOCT relies on phase fluctuation measurements, it should be sensitive to activity-related phase changes in photoreceptor IS as reported\textsuperscript{43} and might give a specific signal related to photoreceptor activity. By switching the illumination wavelength, it could be a promising approach to estimate the distribution of blue cone photoreceptors for example, similar to that which was recently shown with AO-OCT (Jonnal RS, et al. IOVS 2017:ARVO E-Abstract 308). We also expect retinal neurons to experience a change of dynamics during electrical activity, so that dynamic FFOCT might provide truly functional imaging of most retinal neurons. Finally, because phase sensitive OCT and FFOCT can also measure blood flow,\textsuperscript{44} we emphasize the promising future of applying such dynamic phase strategies (and especially high transverse resolution OCT technologies) in retina for studying and understanding neurovascular coupling at the cellular level in vivo.

In conclusion, the major strength of the static and dynamic FFOCT technique is that without depending on contrast agents, and the complex, time consuming experiments their use implies, essentially all of the cells in a living explant can be measured. While specific external markers may be more efficient for a specific scientific question, static and dynamic FFOCT might be more efficient for issues relating to overall retinal changes. In addition, the opportunity to perform time lapse imaging on a living retina for extended time periods opens up the possibility of imaging the explants in culture conditions, and allowing exploration of response to external factors for example, provided a suitable culture chamber is developed (providing similar conditions to those described in Leroux et al.\textsuperscript{18}). Indeed, we are currently investigating disease models of retinal pathology and inflammation using dynamic FFOCT as a tool to measure cell dynamics in pathological tissue. It is hoped that such an approach may provide insight into the evolution of pathology.

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