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Temporally Registered Optical Coherence Tomography and Fluorescence Microscopy for in vitro Detection of Neural Activity

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Temporally Registered Optical Coherence Tomography and Fluorescence Microscopy
for in vitro Detection of Neural Activity

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Md Rezuanul Haque

December 2014

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ABSTRACT OF THE DISSERTATION

Temporally Registered Optical Coherence Tomography and Fluorescence Microscopy for in vitro Detection of Neural Activity

by

Md Rezuanul Haque

Doctor of Philosophy, Graduate Program in Bioengineering
University of California, Riverside, December 2014
Dr. B. Hyle Park, Chairperson

Current methods for investigating activity in neurons largely rely on different varieties of electrodes, which require direct or very close contact, or fluorescence-based techniques, which require chemical or genetic introduction of a voltage- or calcium-sensitive fluorophore. The primary motivation for this work was to develop an intensity (non-phase) based extension of optical coherence tomography (OCT), an optical technology capable of rapid imaging of subsurface tissue structure with micrometer resolution without the need for exogenous contrast agents, for in vitro detection of neural activity. First, a preliminary study of epileptiform activity in murine hippocampal brain slices was used as an assessment of OCT for in vitro detection of neural activity. Elevated potassium was used to induce seizure-like bursts of activity. Decreases in OCT light intensity were found to be correlated with the initial onset of large-scale activation using simultaneous electrophysiological detection with a multi-electrode array. These results motivated the development of a novel spatiotemporally co-registered OCT and fluorescence microscopy (FM) system capable of observing neural activity
simultaneously with both modalities. The combined system was designed to provide sequential OCT volumetric imaging over an extended depth range with fluorescence imaging through a common objective. This allows for the results of analytical methods developed to endogenously detect neural activity based on OCT intensity to be correlated with changes in fluorescence. Finally, the system was used to observe neural circuitry in the brain of *Drosophila* pupae. The fluorescence response of GCaMP-3 labeled bursicon and kinin cells in response to presentation of ecdysis triggering hormone (ETH) is well-characterized. Time sequences of OCT volumetric data acquired over these neurons in *in vitro* adolescent Drosophila brain in response to presentation of ETH were analyzed and compared to simultaneously recorded fluorescence data. Changes in the backscattered OCT intensity in both cell types were found to correlate well with neural activity as identified by fluorescence. These results demonstrate the potential of OCT for *in vitro* detection of neural activity without the need for fluorescence contrast.
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Introduction

Detection of neural activity is critical for understanding how neural processes operate. A great deal of information can be obtained by using traditional electrophysiology but direct or very close contact must be established between the nerve and electrode, which requires a level of invasiveness that may damage the nerve. For fluorescence methods, different dyes or genetically modified indicators are used for activity detection. A non-contact label free technique for detection of neural activity will be user friendly and beneficial for various neural studies. Optical coherence tomography (OCT), being a minimally-invasive technology, is suitable for identification of neural activity in non-contact and label free manner. This dissertation is aimed to assess OCT as a functional tool for detection of neural activity by comparing with standard methods for activity detection. Phase-sensitive OCT has potential of identifying neural activity from a fixed lateral location of neurons through which action potential propagates. To study a group of neurons located in a region, it is necessary to identify activity from different lateral locations in that region. But phase sensitive OCT measurement is limited to one single lateral location due to the fact that lateral scanning introduces significant phase noise which makes phase sensitive analysis to be nearly impossible. But intensity based OCT information can be obtained from different lateral locations by scanning. So the goal of this dissertation is detection of neural activity from a single/ different lateral locations of neurons by intensity (non-phase) based OCT measurements. Firstly, in this dissertation, phase sensitive OCT will be used to identify single action potential propagation in *limulus* optic nerve. For stepwise verification of intensity based OCT as a tool for neural
activity detection, OCT is used to identify optical changes in mouse brain slice during seizure like activities as a preliminary level of assessment. For next level of assessment, a registered spatially and temporally registered OCT and fluorescence microscopy (FM) system was developed and used to identify neural activity in neurons of *Drosophila* pupae by both modalities.

The dissertation was divided in five different chapters. Chapter 1 describes the working principles of OCT. The latter portions of the chapter introduce fluorescence microscopy and explain working principle of fluorescence microscopy.

The second chapter of this dissertation focuses on detection of action potential in *limulus* optic nerve by OCT. Action potential was detected in optic nerve by identifying the thickness change of nerve in nanometer level by phase sensitive measurements of OCT. As phase sensitive OCT collects phase information from different depths of nerve, the OCT phase sensitive data were analyzed to identify neural activity in different axon bundles from different depths of nerve. A correlation algorithm was developed to identify neural activity in different depths of nerve. The results were compared with electrophysiology. The main important points in this chapter are: (i) a common path OCT system has been developed and used for phase sensitive measurements, (ii) functional stimulation was used to induce neural activity instead of electrical stimulation, (iii) the thickness change of ~20-70 nm was detected by OCT in limulus optic nerve during action potential propagation and (iv) a correlation technique was developed and used to identify neural activity in different axon bundles from different depths of nerve. The chapter
explains background of this study, animal model preparation, experimental procedures and the results.

It is not possible to identify neural activity in different lateral locations of neurons by phase sensitive OCT as scanning is not possible by this method while intensity based OCT can obtain intensity information from different lateral locations by scanning. The goal of third chapter of this dissertation is identification of change in OCT intensity during burst of activities, which will preliminarily assess intensity based OCT as a tool for detection of neural activity. Third chapter focuses on identification of change in OCT intensity in mouse brain slice during burst of activity. This chapter explains about animal model preparation, experimental set up of combined microelectrode array (MEA) with OCT, structural imaging of mouse brain slice and the results. The main points of this chapter are: (i) MEA was incorporated with OCT for electrophysiological measurements. (ii) OCT intensity was found to be decreased during onset of bursts of activity. The results verify that intensity based OCT is capable of identifying bursts of activity from group neurons. The limitation of using MEA is, MEA cannot provide electrophysiological information from different depths of sample and also the lateral resolution of MEA is very poor (~ 100µm) compared to OCT resolution (~3-15µm).

By using fluorescence microscopy (FM), fluorescence information can be obtained from different depths of sample within some extent. Addition to depth information, FM provides better resolution and is well accepted as a functional tool for detection of neural activity. So, FM is more comparable with OCT to verify OCT as a functional tool for neural activity detection. In chapter 4, FM was incorporated with OCT for better and next
level of comparison. Fourth chapter describes about developing a combined OCT and FM system. This chapter mainly focuses on: (i) development of hardware and software for spatial and temporal registration and (ii) verification results that confirm that OCT and FM system are spatially and temporally registered.

The overall goal of this dissertation was to assess intensity based OCT system for identification of neural activity and for gold standard comparison FM was incorporated with OCT. The animal model Drosophila was chosen for experiments as fluorescence changes due to neural activity is well known and well characterized for this animal model. In chapter 5, the developed registered OCT and FM system was used to identify optical changes in neurons of Drosophila pupae during induced activity. It was found that OCT intensity reduces during neural activity also identified by fluorescence measurements. The correlated results verify that intensity based OCT has potential for detection of neural activity in label free manner. Fifth chapter explains in details about animal model preparation, experimental procedures and results of these experiments.
Chapter 1: Introduction

1.1 Optical coherence tomography (OCT)

Optical coherence tomography (OCT) was first demonstrated by D. Huang et al. in 1991 in cross-sectional retinal imaging [1]. Since then it has become an important tool in biomedical imaging [2-32]. Optical coherence tomography has gained significant interest in biomedical researches due to its high resolution cross-sectional images of the biological tissues, non-invasive nature which allows to image in vivo imaging and high speed real time 3-D imaging of the samples. OCT is well known as an “optical biopsy” as it is minimally-invasive technique to provide anatomical structure of the tissue in a micron scale. OCT has resolution of 1-10µm and penetration depth of 1-3mm [2-30]. It resides in a critical niche between confocal microscopy and ultrasound methods in respect of penetration depth and resolution (figure 1.1) [33].

OCT has received significant interest in biomedical imaging and is now accepted as one of the major biomedical imaging technique. OCT has shown its contribution in various applications including ophthalmology [3, 5, 7-9], cardiology [6], urology [28], neuroimaging [34, 10-11], dental imaging [12, 13], dermatology [14] developmental Biology [15, 16] oncology [29, 32] tissue engineering [18, 19]. Due to the advancement of OCT, image acquisition speed has been increased significantly and it is now possible to have real time 3-D imaging [35, 36]
1.2 Principle of time domain OCT

In principle, OCT is a Michelson interferometer that uses low coherence interferometry as detection technique. Low coherence interferometry (LCI) technique measures the interference of two optical fields to resolve the difference of light paths that the two beam travel. When coherent light are superimposed on each other, they produce interference. A light source is used which has low temporal coherence but high spatial coherence. This partially coherent light beam is directed onto a beam splitter that splits the light into two portions – a reference beam and a sample beam (Figure 1.2). These two beams travel the set distances in reference arm and in sample arm, respectively. Once they reach the reflectors at the end of each arm, these light beams are reflected back in the opposite direction and they are combined in the splitter. Then the resulting interference fringes are collected by the detection device in the detector arm. LCI uses broadband light source and because of broadband nature, mutual interference between two optical fields only
occurs when the optical path difference between these two fields are within the coherence length of the source.

OCT uses LCI technique and it is analogous to the ultrasound except OCT uses infrared light instead of acoustical waves. As light does not need to travel through a medium the way sound does, no direct contact is required with tissue/transducing medium as like ultrasound. In ultrasonography or ultrasound, sound wave from an acoustic source is transmitted into the tissue structure and the time delays of reflected sound waves are used to measure the depth of the reflecting structures in tissue sample. In OCT, light waves are used instead of sound and because of the fact that light travels at a much faster (~10^6 times) velocity than sound, similar measurement of the time delays of reflected light waves is quite impossible. Therefore, a different detection method is used in OCT where time delays are measured from the interference fringes resulting from the backscattered light.
lights from sample and a reference reflector. A mirror with very high reflectivity (>95\%) is usually used as a reference reflector.

OCT uses low coherence interferometry technique where a low coherent light source is used to send light through a beam splitter which sends part of light to the reference arm and rest of it to the sample arm. When the light waves reflected back from the reference arm and sample arm are collected at the detector, they form interference. These detected interference patterns are used to extract information about the structure of the sample.

If $\lambda_0$ is the central wavelength, $\Delta l$ is the optical path difference between two arms, $E_s$, $E_r$ are electromagnetic waves from sample arm and reference arm respectively. Then the detected signal by interferometer is given by:

$$I_d = E_s^2 + E_r^2 + 2E_s E_r \cos\left(\frac{2\pi \Delta l}{\lambda_0}\right)$$

(1-1)

First two terms of the detected signals are dc term and the interference signal (last term) varies with $\Delta l = l_r - l_s$ periodically. $l_r$ is the distance from beamsplitter to the reference arm and $l_s$ is the distance between beamsplitter to the sample. When a partial coherence light is used with a Gaussian spectral distribution, the detected signal in the interferometer is given by:

$$I_d = E_s^2 + E_r^2 + 2E_s E_r \exp\left(-4ln2 \frac{\Delta l}{l_c^2}\right) \cos(2k_0 \Delta l)$$

(1-2)

Where $k_0$ is the centre wave number $l_c$ is the coherence length of the light source which is defined as:

$$l_c = \frac{4ln2 \frac{\lambda_0^2}{\pi}}{\Delta \lambda}$$

(1-3)
If the sample has different layers, depending on the difference of the path lengths, the depth information can be extracted by scanning over different length of $l_r$.

This low coherence interferometry technique, being a one dimensional measurement, cannot form a cross sectional image of a three dimensional volume. When multiple parallel low coherence interferometry scans are performed over a dimension of a sample by using a galvanometer(x-scan), and then these reconstructed data provides topographic or cross sectional image. This is how OCT images are formed from the lateral LCI scans performed along a dimension of a sample. Another galvanometer is used to scan in lateral direction. Each LCI scan or depth profile is called an A-scan (z-axis) and the lateral scans are termed as B-scan (x-axis). OCT can also provide 3D volumetric images by reconstructing all the cross sectional images or “slices” obtained by scanning in the third dimension (y-axis). This third dimensional scan is called C-scan. A general schematic of OCT system is shown below (Figure 1.3).

![Schematic of a basic time domain OCT system based on Michelson interferometer [1](Figure 1.3)](image-url)
As these sources have very small coherence length (2~20μm), OCT can provide images with very high axial resolution. Alternatively, this means that by using very wideband light source, OCT can provide very strong resolving power in axial direction. The axial resolution of OCT is given by:

$$\Delta z_R = \frac{2\ln 2 \lambda_0^2}{\pi \Delta \lambda}$$  \hspace{1cm} (1-4)$$

where $\lambda_0$ is the centre wavelength of the light source and $\Delta \lambda$ is the FWHM bandwidth in wavelength. This outstanding ability of producing high resolution volumetric images of microstructures has enabled OCT to earn significant attention from the biomedical imaging research community. The figure below shows a cross-sectional image of a finger by OCT.

![Cross-sectional image of a finger by OCT](image)

Figure 1.4: Cross-sectional image (2mmX8mm) of finger by OCT [36]

### 1.3 Spectral domain OCT (SD-OCT)

The OCT principle described above requires the scanning of reference arm back and forth for imaging in axial direction of sample. This is because interference between backscattered light from reference and sample arm is observed only when the path difference between them is within the coherence length of the source, which is termed as “coherence gating”. So, in order to image the entire depth of the sample, reference arm
has to be scanned over time. This general modality of OCT is known as time domain OCT (TD-OCT) [26,27].

One limitation of TD-OCT is its low imaging speed due to the requirement of mechanical scanning of reference mirror. Fourier domain OCT (FD-OCT) is another modality of OCT where no mechanical movement of reference arm is required, i.e. the reference arm length is kept fixed. The detector detects the wave number-dependent current and by using Fourier analysis, the detected output is converted to internal sample reflectivity profile at different depths. Now depending on the detection process, FD-OCT has two different modalities. One is spectral domain OCT (SD-OCT) where a broadband light source is used and the single photodetector is replaced by a spectrometer consisting of a diffraction grating, a focusing lens and an array of detectors (figure 1.5). The

Figure 1.5: Schematic diagram of spectral domain OCT. The detected light is dispersed by the grating (DG) and spectrum is collected by the line scan camera. (FL: Focusing Lens, GS: Galvo Scanner, M: Mirror, LS: Broadband light source, fBS: fiber based beam splitter, col: Collimator, OL: Objective Lens)
spectrometer collects all the spectral components of detected light at the same time and Fourier transform of that spectrum gives the depth profile along the optic axis. In case of SD-OCT, if the electromagnetic field from the reflector arm is defined as \( E_r(k) \) as a function of wave number \( k \) for the source spectrum \( S(k) = E(k)E^*(k) \), the electromagnetic field from the sample arm can be defined as:

\[
E_s(K) = E(k)[\alpha_1 e^{ikz_1} + \alpha_2 e^{ikz_2} + \ldots ]
\]

(1-5)

Where \( E(k) \) is the field strength incident on the sample, and \( \alpha_1 \) and \( \alpha_2 \) are the square roots of the reflectivities at depth locations \( z_1 \) and \( z_2 \), respectively.

The intensity at the detector as a function of \( k \) is defined by,

\[
S_{SD}(K) = S(K)\left[1 + 2\alpha_1 \cos(kz_1) + 2\alpha_2 \cos(kz_2) + \ldots \right]
\]

(1-6)

Where \( \alpha_1 \) and \( \alpha_2 \) are assumed much smaller than 1. The above equation shows that the detected signal is the modulated signal of the source spectrum according to the depth information by \( \alpha \cos(kz) \). Now if the Fourier transform is plotted, according to the reflectivity at different layers, addition to the peak at 0 position due to source spectrum there will be peaks at different depths (\( z_1, z_2 \) etc.) which gives information of different layers. As an example when a reflecting mirror is placed in the sample arm, the spectrum and the Fourier transform of the spectrum provides the depth information shown in figure 1.6.
1.4 Phase-sensitive OCT

Addition to visualizing the structural details in micrometer level, transient change in the structure can also be identified in nanometer level by a special measurement technique of OCT named as phase-sensitive OCT. The phase for any depth at from the detected spectrum is defined as

\[ \varphi(z) = \tan^{-1}\left(\frac{\text{Im}[S_{zd}(z)]}{\text{Re}[S_{zd}(z)]}\right) \]  \hspace{1cm} (1-7)

Common mode noise can be eliminated by calculating phase differences between two layers one of which is considered as reference layer. The phase difference between two different layers can be expressed as:

\[ \Delta \varphi(z) = \varphi_1(z_{\text{ref}}) - \varphi_1(z) \]  \hspace{1cm} (1-8)

If the phase difference is observed over time, we obtain,
\[ \Delta \varphi(z) = (\varphi_1(z_{ref}) - \varphi_1(z)) - (\varphi_2(z_{ref}) - \varphi_2(z)) \quad (1-9) \]

Or, \[ \Delta \varphi(z) = \varphi_2(z_1) - \varphi_1(z_2) \quad (1-10) \]

This phase difference is related to the small displacement given by:

\[ \Delta \varphi(z) = 2\frac{2\pi}{\lambda_0} \Delta z \quad (1-11) \]

Transient thickness change can be detected by looking phase difference over time.

1.5 OCT for neural study

Due to the advancement of the technology, OCT is capable of high speed real time imaging. OCT can generate cross-sectional images in micron scale resolution and can generate 3-D volume by stacking those cross-sectional images. OCT has shown its contribution almost in every field of biomedical applications [3-19]. Due to its potential, OCT is also being used for various neural studies. Phase sensitive OCT provides structural information in the resolution of cellular level where conventional OCT provides structural information in the tissue level. Due to its wide range of imaging capability, OCT has been used to visualize structure as well as for functions from small amount of neurons to tissue level of brain. As for example, by using phase sensitive OCT, Akkin T et al. identified the neural activity in squid giant axon [34] In another study, OCT intensity from rat barrel cortex was found to be changed during forepaw stimulation [124, 125] As OCT is a great tool for providing structural information in micrometer
resolution, OCT has been used for visualize the structure brain tissue non-invasively as well as for distinguish between normal brain tissue with diseased tissues [126-131].

1.6 Fluorescence microscopy (FM)

Fluorescence microscopy (FM) is one of the most rapidly adapted imaging technologies in biomedical sciences. Medical application of fluorescence technique started back to 1924, when endogenous autofluorescence of porphyrins was observed in tumors illuminated with ultraviolet light [45]. Now FM has applications almost in all scale including in microscopic, mesoscopic and macroscopic applications [46-52]. This technique is now being widely used for various applications including immunolabeling, identifying different kind of normal healthy as well as diseased cells/tissues by using specific markers, such as identifying tumor cells[46,47], cancer cells [48,49], different type of body cells as for example neuronal cells[53,54]. Different kind of targeted molecular probes are being developed day by day. One of the recent advancement is in developing quantum dots for various applications and varying its wavelength and size according to the requirement [55]. The most important feature of fluorescence imaging is it provides the biochemical and metabolism information of the tissue [50-52] .Calcium and voltage sensitive dyes are being widely used with neural activity related researches [56-77].
1.7 Working principle of FM

When light of particular spectra hits the fluorophore, the fluorophore absorbs the light and emits another longer wavelength of light (figure 1.7 (right)). The wavelength difference between absorbed light and Flouresced light is called Stokes shift.

![Diagram of fluorescence microscopy](image)

Figure 1.7: The basic components of a fluorescence imaging system (left), Absorption and fluorescence spectra of a fluorescent dye (right) [78]

This stokes shift property made the system valuable in imaging as the fluoresced light is different in color than the excited light which makes the desired cells/tissue/process visible. An excitation filter allows only particular wavelengths to pass through it and blocks other wavelengths of light. A dichroic mirror separates fluoresced light from the excited light by reflecting exciting light and allowing the fluoresced light to pass through the mirror. The fluoresced light is detected by CCD camera. A schematic diagram of the fluorescence microscopy is shown in figure 1.7 (left).

1.8 Fluorescence microscopy for neural study

Fluorescence imaging is one of the most widely used optical methods for neural study. Various dyes and genetically coded indicators are now currently being used for studying
single neuron to group of neurons or different neuronal pathways for identifying neural activity as well for labeling tissues or cells. The major two types of fluorescent dyes used as contrast agents are voltage sensitive dyes and ion sensitive dyes. Voltage sensitive dyes (VSD) exhibits increased fluorescence in presence of action potential. As for example Grinvald et al. in 1981 studied the application of voltage sensitive dyes to detect the evoked activity in rat hippocampus pyramidal neurons (CA1) and their recorded activity matched with the electrophysiological recordings [132]. There are various other studies that also used voltage sensitive dyes as a contrast agent in their neural recording [63,133].

Due to the lack of strength in VSD signals, there are also various studies focusing on ion sensitive dyes which exhibit signal few orders of magnitude higher than VSD signals. Ion sensitive dyes, depending on their chemical structure and properties, target particular ions in the active site of neuronal activity. A transient influx of Ca\(^{2+}\) ions is triggered in neurons and this influx occurs through the voltage gated calcium channels located almost all over the nerve cell. By using Ca\(^{2+}\) sensitive dyes, this Ca\(^{2+}\) influx can be identified. So, propagation of an action potential through the axon or synapse can be detected by observing Ca\(^{2+}\) concentration in that region [70-71,134]. Calcium ion sensitive dyes provides very good signal to noise ratio, more than 100 fold greater fluorescence signal during activity than any other ion and as a result, Ca\(^{2+}\) indicators allow the monitoring of nerve activity even for single neurons due to ubiquitous presence of calcium channels in neuron, activity can be resolved in a population of neurons [71,73]. Although Ca\(^{2+}\)
indicators provide very good contrast in depicting level of activity in different regions of the neural circuit, the technique of introducing these dyes into a specific location remains a challenge as it is more or less invasive in most cases. On the other hand, genetically encoded calcium indicators based on recombinant fluorescent proteins have been developed which are now being for long term investigations for different applications. By using genetically modified indicators, cell specific imaging of neuronal activity has been possible in living animals [74-76]. Green fluorescence protein (GFP) is one of major encoded protein which is used for various applications of neural studies. There are wide varieties of new mutants of GFP developed by the researchers for its wide use and applications.

1.9 Effect of objective lens’s numerical aperture in resolution of optical coherence tomography and fluorescence microscopy imaging

Lateral resolution of a fluorescence microscopy system depends on numerical aperture (N.A.) of objective lens. Lateral resolution of fluorescence microscopy system is inversely proportional to the N.A. of the objective. So, a high N.A. objective is generally used to obtain better lateral resolution by fluorescence microscopy. Similarly, lateral resolution of OCT system is also inversely proportional to the N.A. of the objective. So a high N.A. objective can be used to obtain better lateral resolution in OCT imaging. But high N.A. objective limits in OCT axial field of view. The corresponding relation of axial field of view (FOV_{axial}) to N.A. of the objective can be expressed by
\[ \text{FOV}_{\text{axial}} = 0.565 \, \lambda / \sin^{2}[(\sin^{-1}(\text{N.A}))/2] \]  

(1-11)

where \( \lambda \) is the center wavelength of the OCT light source.

From equation (1-11) it can be stated that high N.A. objective limits in OCT axial field of view. To obtain enough axial field of view in biological sample using high N.A. objective in OCT imaging system, the OCT beam width incident on objective lens, can be reduced, which eventually will allow to use high N.A. objective as a low N.A objective and thus the axial field of view can be increased with sacrificing lateral resolution in OCT imaging.
Chapter 2: Phase sensitive optical coherence tomography for non-contact detection of action potentials in functionally stimulated *Limulus* nerve

Abstract

Phase-sensitive optical coherence tomography was used to identify nerve activity in *limulus* optic nerve in a non-contact and label free manner. A common-path OCT system is developed and used for detection of neural activity instead of using conventional reference arm set up due to the fact that common path OCT provides better phase stability. The developed common path OCT system demonstrated the capability to detect rapid transient structural changes that accompany action potential propagation. In this study, no averaging over multiple trials was required, which indicates that the OCT system is capable of identifying individual action potential propagation from functionally stimulated Limulus optic nerve. The strength of this phase sensitive OCT-based optical electrode is that it does not need to be in contact with nerve and also does not require any exogenous fluorophores. As OCT collects phase information from different depths of sample in same time, neural activity in different axonal bundles from different depths of nerve was also identified by further analysis of phase sensitive OCT measurements. An automated algorithm was developed for detection of neural activity in different depths of nerve. One limitation of this study is phase sensitive OCT data were acquired from same lateral position of nerve over time during action potential propagation. Identification of neural activity from multiple lateral locations of nerve is not possible with phase-sensitive OCT due to increased phase noise introduced for lateral scanning.
2.1 Introduction

Electrophysiological techniques are widely used for detection of neural activity. Although these techniques provide very reliable results, these techniques are invasive and require direct or near contact with the nerve. There are some cases when it is desired not to have any direct contact with electrode; for example, cell adhesion and neurite development in cultured neurons are sensitive to physical contact between electrode and neurons [79]. Also in some cases, as for example, for retina, it is inaccessible for electrodes to be in contact with the nerve. On the other hand, fluorescence methods can be used to identify neural activity, but these methods require exogenous contrast agents such as voltage- or calcium-sensitive dyes or genetically modified fluorescent markers [61, 81-82] to identify neural activity and also the contrast agents might be toxic in some cases. For many applications, a non-contact optical method which can non-invasively detect the nerve activity utilizing only the endogenous markers would be greatly beneficial. It was found in several earlier studies that a nerve undergoes transient structural (thickness) changes in addition to changes in several optical properties, such as scattering, birefringence, fluorescence and absorbance during action potential propagation[34,38,40,41,83-87]. In this study, the transient thickness change of nerve was identified during action potential propagation as an indirect measurement of neural activity.

It was found by several studies that the thickness of nerve changes during neural activity [34, 38, 40, 41, 83-87]. Hill first discovered 220nm thickness changes in diameter in a single cuttle fish nerve fiber of 238μm in diameter for 10,000 impulses [83]. Similar
results were also obtained for crayfish walking leg nerve [34], lobster nerves [38, 41, 84] crab nerves [84-86], garfish olfactory nerves [87], and squid giant axon [92, 93]. Phase-sensitive optical coherence tomography (OCT) has enabled the measurement of sub-nanometer displacement and thickness changes and it has been demonstrated that phase sensitive-OCT is a promising tool for detection of rapid transient structural changes associated with neural activity [1, 34, 38, 92, 93]. Akkin et al. and Fang-Yen et al. in 2004 independently used phase-sensitive interferometry to observe change in nerve thickness during activity. Akkin et al. used a phase-sensitive OCT system to identify transient thickness change in crayfish walking leg nerve during action potential propagation. And transient motion on the order of 0.4-1.0nm (average of 500 responses) of the saline bath/nerve tissue interface on a time scale of 1-2ms [34] was observed. Using similar configuration, Fang-Yen et al. observed 5nm swelling of 10ms duration (single response) on a lobster nerve, indicating a compound response [38]. In another study, Akkin et al. has demonstrated optical detection of nerve activity in squid giant axons [92, 93]. An average thickness change of 1-2nm (average of 500 responses) from a squid giant axon of 360μm in diameter, was observed at room temperature and a maximum thickness change of 21nm (average of 50 responses) was observed at cold temperature with 400mM NaCl added to the seawater [92]. In a different study, Akkin et al. stained a squid giant axon of 410μm in diameter with voltage-sensitive dye and fluorescence intensity from the nerve was found to be decreased by 0.1% - 13% while resulting thickness changes of 1-2nm (average of 250 responses) and a maximum change of 11-13nm was observed[93].
This study was aimed for improvement of the phase sensitive OCT as an optical electrode for neural activity detection with few significant differences in approach compared to the earlier OCT studies. Most of the previous studies with OCT have used either giant axons (diameter of 300-450μm) or nerve fiber bundles as a whole (diameter 100-300μm) to identify the structural changes during neural activity. For next level of assessment of phase-sensitive OCT for neural activity detection, it needs to be used to identify similar detection in nerve axons that are comparable in size to finer nerve endings in mammals. In this study, a lateral compound eye of Limulus polyphemus (horseshoe crab) was used as animal model, specifically, a bundle of few (10-15) axons from its associated optic nerve was used for experiment. The compound eye has hexagonally packed array of ommatidium which generates activity in the optic nerve axons in response to light stimulation. Sizes of these axons are 3-10 μm in diameter [95] and are comparable to axons in most mammalian peripheral nervous system (the largest mammalian axon reported to be of 20μm diameter [96]). Secondly, this study was aimed to use phase sensitive OCT to identify individual impulses. During each stimulation of the compound the eye with light, a train of action potentials are generated in axons and the responses are not necessarily identical in every trial (timing and number of action potentials). Therefore, it would be more meaningful to identify single response instead of averaging of multiple responses. This study was aimed more towards improving the spatial sensitivity of the system in order to detect single action potential propagation from the axons. And the final improvement of this study is that the eye was functionally stimulated instead of conventional electrical stimulation. Functional stimulation removes stimulation
artifacts in optical measurement as well as opens the opportunities of future studies on monitoring functionally stimulated small scale network activity [97] using this OCT-based optical electrode.

2.2 Imaging System

In this study, a regular reference arm configuration as well as a common-path configuration SD-OCT system was used for experiments (Figure 2.1(a) shows schematic of regular arm configuration and 2.1(b) shows schematics of common-path configuration). A Ti:Saph broadband mode locked laser (Femtolasers, Inc., Integral OCT 2709) with a center wavelength of 800nm and a bandwidth of 170nm is used as the light source. The light from the laser is sent to the reference and sample arms by a 2x2 fiber-based beam splitter. The sample arm of regular reference arm set up is incorporated in upright microscope (Olympus BX 61W). In common-path configuration (Figure 2.1 (b)), instead of using regular reference arm, a small reference arm is installed inside the sample arm microscope (Olympus, BX61). This reference arm shares the same path with sample arm until just before the objective lens. A plate beam splitter (Thorlabs, BS11, 70:30) is positioned on a custom-built mount that connects to the objective port of microscope. The optical beam transmitted through the tube lens of microscope is incident on the beam splitter where light is split between reference and sample arm, 70% light is transmitted straight down through the beam splitter and focused onto the sample by an objective (Olympus, UPLSAPO 4x). The other 30% of light is reflected from the beam splitter and directed at the reference arm which is 90° from the sample arm beam. Then
the reflected beam from splitter passes through an achromatic doublet lens (f=30mm, Thorlabs) and finally reflected back by a mirror positioned at the focal distance of the lens. Finally the backscattered lights from both reference and sample arms are collected by a custom built spectrometer. The spectrometer uses a diffraction grating (Wasatch Photonics, 1200lpmm, 830nm), a focusing lens (JenOptik Optical Systems, f=150mm), polarization beam splitter cube (Rocky Mountain Instrument Co., 4 inch Cube) and two line scan cameras (Basler sprint camera, sp4096-140km). The axial resolution of the system was found to be 3μm in air and the lateral resolution to be 5μm with a 4x objective. The maximum acquisition speed of the line scan camera is 144 KHz (A-lines/seconds) which yields a temporal resolution of the order of 7μs. A frame grabber (National Instrument, NI1429) is used to detect the spectrum from line scan camera. Another data acquisition card (National Instrument, NI 6259) is used to generate the trigger pulses to synchronize all devices during acquisition. A PCI-e chassis (Express Box 7, Magma) accommodates all these PCI-e cards and is connected to a workstation laptop computer (Lenovo 710wd) which is used for data acquisition. A multithreaded software program written in Microsoft visual C++ is used to synchronize all the devices and for data acquisition. A desktop computer, with CUDA enabled Tesla GPUs (C1060), is used for post processing. MATLAB 2011b was used for image processing and AMIRA was used for volume rendering and 3D visualization of OCT images.

2.3 Animal model and sample preparation

The lateral compound eye with optic nerve of Limulus polyphemus was chosen as animal model for this study. Medium size (6”-8” in diameter) male Limulus (Marine Biological Laboratory, Woods Hole, MA) with clear (non-injured) lateral eyes was chosen for
dissection. The animal was kept in an ice bucket for 20-25 minutes before dissection and then the cold-anaesthetized Limulus was fixated onto a wooden platform for dissection. One lateral eye with associated optic nerve was carefully extracted from the animal and placed in a Petri dish with Ringer’s solution (430mM NaCl, 9.56mM KCl, 9.52mM CaCl2.2H2O, 9.97mM MgCl2.6H2O, 50μM HEPES and pH maintained as 7.4). Then the sample was brought under stereomicroscope for further cleaning of the sample. After cleaning the sample, the axonal bundles were separated by cutting the sheath of the optic nerve carefully. The sample was then placed on the imaging chamber of the sample arm microscope (setup schematic shown in Figure 2.1(c)) for structural imaging and neural recording. A silver wire suction electrode (A-M Systems) was used for electrical recording. The electrical data from the suction electrode was sent to a differential amplifier (Warner Instruments, DP-301) for filtering and amplification (gain: 10,000) and then sent both to the acquisition computer (for recording) and to an oscilloscope (for display).

2.4 Structural imaging of limulus optic nerve

At the beginning of each experiment, OCT cross-sectional images of Limulus optic nerve sample were obtained. Schematic in Figure 2.2(a) shows OCT scanning to obtain cross-sectional images of sample. After combining cross-sectional images, a 3D OCT image is made of sample. Each of cross-sectional images is taken across the long axis (y-axis) of nerve and the cross-sectional images are in xz-plane where z is the axial or depth direction. Figure 2.2 (b) shows a 3D volumetric OCT image of the optic nerve sample.
One hundred cross-sectional images were taken along 1mm length to obtain 3D OCT volumetric image of the nerve.

![Image](image.png)

Figure 2.2: Structural imaging of Limulus optic nerve using OCT. (a) Schematic showing the optic nerve bundles and scanning axes of OCT to obtain cross-sectional and volumetric images. XZ is the cross-sectional image plane and y-axis represents the third axis of scanning to obtain the 3D volume. (b) Volumetric reconstruction of a 1mm section of the nerve. Scale bar: 100μm

### 2.5 Optical recording of neural activity

The OCT system can generate high resolution images that can distinguish axon bundles as small as 8-12μm. After visual inspection of OCT cross-sectional images of nerve, small bundles with high signal intensity (at least of SNR > 15dB to avoid unreliable phase measurements) were chosen for acquisition. Then selected bundle of optic nerve was brought in the center position of cross-sectional image by moving the stage laterally. The OCT intensity and phase information from same lateral location of the bundle was acquired over time (M-scan). It is important to make sure that the endings of chosen axonal bundles are actually going into the suction electrode so that the electrical
recording can be compared with the thickness changes from these bundles. A 3D volumetric reconstruction of the nerve and adjacent suction electrode tip was made which makes it easier to track the axon bundles. During analysis, phase unwrapping was performed first to account for phase jumps and then the difference between two unwrapped phases was calculated. The change in phase difference ($\Delta \varphi$) over time indicates the change in thickness change due to the change in optical path length ($\Delta p$) between top and bottom layer of nerve. The corresponding relation of phase difference to optical path change can be expressed by $\Delta p = (\lambda_c/2\pi)\Delta \varphi$ where $\lambda_c$ is the center wavelength of the light source. To get rid of common mode environmental noises, differential phase measurement was preferred over comparison to an external stationary reference (e.g. cover slip). As the optical path length is twice the physical path length, the change in optical path length during neural activity can be converted to change in thickness of the bundle ($\Delta z$) by using the relation $\Delta z = \Delta p/2$. Continuously acquired OCT depth profiles (A-lines) from same lateral position over time were analyzed to find
thickness change during neural activity and were compared with electrical recordings. Figure 2.3 shows a representative set of simultaneous electrical and optical recording of neural activity in Limulus optic nerve. The results show that the thickness of nerve changes during action potential propagation confirmed by electrical measurements and a very good temporal correlation was observed between the optical and electrical measurements. The thickness change for an average size Limulus axon (10-15\( \mu \)m in diameter) was found in the range of 25-75nm. These are single shot optical measurements of neural activity.

2.6 Controlled experiment with cold block

A cold block can be used to control action potential propagation through nerve. In a cold block, a small section of nerve is introduced inside the cold block and temperature is gradually decreased within that block by using some cooling medium, such as cold ice-salt solution [29]. When the temperature of limulus nerve falls below 2\(^0\)C, the nerve stops propagating action potential and it is expected that there should be no structural change during that time. The advantage of using cold block is that the whole process of inactivating the nerve is rapidly reversible which means that once the temperature of nerve is brought back in room temperature by circulating warm solution through the cold block, nerve recovers its active state and starts to propagate action potential again. As a control experiment, a cold block was used to control action potential propagation through limulus optic nerve and continuous OCT data were acquired during normal active state,
during blockage of action potential through nerve and finally again recovery from inactive state to active state of action potential propagation.

Figure 2.4: Simultaneous electrical and OCT recordings from functionally stimulated optic nerve during three stages of cold block operation. (a) During normal state (before circulating cold solution through cold block), (b) after circulating cold solution through cold block and (c) after circulating warm water through cold block.
Figure 2.4 shows the representative results of cold block experiments. The optical data shows that during normal state of propagating action potential, the thickness of nerve changes during action potential propagation (figure 2.4(a)), during inactive state of no action potential propagation, there is no thickness change observed in the optical data (figure 2.4(b)) and finally when the nerve was brought to normal condition, the optical data shows of thickness change in the nerve during action potential propagation (figure 2.4(c)).

2.7 Depth resolved detection of neural activity in optic nerve

As OCT collects phase information from different depths of sample in same time, neural activity in different axonal bundles from different depths of nerve was investigated by further analysis of phase sensitive measurements of OCT. Firstly interlayer phase difference was calculated for all depths of OCT depth profiles by subtracting the phase of one depth to the next depth. Then correlation between adjacent interlayer phases over time were calculated and normalized to 10. Interlayer correlation for each time point were calculated by finding the correlation of interlayer phase values between one layer to the next layer for a duration of 16 time points (=1.6 ms) of which 8 time points are previous and 8 time points are after of that specific time point. The time duration for calculating correlation was selected for 1.6 ms just to make sure the duration is almost equivalent to the duration of one action potential (~2ms). The normalized correlated values were thresholded by 4.0, and so, any correlation value greater than 4.0, are taken into consideration. As size of axon is 5-6µm and OCT pixel resolution is 1.73µm, if the
correlation values are more than 4.0 in 4 consecutive layers, it is almost assumed that there is change in thickness of nerve due to neural activity. For final sorting, the total time of positive correlation in adjacent layers is brought under consideration. If the total time of correlated values above of threshold in 4 consecutive layers is less than 0.5ms, the correlation is considered as false positive due to noise. But if the total time of correlated values above of threshold at least in 4 consecutive layers is more than 0.5 ms, it is considered that there is thickness change in the nerve during neural activity and thus those layers and times are marked as positive. By applying this searching algorithm, neural activities were identified in different depths of nerve during action potential propagation. Figure 2.5 shows phase plots in different layers, identified correlated neural activity in binary image and electrophysiology. The optical results show that action potential propagated through two different bundles during first action potential propagation confirmed by electrical measurements, but action potential propagated through only bottom bundle during second action potential propagation. The results clearly show that OCT can identify neural activity in different depths of nerve efficiently by using this developed algorithm.
Figure 2.5: Interlayer phase differences in selected depths of optic nerve (top), correlated neural activity in binary image (middle), electrophysiology (bottom).

Conclusion

Optical detection of neural activity will be very beneficial for many applications where no physical contact between electrode and nerve is desired. In this study, phase-sensitive
OCT has been demonstrated to be capable of measuring the changes in nerve thickness associated with action potential propagation. The optical detection of activity is also verified during deactivation and reactivation of action potential propagation through nerve using cold block. A novel algorithm was developed to find neural activity in different layers of nerve by OCT. Neural activity in different axons located at different depths of nerve was identified by using the developed algorithm. These results have shown that OCT can be used to detect thickness changes during action potential propagation for small sized Limulus axons and to the best of our knowledge, for the first time this study has demonstrated single shot optical detection of neural activity. The novel developed algorithm will help in identifying neural activity efficiently in different depth layers. Future efforts are focused towards the improvement of phase sensitivity of the system to increase the overall accuracy in detection as well as to make the correlation algorithm more robust and automatic so that it is more efficient during identification of neural activity in different depths of nerve.
Chapter 3: Identification of optical changes in mouse brain slice associated with seizure activation using optical coherence tomography

Abstract

Phase-sensitive optical coherence tomography (OCT) is capable of identifying neural activity from a lateral position of nerve by acquiring phase information from that location over time. Identification of neural activity from multiple lateral locations of nerve is not possible with phase-sensitive OCT due to high phase noise induced by lateral scanning. On the other hand, intensity based OCT acquires intensity information from all of lateral points that lie in the scanning range of OCT system. Preliminarily, this study was aimed to assess intensity based OCT for identification of burst of activities from a group of neurons. OCT was used to identify the optical change with seizure activity in mouse brain slice in a controlled way using microelectrode array (MEA) with OCT. OCT intensity was found to be decreased in brain slice during onset of bursts of activity detected by MEA. These preliminary results verify that intensity based OCT is capable of identifying seizure like activity in a label free manner.

3.1 Introduction

Phase-sensitive OCT can be used to identify neural activity from a lateral location of nerve through which action potential is propagated. But to study neural activity from a group of neurons, it is also very important to identify neural activity that propagates in random directions rather than in a controlled and known direction. It is difficult to
identify neural activity in multiple locations of nerves by phase-sensitive OCT as scanning is limited in phase-sensitive OCT measurements due to the fact that high phase noise is induced by lateral scanning. On the contrary, intensity based OCT acquire information from all of lateral points in the imaging field of view by lateral scanning. This study was aimed to assess intensity based OCT for identification of neural activities. As a preliminary assessment, intensity based OCT was used to identify seizure-like activities which are relatively easy to detect.

Epilepsy is a neurological disorder in which normal brain function is disrupted as a consequence of intensive burst activity (known as seizures) from groups of neurons [99]. Exact mechanism of Epilepsy is not clearly known but epileptic discharges are in part initiated by a local depolarization shift that drives groups of neurons [99]. In several studies, it was found that there are changes in optical properties during seizure activity. Scattering properties of the cortical tissues was found to be decreased in mice during induced seizures [99,100] by intrinsic optical imaging. The intrinsic optical imaging provides low resolution in depth sectioning for the sample. So it is not possible to visualize the propagation of optical property change in depth direction by intrinsic optical imaging. OCT is a minimally-invasive technology capable of rapid two- and three-dimensional imaging of 2-3mm of subsurface tissue structure with micrometer resolution. In one study, OCT was used to detect changes in optical properties of cortical tissue in vivo in mice before and during the induction of generalized seizure activity. A significant decrease in backscattered intensity during seizure progression was detected before the onset of seizures [101]. But the timing information of seizure progression was confirmed
by solely observing the animal behavior. There was no direct comparison between changes in OCT properties with electrical activities. Also, the study was not able to distinguish the contribution of change in blood flow with neural activity in changing of tissue optical properties. So, instead of using live mouse, mouse brain slice was used in this study to confirm that the identified optical changes will be only due to the neural activity. As there is no blood flow in the brain slice, there is no possibility of optical change due to the change in blood flow. Also for obtaining more accurate temporal information of bursts of activity, microelectrode array is used to record bursts of activity from brain slice.

In this study, the acute hippocampus region of the mice brain slice was simultaneously monitored using volumetric OCT imaging and MEA recordings before, during and after seizure activities. Backscattered OCT intensity was found to be decreased during onset of seizure activity. The preliminary results demonstrate that intensity based OCT measurement is capable identifying seizure-like activity.

3.2 Optical coherence tomography imaging system and microelectrode array

A Ti:Saph broadband mode locked laser (Femtolasers, Inc., Integral OCT 2709) with a center wavelength of 800nm and a bandwidth of 170nm is used as the light source. Instead of using regular reference arm set up, a common path OCT configuration was used for have less environmental noise in OCT measurements. In common-path configuration, a small reference arm is installed inside the sample arm microscope (Olympus, BX61). This reference arm shares the same path with sample arm until just
before the objective lens. A plate beam splitter (Thorlabs, BS11, 70:30) is positioned on a custom-built mount that connects to the objective port of microscope. The optical beam transmitted through the tube lens of microscope is incident on the beam splitter where light is split between reference and sample arm, 70% light is transmitted straight down through the beam splitter and focused onto the sample by an objective (Olympus, UPLSAPO 4x). The other 30% of light is reflected from the beam splitter and directed at the reference arm which is 90° from the sample arm beam. Then the reflected beam from splitter passes through an achromatic doublet lens (f=30mm, Thorlabs) and finally reflected back by a mirror positioned at the focal distance of the lens. Finally the backscattered lights from both reference and sample arms are collected by a custom built spectrometer. The spectrometer uses a diffraction grating (Wasatch Photonics, 1200lpmm, 830nm), a focusing lens (JenOptik Optical Systems, f=150mm), polarization beam splitter cube (Rocky Mountain Instrument Co., 4 inch Cube) and two line scan cameras (Basler sprint camera, sp4096-140km). The axial resolution of the system was found to be 3μm in air and the lateral resolution to be 5μm with a 4x objective. The maximum acquisition speed of the line scan camera is 144 KHz (A-ines/seconds) which yields a temporal resolution of the order of 7μs. A frame grabber (National Instrument, NI1429) is used to detect the spectrum from line scan camera. Another data acquisition card (National Instrument, NI 6259) is used to generate the trigger pulses to synchronize all devices during acquisition. A PCI-e chassis (Express Box 7, Magma) accommodates all these PCI-e cards and is connected to a workstation laptop computer (Lenovo 710wd) which is used for data acquisition. A multithreaded software program written in
Microsoft visual C++ is used to synchronize all the devices and for data acquisition. A desktop computer, with CUDA enabled Tesla GPUs (C1060), is used for post processing. MATLAB 2011b was used for image processing and AMIRA was used for volume rendering and 3D visualization of OCT images.

Figure 3.1: (a) Schematic diagram of OCT system (PBS: beam splitter, UM: Upright microscope, GS: Galvo scanner, fBS: fiber based beam splitter, LSC: Line scan camera, DG: Diffraction grating, CPRA: Common path reference arm, OL: objective lens, FL: Focusing lens, LS: Laser source), (b) The experimental set up of MEA with OCT

Microelectrode array (MEA) with array of 10X6 electrodes was used for electrical measurements from brain slice. The physical distance between electrode to electrode in MEA was 100µm. One synchronous TTL signal from BNC breaker board is sent to analog input of MEA system for temporal registration between OCT and MEA recordings.
3.3 Animal model and sample preparation

Acute hippocampal mouse brain slices (thickness of around 300µm) were prepared from wild-type (P10-P20 days old) mice (The Jackson Laboratory, Bar Harbor, ME). Mice were anesthetized and decapitated according to UC Riverside IACUC approved protocols. The brain slice was placed on top of microelectrode array (MEA). The hippocampus region of the brain slice was brought under OCT imaging system as well as for MEA recording. ACSF solution was flowed through the slice for control measurement followed by a period for which 10mM KCl solution was flowed through the slice to induce seizure activity (figure 3.1(b)). Simultaneous volumetric OCT and electrical data were recorded during control condition and during seizure activity.

3.4 Structural imaging of brain slice

The hippocampus region of brain slice is brought in OCT field of view for imaging. After suction has been implied to suck in some portion of brain slice to fix the sample in the required position, OCT volumetric images are acquired from a lateral field of view of 1mmX1mm of the sample. Each cross-sectional image is combined of 1024 A-lines. 100 cross-sectional images are acquired for each volume. Figure 3.2(a) shows bright field Image of brain slice on top of MEA. The electrodes are visible in the bright field image. The OCT scanning region is shown in red box. Figure 3.2(b) shows 3D volumetric image of brain slice on top of MEA. The brain slice is clearly distinguishable from MEA. After integrating OCT intensity depthwise from 3D OCT mage of brain slice, the en face OCT image is formed (figure 3.3(a)).
Different features of the hippocampus region especially CA3, CA2 and CA1 region of brain slice is clearly visible in \textit{en face} OCT image of brain slice.

### 3.5 Optical changes in brain slice associated with seizure activity

After processing all raw OCT data to 3D volumetric images for whole experiment, 3D OCT image of brain slice for each time was separated into 4 different layers (depthwise)
(figure 3.4), each layer of ~75 µm thickness in depth. The *en face* integrated images for each layer were calculated by integrating the intensity depth-wise (in z-direction) from each layer. Then the average intensity of the *en face* image was calculated for each time point. The intensity for each layer over time was normalized by the initial average intensity of the *en face* image of that layer. By temporally registering MEA acquisition with OCT acquisition, electrophysiology data are also processed firstly by converting electrophysiology .mcd file to text file and then electrophysiology data for each channel is filtered and processed in Matlab. The normalized OCT *en face* intensity for each layer of brain slice was plotted with electrophysiology data. It was found that all of the MEA channels shows temporally correlated burst of activity. As the top and bottom layer of the brain slice contains dead tissue due to dissection, more emphasis was put to find optical properties on 2nd and 3rd layer of the brain slice as the tissue in these layers are mostly alive. Figure 3.5 shows normalized OCT intensity plot and electrophysiology. The OCT intensity both from second and third layer of the brain slice decreases during onset of burst of activity. The change of OCT intensity corresponds...
very well with onset of seizure activity which verifies that OCT is able to identify burst of activity without using any contrast agents.

Figure 3.5: Backscattered OCT intensity and electrophysiology from brain slice. (a) Normalized OCT intensity from second layer of brain slice, (b) Normalized OCT intensity from third layer of brain slice, (c) & (d) electrophysiology

**Conclusion**

This study was a preliminary step to assess intensity based OCT as a functional tool for identification of neural activity. Epileptic bursts of activity were induced in mouse brain slice and simultaneous OCT and MEA data were acquired during control and epileptic condition. OCT intensity was found to be decreased during onset of burst of activity. The
OCT intensity change corresponds very well temporally with burst of activity. These results confirm preliminary that OCT can be used as a functional tool for identification of burst of activity from a group of neurons in non-contact and a label-free manner.
Chapter 4: Development of a spatially and temporally registered optical coherence tomography and fluorescence microscopy system

Abstract

Change in optical properties in mouse brain slice during seizure like activity was identified by intensity based optical coherence tomography (OCT) (Chapter 3). For standard comparison, microelectrode array (MEA) was used for electrophysiology recordings but the spatial resolution of MEA is very poor (~100µm) with compared to OCT resolution (~3-15µm). On the other hand, MEA acquires electrophysiological information only from a depth location. Fluorescence microscopy (FM) is well known for detection of neural activity from a neuron or group of neurons with very good lateral resolution (~1µm) and it is possible to acquire fluorescence information from different depths of sample. For next level of assessment of intensity based OCT as a functional tool for detection of neural activity, a novel spatially and temporally registered OCT and FM has been developed so that OCT results can be compared with fluorescence measurements. After design and development of hardware and software, combined OCT and FM system was characterized for spatial and temporal registration.

4.1 Introduction

While conventional OCT has a resolution on the order of 2-10 microns, phase-resolved Optical coherence tomography further improves on the axial resolution of this technology from the micron-scale to nanometers by examining changes in the phase of localized interference [1-3,33-38]. Although OCT can generate high resolution cross-sectional
imaging in micrometer scale and can detect structural change information in nanometer scale, it lacks biochemical information of the tissue. On the contrary, Fluorescence microscopy (FM) provides complimentary biochemical, metabolic information of the tissue [102-107]. Due to the potential of assessing complimentary structural and functional information of the tissues, there are various studies of combining optical coherence tomography with fluorescence microscopy/multiphoton microscopy for different types of biological application. Combination of fluorescence imaging with OCT is getting significant interest in the biomedical research due to its potentiality, sensitivity and specificity. Combining OCT with Fluorescence imaging is a pretty new tool in the biomedical field [102-112]. There are several studies on combining OCT with fluorescence imaging to identify or diagnose different biological procedures. As for example, Kuranov et al. combined OCT with laser-induced fluorescence (LIF) using aminolevulinic acid (ALA) to identify the tumor boundaries in tumor [102,103,116]. J. P. Dunkers et al. developed a combined OCT and confocal microscopy system to observe structure & functionality of tissue engineered medical product [112]. Barton et al. developed OCT and laser induced fluorescence spectroscopy for diagnosis of atherosclerotic plaques [105]. Pan et al. developed ALA-fluorescence guided OCT for early bladder cancer diagnosis and they demonstrated that the sensitivity and specificity of fluorescence detection improved significantly by the combined system [104,106]. Tumlinson et al. combined OCT with LIF for spectroscopy imaging for mouse colon imaging [107]. Podoleanu et al. combined OCT with confocal laser scanning ophthalmoscopy (SLO) with integrated fluorescence detection by using indocyanine
green (ICG) for understanding macular diseases in a better way [108-110]. Recently Shuai Yuan et al developed a co-registered OCT and fluorescence molecular imaging (FMI) system and assessed its capability by imaging of colon polyps of the APC\textsuperscript{min} mouse model [116]. Maria Gaertnera et al combined OCT with FM for visualizing lung tissue structure [117]. Similarly, there are some other recent studies on combined OCT with fluorescence imaging to study various properties such as for identifying atherosclerotic plaques [118], monitoring wound healing process [114], visualizing the larval heart [115], assessment of drug delivery and tissue integrity [120], studying morphological changes of lung dynamics [121,122]. These studies showed the great efficacy of combining fluorescence with OCT for various biological applications.

Addition to assessing structural and functional information of the tissue at a single time point, it is very important for various applications to observe the dynamics of structural and functional change and the temporal relation between the changes is also important for various studies. There has been a great deal of interest of assessing OCT as a functional tool for different biological applications, especially using OCT as a tool for neural activity detection in a non-invasive and label free manner. Fluorescence microscopy is well known for identification of neural activity from a neural pathway or from a single cell by using different dyes or genetically encoded indicators. To verify OCT as a functional tool for identification of neural activity, OCT results for identification of neural activity can be compared with fluorescence measurements. For either acquiring information of structural and functional change of a biological sample over time or for
assessing OCT as a functional tool for neural activity detection, a temporally registered optical coherence tomography and fluorescence microscopy system is highly required. To the author’s best knowledge, there is no such previous study of development of a temporally registered optical coherence tomography and fluorescence microscopy system.

A spatially as well as temporally registered OCT and fluorescence microscopy system has been developed in this study. The combined OCT and FM system was designed such a way that OCT and fluorescence light paths follow same optical path before and after of the objective, which provides partially registered OCT and FM images. Spatially registered OCT and fluorescence images are made after post-processing. For temporal registration between OCT and FM system, every components of OCT and FM system is controlled and synchronized by external trigger pulses. A multi-threaded C++ program was developed for generating synchronous trigger pulses as well as for OCT and FM data acquisition.

4.2 System development

Optical coherence tomography (OCT)

Figure 4.1(a) shows schematic diagram of combined optical coherence tomography and fluorescence microscopy system. The OCT system uses Ti:Saph broadband mode locked laser (Femtolasers, Inc., Integral OCT 2709) with a center wavelength of 804nm and a bandwidth of 170nm. The OCT sample and reference arm is incorporated in an upright
microscope (Olympus, BX61W). A galvanometer based 2 axis optical scanner (Cambridge Technology, Inc., 6210H) is used for raster scanning across the sample. Instead of regular conventional reference arm, a near common path reference is developed to reduce noise variability from the OCT system. The near common path reference arm shares the same path with sample arm until just before the objective lens. A plate beam splitter (Thorlabs 70:30) on a custom-built mount splits the incident light from tube lens between reference (30%) and sample (70%) arm. The sample arm uses 20X water-immersion objective with N.A of 0.5. A 20X objective is incorporated in near common path reference arm to compensate the dispersion from the sample arm. The light reflected back from near common path reference arm and sample arm are recombined and collected in the spectrometer. The spectrometer consists of a diffraction grating (Wasatch Photonics, 1200lpmm, 830nm), a focusing lens (JenOptik Optical Systems, f=150mm), polarization beam splitter cube (Rocky Mountain Instrument Co., 4 inch Cube) and two line scan cameras (Basler sprint camera, sp4096-140km). The detected spectrums in the cameras are sent to the computer through a frame grabber (National Instrument, NI1429). Another data acquisition card (National Instrument, NI 6259) is used to run two BNC breakout boxes (BNC 2110 and 2120) which generate the controls signals for the OCT line scan camera, scanning mirrors, fluorescence stimulation shutter and fluorescence CCD. The maximum acquisition speed of the OCT line scan camera is 144000 lines/seconds and yields a temporal resolution of the order of 7µs.
The OCT beam diameter of the incident light to objective lens is set to be 2.40mm to underfill the back aperture of the objective lens (back aperture diameter of objective is 10.44 mm) by 24% so that OCT system has good penetration depth in biological tissue with 20X (NA = 0.5) objective while keeping good lateral resolution both in OCT and fluorescence system.

Figure 4.1: Schematic of combined spectral domain optical coherence tomography (SD-OCT) and fluorescence microscopy system. LS: light source (Ti:Sapph 800nm laser), I: isolator, PM: polarization modulator, fBS: fiber-based beam splitter, M: mirror, GS: galvo scanner, UM: upright microscope, pBS: plate beam splitter, nCPRA: near common-path reference arm, OL2: 20X objective lens (N.A 0.4), OL1: 20X water immersion objective lens (N.A. 0.50) DG: diffraction grating, FL: focusing lens, PBS: polarization beam splitter, LSC: line scan camera, S: moving stage, F: filter, FD: Fluorescence detector (CCD), ELS: fluorescence excitation light source, SR: Shutter

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**Fluorescence microscopy (FM)**

The excitation light from the excitation light source (Lambda XL (Sutter Instrument), spectrum: 300-700nm) is guided to a stimulation shutter through a liquid light guide. The stimulation shutter is configured to control the exposure time of excitation light. After the excitation light passes the shutter, it is filtered by excitation filter and is then combined with OCT light in the microscope by dichroic mirror (DM2 in figure 4.1) and is finally focused in the sample by objective. The fluorescence light from sample is separated from OCT light by another dichroic mirror (DM1 in figure 4.1) and passed through the emission filter and is detected by CCD camera. The FM system uses high gain Electron Multiplier-CCD camera (Hamamatsu: C9100-02) for imaging the field of view (FOV) of 450µm X450µm. The CCD camera has pixels of 1000 X 1000 and it can acquire images at the maximum rate of 16.7 frames/s and the acquisition can be accelerated up to 242 frames/s with 16 x 16 binning option. The detected signal by fluorescence CCD camera is sent to the computer through frame grabber (Active Silicon). The filters were set for imaging GFP/GCaMP labeled samples (excitation filter: 488/35nm, emission filter: 535/50nm, Chroma Inc). For different fluorophores, different filters can be used.

4.3 OCT and FM system characterization

**Resolution and field of view**

An USAF target was imaged by OCT as well as by FM system for finding lateral resolution for both of imaging system. An *en face* OCT image was created from 3D OCT image of USAF target (figure 4.2). First order intensity derivative along one lateral
dimension in OCT *en face* and bright filed image were calculated and plotted. The intensity derivative plot has Gaussian shape at the edge of the bars of USAF target.

![Figure 4.2](image)

Figure 4.2: (a) Bright field image of USAF target. (Scale bar 45µm), OCT scanning region was shown in red box, (b) *En face* image of USAF target (Group 6 elements 1) Scale bar 7.81µm)

Lateral resolution was calculated by finding FWHM of the Gaussian section. With 20X water immersion objective, the lateral resolution for OCT system was found to be 4.34µm and for FM system it was found to be 1.86µm. On the other hand, axial resolution of OCT system was found to be 1.72µm in air. The field of view for FM system was found to be 450µm X 450µm and for OCT system it was found to be 90µm X 90µm with 20X water immersion objective.

**System noise characteristics**

For finding noise characteristics in FM camera, a transparent glass was kept in the FM field of view and illumination light intensity was increased gradually. Then the acquired images were processed and the standard deviation was calculated for different level of intensity. Finally the intensity values were converted to no of photons and the standard
deviation was converted to number of electrons for the FM camera. The noise characteristic curve shows that the FM camera is shot noise limited.

Similarly for OCT camera, the intensity in the reference arm was gradually increased by allowing more light through a neutral density filter. Then the standard deviation was calculated for each level of intensity. The standard deviation was plotted against the input intensity level. The OCT noise characteristic plot also shows that it is shot noise limited.

4.4 Spatial registration between OCT and FM images

The image acquisition technique between OCT and fluorescence microscopy is different (figure 4.4). OCT is a point scanning method and it acquires information from each lateral location at a time while FM acquires full en face image from the field of view at a time. For spatial registration between OCT and FM images, firstly a 3D OCT image is processed and then an en face OCT image is made to compare with FM/bright field image of the sample. For finding spatial registration between OCT and FM system, an
USAF target was used as a sample and was imaged both by OCT and FM system. Bright field image of USAF target is shown in figure 4.5(a).

Figure 4.4: Imaging planes of OCT (xz plane) and fluorescence microscopy (xy plane)

Figure 4.5: (a) Bright field image of an USAF target (OCT scanning region is shown in red box), (b) cropped and zoomed at group 7 elements 1 of bright field image of USAF target, (c) En face OCT image of the same USAF target. Selected common features for spatial registration are shown in red arrows
An *en face* OCT image was made of USAF target from 3D OCT image of USAF target (figure 4.5(c)). After visualizing the images, it was assumed that global rigid transformation would be sufficient for spatial registration between the OCT and FM images. 4 common features were identified both in bright field and OCT en face image. The features are shown in red arrow in figure 4.5. After selecting the common features between the images, rotation angle and the scaling factors between OCT and FM pixels were calculated for both lateral directions. The transformation parameters were calculated and OCT image is transformed accordingly and color coded as blue (figure 4.6(b)). Same region of interest is selected in bright field image and cropped from the full bright field image.

![Figure 4.6](image)

Figure 4.6 : (a) Bright field image of USAF target (OCT scanning region is shown in red box), (b) Red color-coded bright field image of cropped section shown in red box in a, (c) Blue color coded OCT *en face* image of USAF target, (d) Overlaid OCT *en face* and bright field image of USAF target (Scale bar: 3.91µm)
The cropped bright field image is color coded in red (figure 4.6(a)) and overlaid with en face OCT image (figure 4.6(c)). From the overlaid image it is clearly visible that en face OCT and FM images are spatially correlated very well.

### 4.5 Temporal registration

A multithreaded software program written in Microsoft Visual C++ is used to synchronize all the devices during acquisition. All of the devices are temporally synchronized by external triggers. The wave pulses are calculated and programmed in acquisition program which drives NI acquisition card (NI-6259) to generate the trigger signal through BNC breaker boards (BNC 2110 and 2120). Each of breaker boards has two analog output channels, two out of four analog output channels create trigger pulses for scanning x and y galvo, one analog output is used to create pulses for OCT line scan cameras, and the last analog channel outputs trigger pulses for fluorescence CCD camera as well as is fed to TTL input of the function generator (figure 4.7). The TTL output of

![Figure 4.7: Schematic diagram of hardware set up for generating trigger signals. AO: Analog output, FG: Function generator](image)
function generator is connected to shutter controller to control the exposure of stimulation shutter. The function generator is programmed to create synchronous TTL pulse for any desired exposure setting for the stimulation shutter. Figure 4.8 shows the timing diagram of all trigger pulses which synchronize the devices and ensures temporal registration between OCT and fluorescence system.

4.6 Verification of temporal registration between OCT and FM acquisition

To verify that the OCT and FM acquisition are temporally registered, an USAF-1951 target was used as a sample and was kept on a 2D translational stage under the
microscope. While acquiring simultaneous bright field and OCT images, the USAF target was moved back and forth in imaging field of view by a 2D translational stage. The OCT reflected light from the bars have different intensity than the background and similarly, the bright field intensity from the bar in bright field images is different than

Figure 4.9: (a) Normalized FM and OCT intensity when rectangular box between group 4 and group 5 of USAF-1951 target is brought in and moved out of the imaging field of view by a translational stage. (b) A zoomed in version (marked in red box in 4.9(a)) of intensity plots for finding temporal resolution of combined OCT and FM system. The temporal resolution of combined OCT and FM system was found to be 60ms.
the background. So moving in / out the black bar of USAF target in imaging field of view introduces intensity variation in both OCT and FM images. The exposure for fluorescence CCD camera was set to 60ms which is the minimum exposure time possible for fluorescence camera for full field of view and OCT images were acquired at line rate of 10 KHz. The time required to acquire one OCT cross-sectional image was 60ms. The OCT intensity for each time point is calculated by integrating the intensity of reconstructed OCT image of USAF target at that time. Similarly, bright field intensity for a time point was calculated by integrating the intensity in same OCT scanning region in bright field image of USAF target. Then the OCT and bright field intensities are plotted over time. Figure 4.9(top) shows FM intensity and 4.9(bottom) shows OCT intensity over time. The plots show a good temporal correlation in the intensity changes between the images of OCT and fluorescence system. The temporal resolution of combined OCT and FM system was found to be 60ms.

4.7 Biological samples imaged by OCT and FM system

Imaging mouse brain slice

Mouse brain slice of thickness of 300µm was imaged with combined OCT/FM system. The bright field image on top of MEA is shown in figure 4.10(a). The reconstructed 3D OCT image of brain slice on top of MEA is shown in 4.10 (b). From 3D OCT image, it is clearly visible that MEA can be separated from brain slice in 3D OCT image which is not possible in bright field imaging system.
Figure 4.10: (a) Bright field image of a brain slice on top of microelectrode array, (b) 3D OCT image of brain slice on top of MEA.

Figure 4.11: (a) Bright field image of epithelial chick cell, (b) topographic image of chick cell by optical coherence phase microscopy, (c) Bright field image of cultured neuron, (d) topographic image of neuron by optical coherence phase microscopy (scale bar: 10µm)
Morphology of chick cell/neurons

Optical coherence tomography provides structural information while fluorescence microscopy provides biochemical, functional information of biological sample. Due to the potential of the combined OCT and FM system, it can be used for various applications. OCT system can be used to observe structures of a cell or tissue while fluorescence images can be used to verify the cell or tissues by labeling the cell/tissue with/without fluorescent dyes. The morphology of chick cell/neuron was visualized by phase sensitive OCT (with high N.A. objective) a.k.a optical coherence phase microscopy (OCPM) and was compared with bright field images. Figure 4.11(b) shows morphology of chick cell by OCPM, corresponding bright image is shown in 4.11(a). Similarly figure 4.11(d) shows OCPM image of neuron and figure 4.11(c) shows bright image of same neuron.

Imaging CNS of Drosophila pupae

The CNS of Drosophila pupae was image by combined OCT and FM system. Initially bright images were taken by FM system. The fluorescence and OCT images were taken after setting the filters set for fluorescence measurements. The bright field, fluorescence and reconstructed 3D OCT images of CNS are shown in figure 4.12. Fluoresced bursicon and kinin neurons are shown in arrows in fluoresced image.
Figure 4.12: Bright field image of GCaMP labeled CNS of *Drosophila* pupae on top of a petridish, (b) Fluorescence image of same animal. Fluoresced bursicon (marked in blue arrow) and kinin (marked in orange arrow) neurons are clearly visible, (c) 3D OCT image CNS on top of Petridish from the lateral region shown in red box in figure a and b, (d) Rotated 3D OCT image of figure (c). One of projection in CNS is shown with an arrow both in bright field image and 3D OCT image. (Scale Bar: 45µm)

**Imaging GFP labeled mouse brain slice**

A GFP labeled mouse brain slice (thickness of 300µm) was imaged by fluorescence microscopy system to see if it was possible to visualize GFP labeled primary neurons with the developed system. Figure 4.13 shows GFP labeled mouse brain slice using 4X and 40 X objective.
Figure 4.13: Fluorescence image of GFP labeled mouse brain slice with 4x objective (left) and 40X objective (Right). Scale bar: 100µm (left figure) and 20µm (right figure)

**Imaging cultured epithelial cells**

Cultured epithelial cells were imaged by developed fluorescence system to see if the cells were alive or not grown in a newly installed cell culture system (figure 4.14).

Figure 4.14: Bright field image cultured epithelial cells (scale bar: 10µm)
Conclusion

In this study, a spatially and temporally registered OCT and fluorescence imaging system has been developed. To our best knowledge, this is the first study of developing temporally registered OCT and fluorescence imaging system. Both of the imaging systems were temporally registered by external trigger pulses. An acquisition software was developed in C++ for synchronous acquisition. After developing hardware and software for the registered system, it was verified that the two imaging systems are spatially as well as temporally registered. The temporally registered OCT and FM system will allow us to assess OCT as a functional tool for detection of neural activity from a neuron or a group neuron. Moreover, we believe the registered OCT and FM system will greatly benefit in various other studies including understanding the mechanism of structural change with functional change of tissue for normal condition as well for different disorders like seizure, cancer etc.
Chapter 5: Identification of optical change in CNS of *Drosophila* by registered optical coherence tomography and fluorescence microscopy

Abstract

This dissertation was aimed to verify OCT as a functional tool for *in vitro* detection of neural activity in a non-invasive and label free manner. Preliminarily, OCT was used to identify optical change in mouse brain slice during seizure like activity from a group of neurons (chapter 3). A temporally registered OCT and FM system has been developed for identification of neural activity by both of modalities (chapter 4). As a final step of assessing intensity based OCT for detection of neural activity, the developed registered OCT and FM system was used to identify neural activity in single neuron. The fluorescence response of GCaMP-labeled bursicon and kinin neurons in CNS of *Drosophila* pupae in response to presentation of ecdysis triggering hormone (ETH) has been well-characterized [123]. Registered OCT and FM system was used to image CNS of *Drosophila* pupae prior to and after ETH presentation. Backscattered OCT light intensity from bursicon and kinin neurons was found to be decreased during neural activity as identified by fluorescence measurements. These results demonstrate that intensity based OCT can be used a functional tool for detection of neural activity without the need for fluorescence contrast.

5.1 Introduction

Electrophysiological methods are widely used for detection of neural activity in nerves but these techniques are invasive and require direct or near contact with the nerve and so
these techniques are not user friendly. In some cases, it is highly desired not to have any direct contact with electrode; for example, cell adhesion and neurite development in cultured neurons are sensitive to physical contact between electrode and neurons [79]. Also in some cases, as for example, for retina, it is inaccessible for electrodes to be in contact with nerve. On the other hand, fluorescence methods can be used to identify neural activity, but these methods require exogenous contrast agents such as voltage- or calcium-sensitive dyes or genetically modified fluorescent markers [61, 81-82] to identify neural activity. A non-contact, label free method for detection of neural activity will be great beneficial for many applications. Optical coherence tomography is an ideal tool for identification of neural activity from nerve in a non-invasive and label free manner. In several studies, the transient thickness change of nerve was identified during action potential propagation by phase-sensitive OCT as an indirect measurement of neural activity [1, 34, 38, 92, 93]. One limitation of these studies is phase sensitive OCT data were acquired from same lateral position of nerve over time during action potential propagation. Identification of neural activity from multiple lateral locations of nerve is not possible with phase-sensitive OCT due to increased phase noise introduced for lateral scanning. To study a group of neurons or a neuronal network, it is necessary to identify neural activity from different lateral locations. Opposite to the phase-sensitive OCT measurements, intensity based OCT can be used to obtain information from different lateral locations of nerve/network by scanning. Previously, intensity based OCT was used to identify seizure like activities which are easy to detect (chapter 3). For next level of assessment, intensity based OCT was used to identify more physiological neural activity
in this study. Fluorescence microscopy was incorporated with OCT to compare OCT results with fluorescence measurements during neural activity (chapter 4). In this study, the registered OCT and FM system was used to identify optical changes from CNS of Drosophila during neural activity. The OCT intensity changes were correlated with fluorescence intensity changes during neural activity.

5.2 Animal model and experimental set up

It has been shown that the fluorescence intensity in GCaMP-labeled bursicon and kinin neurons in central nervous system (CNS) of Drosophila pupae change in response to presentation of ecdysis triggering hormone (ETH). We use the same animal model for our study. The brain of Drosophila pupae was extracted from the animal and the brain was attached in a petridish and buffer solution is added in the petridish. Then the sample is brought under microscope for simultaneous OCT and FM acquisition (figure 5.2). Then the objective is focused for brightly fluoresced neurons. The fluorescence exposure time for the fluorescence camera was set to 1s and the opening time of shutter that controls the excitation light was set to 160ms. For each of FM image acquisition, the shutter was kept open for first 160ms and closed for rest of 840ms. The OCT camera acquisition was set was set to 10000 depth profiles/s. 1024 A-lines and 100-B lines were scanned for 90µmX 90µm field of view. Baseline OCT and FM images were acquired for 5-7 minutes. Then 150nM/600nM ETH is injected in the solution carefully so that the Sample does not move.
Figure 5.1: (a) Life cycle of *Drosophila*, GCaMP-3 labeled brain of *Drosophila* pupae is selected as animal model for experiment, (b) Schematic diagram showing peptidergic ensembles producing ETHR-A. (Neurons of interests are bursicon and kinin neurons) [123,136]

Figure 5.2: Experimental set up for imaging CNS of *Drosophila* pupae by combined OCT and FM system. The sample is kept on a petridish(P). (The schematic complete diagram of combined OCT-FM system was shown in chapter 4) (pBS: Plate beam splitter, OL: Objective Lens, UM: upright microscope, DM: Dichroic mirror, F: Filter)

Simultaneous OCT and FM data are acquired for another 25-35 Minutes after ETH
presentation.

5.2 Structural imaging of brain

Bright field images were acquired from a dissected brain of *Drosophila* pupae. Bright field images were acquired by using white light that transmits through the sample from the halogen lamp of the microscope. Figure 5.3(a) shows a bright field image of CNS. Then simultaneous OCT and fluorescence images were acquired from same field of view. The exposure time for excitation light source was set to 160ms. The exposure time of fluorescence CCD camera was set to be 1s. Figure 5.3(b) shows fluorescence image of the CNS of Drosophila brain. Some of fluorescence bursicon neurons are marked blue arrow and kinin neurons in orange arrows in figure 5.3(b). The OCT scanning region in fluorescence image is shown in a red box. The scanned OCT cross-sectional images of the CNS on top of petridish are combined and a 3D image is reconstructed (figure 5.3(c)). From the 3D image, the CNS is clearly visible and separable from petridish. Also the elongated projection from CNS is clearly visible in the 3D OCT image of CNS. As OCT provides depth resolved images, it is easily possible to separate different depths of the tissue and to analyze results for different depths of the sample separately.
5.3 OCT and FM intensity change during neural activity

To study the optical changes in backscattered OCT and fluorescence light from CNS, the acquired OCT and fluorescence data were analyzed as follows. Firstly, for each of fluorescence cell, fluorescence images were shifted and corrected over time for any
lateral movement by performing local registration for each of fluoresced neuron. Region of interest (ROI) corresponding to each cell is selected (blue circles in figure 5.4(a)) and the fluorescence intensity for each cell is calculated by summing the intensity of that region of interest. OCT images are also corrected for lateral movement for each cell using the same specific fluorescence shift correction which was used to correct for lateral movement in fluorescence images over time. Then OCT lateral ROI is selected same as fluorescence ROI for each cell after performing spatial registration. Intensity from that lateral ROI over whole depth of the CNS is integrated to calculate intensity for each time point. The fluorescence and OCT intensity corresponding to the different bursicon neuron over time is shown in figure 5.4. The timing of ETH presentation is shown with arrow. The fluorescence and OCT intensity changes around 5-7 minutes after ETH injection. There is a very good correlation between OCT and fluorescence intensity changes. It was well characterized in previous studies that introducing more concentrated hormone initiates early fluorescence change in the cells and we observe the similar results. When 150nM of ETH was introduced, the fluorescence change starts around 7 minutes after hormone presentation (figure 5.4(c)), and when 600nM of ETH was used, initiation of fluorescence change starts within 5 minutes of ETH presentation (figure 5.4(e)). Initiation of OCT intensity change also occur within 5 minutes after 600nM ETH presentation (figure 5.4(f)) while the OCT intensity starts to change within 7 minutes when 150nM of ETH was presented (figure 5.4(d)). A very good temporal relation was found between the OCT and fluorescence changes.
Figure 5.4: (a) GCaMP labeled CNS of *Drosophila* pupae A. (b) GCaMP labeled CNS of *Drosophila* pupae B. (OCT scanning region is shown in red box and the focused cell of interest is shown in blue circle.) (c) Normalized fluorescence intensity in bursicon neuron of animal A (blue circle in figure a). (d) Normalized OCT intensity of animal A in same region of interest as shown in figure (a). (e) Normalized fluorescence intensity in bursicon neuron of animal B (blue circle in figure b). (f) Normalized OCT intensity of animal B in same region of interest as shown in figure (b). (The time of ETH presentation is shown in arrow at time = 0 mins)
5.4 OCT intensity from bursicon and kinin neurons

More importance was put on imaging bursicon neurons during experiments and so the FM imaging depth of focus was set for bursicon neurons. So fluorescence change was expected from bursicon neurons only and not from kinin neurons as kinin neurons do not lie in same plane rather they lie typically 40-50µm below of bursicon neurons, the focusing depths for kinin neurons were found to be ~50 µm below of bursicon neurons. The fluorescence intensity from bursicon and kinin neurons are shown in figure 5.5(b,d). Although fluorescence change in kinin neuron was not identified due to different focusing depth compared to bursicon neuron, OCT intensity corresponding to kinin neuron changes significantly. With compared to OCT intensity change in lateral region of bursicon neuron, the OCT intensity change corresponding to kinin neuron is more and obvious (figure 5.5(e)). The location of kinin neuron was out of OCT scanning range for other animals and so it was not possible to analyze OCT intensity for kinin neuron for other animals.
Figure 5.5: (a) GCaMP labeled CNS of a *Drosophila* pupae bursicon neuron is marked as region of interest (ROI) #1 and kinin neuron is marked as ROI#2 with blue circle (OCT scanning region is shown in red box and FM system was focused for bursicon neuron (ROI#1)). (b) Normalized fluorescence intensity in bursicon neuron (ROI#1), (c) Corresponding normalized OCT intensity in bursicon neuron (ROI#1). (d) Normalized fluorescence intensity in kinin neuron (ROI#2), (e) Corresponding normalized OCT intensity in kinin neuron (ROI#2). (The time of ETH presentation is shown in arrow at time = 0 mins)
5.5 Depthwise OCT intensity variation in CNS corresponding to bursicon and kinin neuron

Opposed to normal fluorescence imaging, OCT can provide information from different depths as OCT is capable of 3D imaging of the biological sample. As bursicon and kinin neurons lie in different depth planes in CNS mainly in mid and bottom region of CNS respectively, OCT data was also analyzed for 3 different depths to identify OCT intensity variation corresponding to different depths. Firstly 3D OCT image of CNS is sectioned in 3 different layers (each of 40µm thick). Then the OCT intensity for each layer was calculated by integrating the intensity of the lateral pixels corresponding to same lateral position as fluorescence ROI and for all axial pixels in that layer. The OCT intensity decrease was found mainly middle and bottom layer of CNS (Figure 5.6(b,d)). OCT intensity change in three different layers corresponding to kinin neuron is more obvious and separable than OCT intensity change in those three layers corresponding to bursicon neuron. As the kinin neurons are mostly at the bottom of CNS, the OCT intensity corresponding to kinin neuron changes in bottom layer more significantly than in the middle layer (figure 5.6(d)). For bursicon neuron, the OCT intensity change is observed between middle and bottom layer (figure 5.6(b)).
Figure 5.6: (a) Normalized fluorescence intensity in bursicon neuron (ROI#1 of figure 5.5(a)), (b) Corresponding normalized OCT intensity in depthwise discriminated 3 layers (thickness of each layer is 30µm) (c) Normalized fluorescence intensity in kinin neuron (ROI#2 of figure 5.5(a)), (d) Corresponding normalized OCT intensity in depthwise discriminated 3 layers (thickness of each layer is 30µm). (The time of ETH presentation is shown in arrow at time = 0 mins)

Conclusion

In this study the registered OCT and FM imaging system was used to identify the change in optical properties in bursicon and kinin neurons of *Drosophila* pupae after presentation of ecdysis triggering hormone (ETH). In previous study, the fluorescence change in bursicon and kinin neurons of *Drosophila* due to presentation of ETH was well characterized. In this study, the change in backscattered OCT light intensity from
neuronal cell was identified and was correlated with fluorescence intensity change from the same neuronal cell. OCT intensity decreases within 5-7 minutes of ETH presentation while fluorescence intensity increases within 5-7 minutes of ETH presentation. Both of OCT and fluorescence intensity change shows a very good temporal relation. The temporal correlation between fluorescence intensity and OCT intensity changes indicate that OCT can be used as a potential tool for neural activity detection in a non-contact and label free manner.

While conventional fluorescence system provides fluorescence information from a depth at a time, OCT can provide information from different depths at the same time. By utilizing depth discrimination capabilities of OCT, the change in backscattered OCT intensity from different depths of CNS was identified and it was found that the intensity change correlates well with the physical depth location of neuron in CNS. As bursicon neuron lies mostly between top and middle of CNS, we see a good OCT intensity change in those layers when the lateral region of interest was chosen for bursicon neuron. On the other hand, as kinin neurons are mostly at the bottom of CNS, we observed a significant OCT intensity decrease at the bottom of CNS when the lateral region of interest was chosen for kinin neuron.

In summary, the optical change detected by OCT intensity measurement during neural activity demonstrates intensity based OCT measurements can be used as a tool for neural activity detection in a label free manner.
Conclusion

Detection of neural activity in non-invasive and label free manner is user friendly and beneficial for various neural studies. This study was aimed to assess intensity (non-phase) based OCT as a functional tool for detection of neural activity in non-invasive manner and without using any contrast agents. Neural activity from a fixed lateral location of *limulus* optic nerve was identified by using phase-sensitive OCT. With phase sensitive OCT, it is not possible to identify neural activity from different lateral locations of nerve by lateral scanning as scanning introduces significant phase noise which makes phase sensitive analysis to be nearly impossible. On the other hand, intensity based OCT information can be obtained from different lateral locations by scanning. So this dissertation was aimed to identification of neural activity by intensity based OCT. For preliminary assessment, intensity based OCT was used to identify optical changes in mouse brain slice during seizure likes activities. OCT intensity was found to be decreased during onset of burst of activity. These preliminary successful results motivated for next level of assessment of intensity based OCT for neural activity detection. Fluorescence microscopy (FM) was incorporated with OCT for better comparison and next level of assessment. A registered spatially and temporally registered OCT and fluorescence microscopy (FM) system was developed. The spatial and temporal registration between OCT and FM were verified by experiments. For final assessment of intensity based OCT for identification of neural activity, the developed registered OCT and FM system was used to identify optical changes in neurons of *Drosophila* pupae during induced activity.
Consistent with preliminary results, OCT intensity in CNS of *Drosophila* was also found to be decreased during neural activity identified and verified by fluorescence measurements. These results further verified that intensity based OCT can be used for detection of neural activity in non-contact and label free manner.
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