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Abstract. The cytoarchitecture of the human brain is of great interest in diverse fields: neuroanatomy, neurology, neuroscience, and neuropathology. Traditional histology is a method that has been historically used to assess cell and fiber content in the ex vivo human brain. However, this technique suffers from significant distortions. We used a previously demonstrated optical coherence microscopy technique to image individual neurons in several square millimeters of en-face tissue blocks from layer II of the human entorhinal cortex, over 50 μm in depth. The same slices were then sectioned and stained for Nissl substance. We registered the optical coherence tomography (OCT) images with the corresponding Nissl stained slices using a nonlinear transformation. The neurons were then segmented in both images and we quantified the overlap. We show that OCT images contain information about neurons that is comparable to what can be obtained from Nissl staining, and thus can be used to assess the cytoarchitecture of the ex vivo human brain with minimal distortion. With the future integration of a vibratome into the OCT imaging rig, this technique can be scaled up to obtain undistorted volumetric data of centimeter cube tissue blocks in the near term, and entire human hemispheres in the future. © 2015 SPIE Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.NPh.2.1.015004]

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1 Introduction

Cellular features of the human brain are not fully observable with current in vivo imaging technologies [i.e., magnetic resonance imaging (MRI) and positron emission tomography]. The importance of these features is raised by the observation that many diseases are defined by tissue properties or neuronal loss that are not visible individually in living humans. The quantification of stereologic factors such as counting neurons typically performed in postmortem histology helps define disease diagnosis and severity such as Alzheimer’s disease.1–8

Traditional histology provides the ground truth for neuroanatomy and neuropathology, and remains by far the most common way to visualize neurons and axons. Recently, a three-dimensional (3-D) model of a human brain, called BigBrain, at a nearly cellular resolution of 20 μm and based on the reconstruction of 7404 histological sections was created.9 Each of the 7404 slices were beautifully stained and digitized. However, traditional methods are labor intensive and introduce irredeemable distortions due to cutting, mounting, and staining. Distortions lead to challenges in registering histology slices back to minimally deformed volumetric data such as blockface images, ex vivo MRI, and in vivo MRI.10–14 and in registering each slice to its neighbors to generate undistorted 3-D volumes.

Optical imaging has emerged as a promising alternative to traditional histology. For example, two-photon microscopy provides undistorted high resolution volumetric imaging of brain tissue over several hundreds of microns in depth.15–17 In standard microscopy, fluorescent dyes label structures or proteins of interest. When coupled with a vibratome, larger volumes are imaged, such as a full mouse brain.16,17 To improve the depth penetration of light, tissue can be cleared using techniques such as CLARITY20 and rendered optically transparent while retaining the structural anatomy. The tissue can then be stained, imaged by two-photon microscopy, and repeated, which allows undistorted results, automatically registered with diverse and specific immunocytochemical information. All these methods, however, rely on staining that contains multiple steps and can be a long process for several cubic centimeters of tissue blocks. In addition, current CLARITY techniques can only clear a few hundreds of microns or at most a millimeter or so of the myelinedense human tissue, making it currently infeasible to image large sections of the human brain. Optical coherence tomography (OCT)21 is versatile and generates cytoarchitecture and myeloarchitecture-like images.12,22–24 OCT relies solely on the intrinsic optical properties of the tissues, mainly those of neurons and myelinated fibers.22,24–26 No dye is required and the tissue block is directly imaged, no sectioning is necessary prior to image acquisition. This last point is critical as imaging before cutting implies that the vast majority of distortions that plague standard histology are avoided in the OCT images. It is important to note that while optical coherence microscopy has been performed in vivo or on fixed rodent tissue previously,22–24 fixed human tissue has been far less studied. In large part, this is due to the challenges one faces in analyzing human tissue that

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pose significant hurdles. For example, the time period between
death and fixation, called the postmortem interval (PMI) ranges
domains, while in rodents it is essentially
during this interval, there are significant autolytic pro-
tic processes that degrade tissue quality, making subsequent analyses
more difficult and variable. Similarly, rodent brains are typically
perfusion fixed through their vascular system, providing rapid
and homogeneous fixation of the tissue that is not possible in
humans. In contrast, full fixation in humans requires a month
or more of immersion in fixative, and fixation time is, therefore,
variable across the brain, with deep white matter (WM) regions
fixing last. Further, environmental and dietary variability is
enormous in humans vis-à-vis rodents, in which living condi-
tions and food supply are tightly controlled. Finally, rodents
are typically sacrificed early in their normal lifespan, as opposed
to most human tissue that comes to autopsy only at old age or as
a consequence of some traumatic or pathological cause of death
that can degrade tissue quality. Here, we demonstrate micron-
resolution OCT to image individual neurons in human ex vivo
tissue at various depths in the first 50 μm, and validate this im-
aging technique with Nissl staining of the same 50-μm thick
tissue. We used layer II of entorhinal cortex (EC) samples of
human brain. Images obtained by both modalities (OCT acquis-
tion and digitized Nissl stained sections) were registered using a
nonlinear transform. Once registered, the degree of overlap was
assessed to quantify the agreement between the neuronal content
obtained with OCT and with the gold standard Nissl stain.

2 Optical Coherence Tomography

For this study, we used a spectral domain optical coherence
tomography/optical coherence microscopy (OCM) that was
described in Ref. 22. The broadband light source is provided
by a superluminescent diode (LS2000B SLD, Thorlabs Inc.,
Newton, New Jersey) with a center wavelength of 1310 nm
and a full width at half maximum of about 200 nm, which
yielded an axial resolution of 4.7 μm in air (3.5 μm in tissue).
The spectrometer consisted of a grating and a 1024 pixel
InGaAs line scan camera (Thorlabs Inc.), which provides a
depth of field of 2.2 mm in air (1.5 mm in tissue) and an
axial pixel size of 2.9 μm. In the sample arm, two objectives
were used: a 10× water immersion objective (Zeiss N-
Achroplan 10× W, NA 0.3) that gives the global laminar struc-
ture of the cortex, and a 40× water immersion objective
(Olympus LUMPLANFL/IR 40 W, NA 0.8) that allows the im-
aging of individual neurons. The lateral resolution of the two
objectives was 2.5 and 1.25 μm, respectively. Each volume con-
isting of 1024 frames with 1024 axial scans per frame, was
acquired over a field of view (FOV) of 1 mm × 1 mm and
400 μm × 400 μm, respectively, corresponding to a lateral
pixel size of 0.97 and 0.39 μm for the 10× and 40×, re-
spectively. To cover the whole sample, the tissue was placed on a
manual xy translation stage (Optometrix, 1 in. displacement).
The x and y displacements allow for overlap between the vol-
umes in order to reconstruct the sample by stitching the images
using a Fiji plug-in based on the Fourier shift theorem.27

3 Tissue Samples

Three human brains were obtained from the Massachusetts
General Hospital Autopsy Suite (Boston, Massachusetts). The
demographics were as follows: mean age 50.3 ± 11.0 y.o.,
1M/2F, PMI <24 h, neurologically normal. Each brain was
immersed in 10% formalin for at least 2 months until thoroughly
fixed. A subregion within the EC of each brain was then blocked
in several square millimeters of en-face area (approximately
3 × 5 mm²). The samples were then embedded in melted oxi-
dized agarose and covalent cross-linking between tissue and
agarose was activated using borohydride borate solution.18

4 Histology Protocol

A vibratome (TissueCyte 1000, TissueVision), described in
Ref. 18, was used first to flatten the samples and subsequently
section 50 μm slices after the imaging over this depth was per-
formed. The agarose from the sections was removed by heating
the phosphate buffer above the agarose melting temperature
(above 50°C) and rinsing the slices for about 3 s. The sections
were then mounted onto gelatin dipped glass slides and stained
for Nissl substance, revealing mainly neuronal and glial cells
details in our previous paper. The stained slices were digitized
with a camera mounted on an 80i Nikon Microscope (Microvideo
Instruments, Avon, Massachusetts) with high magnification
(20×), giving a pixel size of 0.37 μm. We used the image series
workflow (“SRS Image workflow”) provided by Stereo
Investigator (MBF Bioscience, Burlington, Vermont) to automati-
cally mosaic the entire slice, and the tiles were then stitched with
the same Fiji plug-in used for the OCT images. Figure 1(a) shows
a Nissl stained section of EC where layers I–VI as well as WM
tabers were labeled. We confirmed that layer II exhibits large neurons in
island formation in our samples.

5 OCT Acquisition

As shown in our previous publication,14 to obtain the overall
laminar structure of the EC using the 10× objective, the average
intensity projection over 300 μm in depth from the surface
of each volume was performed, filtered28,29 and intensity adjusted
to enhance the contrast. The images were then stitched together
to obtain the full cortical ribbon of the sample. Figure 1 shows
the cortical layers in EC that are easily observable with OCT
imaging [Fig. 1(b)] and corresponding Nissl [Fig. 1(a)].
Layers exhibit different intensities related to their cellular archi-
tecture and myelin content.30 To evaluate the capability of OCT
to accurately resolve individual neurons, we performed high-
resolution imaging on layers I–III in EC. The 40× objective pro-
vides a depth of focus of about 10 μm. Therefore, at each xy
position of the sample, we acquired five volumes, every
10 μm in depth, starting at 5 μm under the surface until the
first 50 μm of the sample was imaged. The framed inset in
Fig. 2 shows the depth schematic where the dashed lines re-
present the five different focus depths from which we acquired
data. Contrary to what has been reported concerning freshly
resected brain tissue or rodent brains23,24,31 in which neurons
exhibit a lack of backscattered light (shown as black spots in
the images), neurons in ex vivo fixed human brain highly back-
scatter the light compared to the surrounding tissue, resulting in
high intensity (white spots). Thus, for each of the five volumes,
the maximum intensity projection (MIP) over 10 μm around the
focus plane (hatched regions around the focus planes) was per-
formed to highlight the neurons. Each tile was then filtered and
intensity adjusted. For each different depth, the tiles were
acquired across the whole region of interest and were stitched
using Fiji to provide the full FOV. Figure 1(d) shows an exam-
ple of a high resolution OCT image corresponding to the Nissl
stained slice and lower resolution OCT image. For the full corti-
ical lamina, only one focus depth (25 μm, MIP over 10 μm
around the focus) was imaged and presented. Neurons of layers
Fig. 1 Cortical layers of entorhinal cortex (EC) observed using Nissl staining (a) and average intensity projection of lower resolution optical coherence microscopy obtained with a 10× objective (b). Layers I–IV have been labeled, as well as the white matter. The individual neurons can be observed by OCT using a higher magnification (c). The insets (d-i) show the different structures observed by OCT (e, g, i) and its correspondence in the Nissl stained slice (d, f, h): vessels found in layer III (d, e), neurons of layer II (f, g), and neurons of layer IV (h, i). Myelinated fibers care also visible in the OCT images. Scale bar: 500 μm.

Fig. 2 Images obtained of the same sample at five different focus depths (a-e). Dashed lines of the framed inset represent the different focus. The MIP was performed over the 10 μm around the focus (dashed regions of the inset). (f) Composite image representing the neuron content over 50 μm in depth and (g) the corresponding Nissl stained slice of 50 μm section thickness. Scale bar: 500 μm.
II and IV were visible, as shown in the inset of Figs. 1(g) and 1(i) and correspond to neurons observed on the Nissl stained slices [Figs. 1(f) and 1(h)]. Figures 2(a)–2(e) show the images obtained at the five different depths. Next, the five full images corresponding to each depth were stacked and the MIP in depth was performed to visualize all the neurons contained in the 50-µm thick slices of the Nissl stained image. The resulting OCT image is shown in Fig. 2(f) and the corresponding Nissl stained slice in Fig. 2(g). The modular organization of layer II is clearly visible both in the OCT and the Nissl stained images. The dendrites arising from the neurons are visible on both images. However, on the OCT image, tangential fibers in layer I can also be observed.

### 6 Registration and Segmentation of Neurons

To assess the colocalization of the neurons between histologically stained slices and OCT images, the images were registered. Histology protocols can suffer from irretrievable distortions such as tears due to the sectioning, shrinking occurring during the drying process, and geometric warping during mounting. In spite of precautions taken to avoid distortions during the tissue processing, artifacts cannot be avoided completely in large human tissue samples. (For animal histology, these distortions can be reduced significantly just over

Next, the neurons were segmented. The neuronal segmentation in the Nissl-stained images was done using adaptive thresholding based on the implementation provided in the freely available OpenCV library. The thresholds used in the adaptive threshold at $(x, y)$ were the mean value of the $S \times S$ neighborhood of $(x, y)$ minus a constant $C (S = 105, C = 45)$ in Nissl-stained images. The segmentation was manually edited to add neurons whose contrast was insufficient to be identified automatically, and to remove glia and vessels that were segmented incorrectly. OCT images reveal more than just neurons. Vessels, dendrites, and possibly axons are also present in OCT images, for example, and exhibit the same kind of contrast. Moreover, the images are noisier than the Nissl stained slices. We first reduced the noise on each of the images obtained at the five different depths using a nonorthogonal wavelet algorithm optimized on a region of the image containing mainly noise (no visible neurons, fibers, or vessels). We then used adaptive thresholding $(S = 105$ and $C = 25)$ to segment the neurons. As we did for the Nissl stained slices, we manually edited each segmentation to remove nonneuronal features and to add missing neurons. Finally, those five segmentations were overlaid to generate the final OCT segmentation corresponding to the 50-µm Nissl stained slice.

### 7 Results and Discussion

Figure 4 shows the registered Nissl stained slices (left panel), OCT images (center panel), and overlap of the segmented neurons found in layer II, delineated on the Nissl and OCT images by the lines (right panel) for the six tissue samples studied: green for Nissl, red for OCT, and yellow for the overlap. For each brain sample (A, B, and C), two slices were imaged, sectioned,
and stained (1 and 2: two different slices from the same case). The agreement between the cytoarchitecture observed by the traditional Nissl stain and the OCT imaging appears excellent in case A, slice 1 (A1) and 2 (A2), good for slice B1 and case C (C1 and C2), and finally fair for B2. The agreement was visually assessed by CM. Looking closely at the overlay [Fig. 4 (left)], we can see that the shapes of the corresponding neurons are overall visually the same. However, a slight shift is often

Fig. 4  Colocalization of the neurons in layer II of EC for six different tissue samples: registered Nissl stain (left), OCT image (center), and the overlay of the segmented neurons (right): green for Nissl, red for OCT, and yellow for the overlap. Scale bar: 500 μm.
observed showing the limit of the registration between OCT images and histological slices that underwent multiple physical transformations (slicing, mounting, and drying).

At least two possible reasons may explain the varied qualitative colocalization across samples. The first explanation lies in the registration of the Nissl stained slice to the OCT image, which depends on tissue integrity, sectioning, and mounting on the glass slides. All the slices but one (B2) were well prepared, with no major distortions observed and homogeneous tissue thickness. We observed that slice B2 was thinner on its right side. As a result, the density of neurons was lower on this side (Fig. 4). This thickness discrepancy within tissue is due to mispositioning of the sample with regard to the vibratome blade plane. In our present protocol, the vibratome is not yet integrated with the OCT rig and is a separate apparatus. The sample, once flatfaced, is moved to the OCT system for the acquisition and returned to the vibratome for the sectioning. When the sample is not placed exactly in the same position relative to the blade (difference in height and/or tilt occurs), the thickness is not uniform. In the future, the vibratome will be integrated with the OCT rig to remove this complication.

The second explanation is the OCT imaging itself. Ideally, we focus the light at the depth of 5 μm for the first image and then move the sample in 10 μm increments until the first 50 μm of the tissue is imaged, corresponding to the stained tissue. At the beginning of each experiment, we visually position the sample so that the light is focused at the surface and then the sample is moved up 5 μm. Exact positioning of the sample is challenging since the axial pixel size is 2.9 μm. After visually noting the discrepancy in the neuronal content agreement between modalities, we decided to evaluate the position of the light focus with respect to the surface. The surface and the focus planes were fitted by a third degree polynomial surface on every volume acquired at the five different focus depths on the region of interest (layer II). For the first theoretical depth (5 μm), the focus plane and the surface plane are too close to clearly differentiate them. We used the four subsequent theoretical depths to evaluate the experimental depth of focus and use a linear regression to assess the first experimental depth. Table 1 shows the results in the last column. Moreover, we used a built-in algorithm (bwlabel) in the MATLAB programming environment to count the number of neurons on the OCT segmentation image (binary image) obtained at the five different depths (neurons on OCT) and the number of those segmented neurons that are corresponding to neurons on the Nissl segmentation image (overlap on OCT). Finally, we used the same algorithm to assess the total number of neurons segmented on the final OCT segmentation (after overlaying the segmentation of the different depths) and the Nissl segmentation. For each slice, Table 1 reports the intended focus depth, the number of neurons on the OCT at that depth, the number of overlapping neurons, and the percentage of overlapping neurons normalized by the total number of neurons in OCT at each depth. Finally, we also noted the total neurons on OCT and Nissl staining in Table 1. The total number of neurons observed on the final image of OCT (for the 50 μm) is underestimated due to the presence of different neurons at different depths. It does not reflect the number of neurons we would obtain by adding the numbers of neurons at each depth. However, this total number of neurons is in excellent agreement with the number of neurons found on the Nissl stained images (with the exception of case B1, which will be explained in the next paragraph). Contrary to OCT,

<table>
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<th>Case</th>
<th>Depth (μm)</th>
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<th>Percentage (%)</th>
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neurons at different depths cannot be differentiated automatically from the Nissl images—an important advantage of OCT. For case A, the focus position was good, between 8 and 11 μm under the surface. The overlap between OCT and Nissl is above 69%. The overlap is a little lower for the last depth, which could be attributed to the slight mispositioning of the initial focus depth. Slice B1 shows a good overlap over the five different depths, over 70% even though the initial focus depth is about 18 μm. The thickness of this slice was checked using a microscope (with Stereo Investigator, MBF Bioscience) and appears to be thicker than 50 μm. This is confirmed by the fact that the total number of neurons found on the Nissl stained slices is about 1.5 time higher than the one reported on the OCT images. For the remaining slices, those numbers are comparable. B2 exhibits excellent overlap for the first two depths (over 90%), and a subsequent decrease for the next depths, explained by the deeper initial focus depth, around 20 μm and the uneven sectioning (apparent tilt on the sample during the sectioning) of the tissue as discussed previously. Slices C1 and C2 show a good agreement also, the overlap being above 68% except for one depth. This discrepancy could be due to a false positive segmentation on the OCT images.

8 Toward Neuropathology and Whole Brain Imaging

Even though OCT does not have the molecular specificity of histology, this technique shows a variety of features, such as healthy neurons, vessels, and possibly axons as can be seen in the insets of Fig. 1. To assess the connectivity in the human brain more accurately, we will add polarization imaging to our OCM (polarization sensitive OCM) as shown in Wang et al.27,28 and obtain fiber orientation in addition to the backscatter contrast shown in this study. OCT may also be applicable to neuropathology, such as for the diagnosis of Alzheimer’s disease, traumatic brain injury, tumor,1 and cerebral amyloid angiopathy among others. For example, OCT can visualize amyloid plaques, as was shown in Bolmont et al.39 in a mouse model of Alzheimer’s disease. As shown in Fig. 4, each brain exhibits slightly different contrast when imaged by OCT depending on its contents. Case A very clearly shows the processes of the neurons, whereas case B is heavily myelinated (fibers running from WM to pial surface, as well as transversely). The automatic segmentation of these cortical features is a challenge that we are investigating.

By improving and expanding our OCT system and postprocessing, larger brain regions can be imaged with minimal distortion. We have demonstrated that OCT can visualize neurons in depth by imaging at different focus planes under the tissue surface. To reduce the imaging time required to obtain volumetric data, an extended focus depth can be implemented both on the OCT setup itself by using a Bessel beam illumination36,41 or phase apodization,42 and in postprocessing by implementing digital refocusing.33-44 In the future, we plan on increasing the speed of data acquisition by using a camera with a 150 kHz scan rate (GL2048 from Sensors Unlimited). By coupling the vibratome to the OCT as suggested in Wang et al.,44 several cubic centimeters of tissue can be imaged with negligible distortion. The sectioning of the tissue will then be homogeneous and only dependent on the z-stage and the vibratome precision, which is better than 2 μm.39 Detection of the focus depth will also be implemented in our acquisition software to control the initial position of the sample with respect to the light focus.

9 Conclusion

In this study, we showed that OCT can discriminate healthy neurons in ex vivo fixed human EC. This technique has been validated by the histological Nissl staining. The same 50 μm of tissue was imaged by OCT and then stained with Nissl. The modalities were registered using a nonlinear transformation, neuronomally segmented, and the overlap was quantified. The results showed good colocalization. Moreover, we demonstrate that OCT can discriminate the neurons in depth.

In conclusion, we demonstrated that OCT/OCM is a promising technique to image the postmortem human brain at the level of single neurons. One critical advantage of OCT over Nissl staining is the minimal distortion of tissue, since the blockface tissue is imaged prior to sectioning. OCT paves the way to undistorted, high resolution, 3-D visualization of the cytoarchitecture in the human cortex. We anticipated that OCT can have a far-reaching impact in both basic neuroscience and clinical neuropathology.

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