MULTIPLE MODALITY OPTICAL NEURAL IMAGING WITH VCSELS

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
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Abstract

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2011

I investigate the use of vertical cavity surface emitting lasers (VCSELs) for portable optical neural imaging. In particular, I attempt to use one laser source to produce the different illumination needed for three techniques, imaging flow, oxygenation, and fluorescence. Our group’s goal is to combine these techniques using a single source in a simple imaging format, which can be modified for portable use in awake animals. We exploit tuning properties of VCSELs to implement two distinct operation schemes with different illumination characteristics. Single mode operation provides high speckle contrasts, used to map flow speeds in laser speckle contrast imaging (LSCI). Sweep operation provides low noise, allowing us to image small signal changes with intrinsic optical signal imaging (IOSI), which relate to blood oxygenation. Finally, we use our VCSEL devices for on-chip integrated fluorescence sensing. Future work aims to develop a fully portable format, using CMOS detectors and integrated circuit control.
Acknowledgements

There are many people who have been helpful over the last two years, contributing to the studies which comprises this thesis. I would like to acknowledge them all, as without them my work would have not been possible. First and foremost, I would like to thank my supervisor, Ofer Levi, as well as my co-graduate student Dene Ringuette, for working together with me in all of these endeavours and allowing our experiments and ideas to come to fruition. Ofer’s guidance and motivation has been an ongoing source of inspiration, and without him none of this work would exist. His knowledge and enthusiasm for a wide range of topics have helped every aspect of our studies, and helped me develop my own skills as a researcher. Dene has been an excellent lab mate over the past two years, and working with him as a sort of tag team in animal surgeries and experiments has been enjoyable and productive. It’s been good to have someone nearby to help check myself and give biological insight to an engineer lost in a world of anatomy and chemistry.

Thanks also to Elizabeth Munro, who got here before us and started it all. When we started in the lab it was Liz who taught us everything, from surgical skills to understanding tissue modelling and analysis. Even up until the end, her help with ASAP simulations was useful to finally crunch the numbers we could guess but never verify. To Ryan Schilling, Deniz Aydin, and Mohammed El Behairy, it was good having you on the other side of things in our group, to share conversation, meals, and countless late nights in SF4108. To Tom O’Sullivan and the rest of the Stanford team, thanks for assisting us in fabrication and developement. Tom has been extremely helpful in establishing the setup we use for fluorescence measurements, as well as packaging and testing devices.

To the incoming grad students, Costa Nicolau and Yaaseen Atchia, we’ve had a fun summer but now it’s time for you really get down to work! But seriously, I wish you both luck in your degrees, and hope you can work with the knowledge we’ve accumulated, and will leave for you on The Wiki. Thanks also to the excellent summer students we’ve
had over the years. Xiaofan Jin, Duo Yao, and Justin Wee have all been important in
developing the software tools we use to record and analyze data in real time.

To our collaborators in Dr. Gang Zheng and Dr. Brian Wilson’s groups, thanks for
providing the resources and help necessary to complete our fluorescence sensing studies.
Thanks to Natalie Tam for helping me with early dye measurements, and to Tony Liu for
suggestions on imaging dye in liquid phantoms. Thanks to co-op student Joseph Gold
for his long hours spent conducting animal studies with me. And finally thanks to Tracy
Liu, for useful discussions and advice, and generally knowing everything about in vivo
fluorescent measurements.

Thank you also to our collaborators Dr. Bojana Stefanovic and Adrienne Dorr at
Sunnybrook hospital, for assisting us with two-photon imaging and vascular mapping, and
to Dr. Peter Carlen and Dr. Alex Tonkikh at Toronto Western hospital. Though most of
this work hasn’t reached completion for this thesis, we have a lot of planned experiments,
and these studies we have started have given tremendous insight and prompted new
questions as well. Thanks as well to Joe Hayek of Dr. Carlen’s lab, for teaching us his
ischemic surgery model, which we have found very useful.

To the helpful people at QImaging, thanks for ongoing support of our camera and
software needs. To Mic Chaudoir, your visits were always welcome for discussion of our
system and brainstorming how to take things further.

Finally, I’d like to thank my friends and family for being there for me when I needed
it. Mom, Dad, David and Aaron have always been reliable supports and given me the
confidence and desire to strive for more. And last but certainly not least, thanks to
Monica, for being the person who probably has seen me most in the last two years and
not gotten sick of me, for being someone that I know can count on for anything, and for
being a wonderful person in more ways than I can count. Thank you for standing by me
and being my best friend.
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Chapter 1

Background and motivation

The potentially powerful techniques of optical imaging have become increasingly prevalent options in biomedical research in recent years. For discerning functional along with anatomical information at high speed, high resolution and low system complexity, optical modalities are becoming established as a potential alternative to more developed and costly techniques such as fMRI, SPECT or PET [1]. The process of bringing these modalities to the mainstream in biomedical imaging has been aided by the gradual improvement of flexible illumination sources and detectors, boosting detected signal to noise ratios to a level ideal for these techniques. Further research into the fundamentals of optical interactions has given us unprecedented understanding of how light and tissue interact, leading to improved sensitivity and accuracy in accounting for changes in tissue parameters.

In particular, intrinsic optical signal imaging (IOSI) and laser speckle contrast imaging (LSCI) are two techniques which complement each other strongly, providing data on blood oxygenation levels and blood flow/movement respectively. IOSI relies on changing reflectance levels at one or several wavelengths to quantify hemoglobin variances. Total hemoglobin changes can be monitored by single wavelength illumination [2]. As both oxygenated (HbO) and deoxyhemoglobin (HbR) exhibit low absorption to red light, illu-
mination at these wavelengths is ideal to quantify small percentage changes in reflectance, and relate them to changing blood levels. Using known spectral differences in red and near infrared wavelengths, fitting of absorption spectra at two or more wavelengths can be used to compare HbR to HbO levels [1, 2]. In contrast, LSCI makes use of the coherence decorrelation caused by moving scatterers to resolve flow and motion. The stationary speckle pattern of a coherent source is disrupted in the presence of moving particles, and over the time of an image exposure, interference averaging leads to a reduction in speckle contrast in the surrounding region [3]. Thus, low contrast regions are associated with movement, and a number of models are being currently developed to accurately quantify velocities [4]. If we implement these techniques with the addition of anatomical and functional imaging through fluorescence, a wide range of biological data can be obtained by comparatively simple optical techniques. Several systems have attempted to incorporate these techniques in tandem to varying levels of success [2, 5, 6]. The main limitation in producing an elegant solution combining functional and flow information is based on the disparity between illumination requirements for each technique. This is one of the main difficulties which this thesis tries to address. By understanding the needs of each technique, we attempt to produce a simple, cost effective system which can be used to easily record useful anatomical and hemodynamic information in neural tissue. It is our goal to use these results to bring optical techniques to a more portable platform, and facilitate future use of these techniques in a medically relevant context.

1.1 Optical neural imaging techniques

Using light to image tissues at a level beyond simply the anatomical has been a common goal for researchers. Many techniques exist today that take advantage of the various properties of tissue that effect light, and take advantage of a wide range of optical effects to produced different results. Whatever technique is used, the basic interactions remain
the same in all cases. What differs is the way that the illumination source is used, and how it is detected.

### 1.1.1 Light interaction with tissue

Optical imaging techniques attempt to extract useful information on the basis of light interaction with tissue. When a photon interacts with a particle in tissue, there are generally two behaviours which may occur: the photon may be absorbed, or the photon may be scattered. Absorption involves the photon being changed into energy which alters the internal state of the incident particle. Scattering normally does not change the energy of the photon, but simply changes its direction. Based on these two phenomena, we can model light interactions using two parameters: $\mu_a$ and $\mu_s$, the absorption and scattering coefficients. Each of these quantities has units of length$^{-1}$, as they represent the proportion of particles respectively absorbed or scattered per unit length. The effect of absorption is typically modelled using the Beer Lambert law:

$$I(z) = I_0 e^{-\mu_a z}$$

where $I(z)$ is the intensity of light at depth $z$ [7].

In a highly scattering medium, a modified version of the Beer Lambert law can be used to account for the combined effects of scattering and absorption. This version is reliant on a limit known as the diffusion approximation, which is valid when scattering is much stronger than absorption. Another important property in this approximation is the anisotropy of scatterers, $g$. Anisotropy is a measure of the proportion of scattering events which produce forward scattering angles. Specifically, $g$ is defined as the expectation value of the cosine of the angle between incident and scattered photons, and is generally taken to be 0.9 in most tissues. A reduced scattering coefficient can be defined as

$$\mu'_s = (1 - g)\mu_s$$

(1.2)
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This represents the proportion of illumination that is attenuated by scattering events per unit depth. We can now define a new attenuation constant,

\[ \mu_t = \mu_s' + \mu_a \] (1.3)

The reciprocal of this quantity is the effective mean free path in tissue.

Quantification of the diffuse reflectance measured is dependant on the geometry of the source and imaging apparatus. Different orientations will produce different results. In the case, for example, of a collimated or point source incident on tissue, and a detector measuring reflectance at a lateral distance \( r \) from the source, the reflectance is directly dependant on the attenuation coefficients and on \( r \). The reflectance will decay exponentially as radius is increased, at a rate of [9]:

\[ \mu_{eff} = \sqrt{3\mu_a(1 + \mu_s'/\mu_a)} \] (1.4)

In the case we are concerned with, specifically measurement of the diffuse reflectance from a wide beam incident on tissue, approximations can be made by viewing any absorption as caused by interactions along the mean optical path in, and then out, of tissue. Total attenuation through the tissue can be accounted for using tissue modelling based on the parameters of \( \mu_s, \mu_a \) and \( g \) [10]. The effective path length \( L \) in tissue becomes the quantity we need to evaluate total attenuation by absorbers, and depends on system geometry, as well as tissue parameters. An estimate can be made taking these into account, given by [9]:

\[ L = \frac{A}{\sqrt{3(1 + \mu_s'/\mu_a)}} \] (1.5)

A is a path scale factor, typically estimated to be about 7 in tissue. \( L \) itself is usually better determined using Monte Carlo simulation. The modified Beer Lambert law can then be written as [7]

\[ log\left(\frac{I_0}{I(L)}\right) = \mu_a L + G \] (1.6)
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G here is a geometry dependant factor. Seeing as \( \mu_s \) is generally constant in tissue for a given wavelength, the modified Beer Lambert law can be used to quantify changes in absorption, caused by concentration changes in substances such as hemoglobin or water.

1.1.2 Intrinsic Optical Signal Imaging (IOSI)

IOSI is a technique which is used to measure changes in neural activity by measuring reflected light (Fig. 1.2). The technique is often compared in both literature and in experiment to functional magnetic resonance imaging (fMRI), a more established technique often used clinically to measure neural activity. The underlying physiological basis for IOSI is precisely the same as that for fMRI: neural activity can be closely correlated with hemodynamic changes in the surrounding vasculature [11, 12]. The nature of this association has been studied extensively, although there are still outstanding questions [13, 14, 15]. In general, an increase in cerebral oxygenation and blood flow is seen in regions of heightened activity. These functional changes are, however, of a slower time scale than the activity itself. fMRI takes advantage of the speciation of hemoglobin, based on whether oxygen is (HbO) or is not (HbR) bonded. The MRI signal varies due to the different magnetic properties of the different Hb species. Through careful quantification of these changes, regions of higher oxygenation can be isolated, and therefore be identified as showing heightened activity. In IOSI, rather than magnetism it is the spectral differences between the two species which can be measured and used to quantify changes (Fig. 1.1). The main assumptions applied here are that the primary absorbers in the desired spectral range are HbR and HbO, and that scattering is essentially uniform throughout the tissue [5]. By using two wavelengths of light with known absorption in hemoglobin, a linear model using the modified Beer-Lambert law can be applied to discern any changes in the concentration of both HbO and HbR [16, 17].

One limitation in IOSI comes from the effects of scattering and absorption through tissue such as skull. Very little light is able to penetrate deeply through bone. As a
Figure 1.1: (a) Illustration of hemoglobin in its deoxygenated form. (b) Hemoglobin in its oxygenated form. There are slight conformational changes. (c) Absorption spectra showing differences between HbR and HbO. [18, 19]

Figure 1.2: Intrinsic signal imaging of whisker stimulation in a rat. A slight decrease in reflectance is seen before the large increase of the main response pulse.

result, spatial resolution and image quality is greatly limited when the skull is left intact [20, 21]. Wavelengths in the near infrared (NIR) range are able to penetrate deeper than others, and only at these wavelengths is it possible to obtain useful information while imaging through skull. Advanced techniques using dense arrays of sources and
detectors in the NIR range are able to map hemodynamic changes through the skull with limited resolution [22]. This is often referred to as diffuse optical tomography (DOT) or near-infrared spectroscopy (NIRS) (Fig. 1.3). For higher spatial resolution and greater accuracy, the removal of skull is mandated. Another option which allows increased imaging capabilities through intact bone is skull thinning. This technique has been used in animals to reduce the overall invasiveness of IOSI [23]. The desired region of skull may be thinned surgically to a very fine layer through which the cortex and vasculature can be seen with better resolution. In this case, animal survival is more feasible, and longer term studies can be completed with repeated measurement.

Researchers have shown that sensory stimulation can result in reflected amplitude changes on the order of 0.1% in rodents, corresponding to hemoglobin concentration changes of fractions of a milliMolar [24, 15]. Significantly greater changes can be found in primates and larger animals [25]. It is clear, then, that in order to see signal changes in rodents, very stable illumination sources are needed. Generally, high power, stable LEDs are used for their relatively narrow spectra and low noise. However, with typical LED spectral widths in the 10-20 nm range, even narrower sources would be preferable to accurately account for Hb absorption. Recently, research has suggested that typical laser devices produce illumination with spatial and temporal noise much greater than the desired level of 0.1% [26]. This noise arises from the fact that lasers produce spectrally pure, or coherent, illumination. Coherent illumination causes scattering light to interfere with itself, resulting in alternating dark and light regions within an image, which manifest as speckle noise. Some researchers have attempted to use laser devices in spite of this limitation, by implementing averaging techniques or otherwise reducing the apparent noise of the source [5]. One goal of this thesis is to investigate methods of reducing speckle in lasers by decreasing source coherence, in order to allow these devices to be used for intrinsic signal imaging.
1.1.3 Laser Speckle Contrast Imaging (LSCI)

While the use of coherent illumination is regarded as a limitation in the context of IOSI, the interference noise which results is often used instead as a way to obtain other information about tissue. LSCI is a technique which allows us to take a speckled image and use image processing techniques to extract information on the speed of moving particles. Fig. 1.4 shows a simulated example of speckle contrast imaging in action. The principle of LSCI arises from the fact that speckle is a property which can be effected by the imaging system, illumination source and imaged subject. For uniform illumination of an isotropic sample, the speckle pattern will be essentially uniform. If we calculate the standard deviation divided by mean (a calculation known as the speckle contrast) in any two regions, we would expect to obtain the same value. In practice there are statistical variations, but on average the values are identical. If, instead of a stationary image, there are moving elements within the frame, the speckle contrast about these elements will be reduced. This is caused due to averaging of many individual speckles, essentially as if the pattern were blurred out. Faster scattering elements will cause greater speckle reduction. In this way, we can map speckle contrast to speed within an image [27]. An in vivo example of this is shown in Fig. 1.5, where darker vessels correspond to higher
blood flow.

Figure 1.4: Simulated speckle data. When a region of a laser illuminated image contains moving scatterers, the speckle pattern is seen to vary over time. The resultant blurring seen during an image exposure time can be used to produce a contrast map, where speckle intensity is linked to blood flow. By averaging many frames, statistical variance in the pattern is reduced, producing a clear flow map.

From the earliest descriptions of LSCI, it was known that the resulting contrast map is dependent on the image exposure time. Generally, higher exposures will produce greater speckle reduction. Recently, this property was used to increase accuracy in evaluating flow by using a range of exposure times. A single long exposure frame can be used to estimate relative flow speeds in nearby elements, but multiple exposures over many orders of magnitude can be used to absolutely calculate speckle decorrelation times, which are inversely proportional to velocity [28]. Further advantages have been found from the discovery that multiexposure models can be used evaluate flow in the presence of heterogeneous scatterers, where there are stationary scattering elements in conjunction with the moving particles [4]. Many of the results in modeling and quantifying LSCI come from Dynamic Light Scattering (DLS), which monitors interference changes temporally, rather than spatially. The same theory applies in each case under the assumption of ergodicity, which implies that the statistics of spatial sampling match those of temporal sampling.
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1.1.4 Fluorescence imaging

A final versatile tool which is used in medical imaging frequently is fluorescence. 'Fluorescence imaging' is a term that describes a wide range of techniques which are based on the same fundamental interactions. The process of fluorescence production is outlined in Fig. 1.6. When light is absorbed by certain substances commonly known as fluorophores, its energy raises the substance to an excited state. Once excited, the fluorophore will tend to return to an unexcited state. In the process of returning to ground state, the fluorophore will re-emit the incident light with some of its energy lost through other processes, such as heating. This emitted light will have lower energy, and thus longer wavelength, than the incident light. This process is known as fluorescent emission.

In biomedical imaging, fluorescence becomes important when we take tissue and treat it in some way with a fluorophore. Generally, we would like the tissue to preferentially exhibit fluorescence in specific regions relating to function or anatomy. When tissue is in this way functionally or anatomically tagged, we can illuminate a sample with uniform light and detect signal localized to the specific regions we are interested in. There are several ways in which this specificity can be achieved.
Figure 1.6: The principal of fluorescence: light is absorbed by a substance, exciting the fluorophore to a higher state, which then re-emits light of a lower energy.

Exogenous fluorescent markers which can be introduced to an organism or tissue are often in the form of a dye. These dyes can be attached to other molecules which have interesting physiological properties. Through attachment of a dye to the functional group of a protein, the resulting marker or probe can be selectively uptaken in specific tissues or cells [29]. A wide range of markers can be used to selectively tag many tissues, allowing detailed functional and anatomical imaging. More advanced markers include the group of probes known as molecular beacons. These probes contain a fluorophore connected to one end of a strand of nucleic acid, and a molecule known as a quencher at the other end [30]. The structure of such a probe is shown in Fig. 1.7. When the fluorophore is in close proximity to the quencher, any fluorescent emission will be absorbed by the quencher, resulting in a lack of signal. The strand is looped around such that the two ends normally meet, and fluorescence is quenched. When the beacon is introduced to tissue expressing the strands complimentary DNA or RNA sequence, the loop is cleaved and the fluorophore end is freed. Only in its cleaved form will the beacon exhibit fluorescent
signal, leading to strong marker specificity.

Figure 1.7: Molecular beacons consist of a looped strand of nucleic acid with a fluorophore and quencher on either end. Only once the strand is cleaved, fluorescent signal can be produced.

Many common proteins or biological substances tend to fluoresce weakly on their own. This property is often referred to as autofluorescence, and manifests as a background noise which we generally would like to eliminate as much as possible [31]. Genetic techniques recently developed can be used to cause organisms to produce much stronger fluorescence endogenously. Proteins such as Green, Red, and Yellow fluorescent protein (GFP, RFP, YFP) are mutated from luminescent organisms such as jellyfish, and can be introduced into other organisms to be expressed only in certain tissues [32]. This allows researchers to image fluorescence in cells without the need of introducing a dye or marker from outside. Although these proteins are very useful in anatomical imaging, they prove limited in many functional imaging contexts. If we wish to track cellular changes or tissue development, we can only see fluorescence changes as quickly as the proteins can be synthesized. In cases for which we want to see real time dynamics of metabolic behaviour, the slow time
One important application of fluorescence imaging is measurement of cancerous tissues. In many cases cancers will express specific proteins, metabolically uptake dyes at higher rate, or otherwise exhibit properties that allow them to be preferentially tagged with dyes and markers \[33, 34\]. Using fluorescence, we can precisely tag and image tumours and view their progress over time[35]. This can allow a surgeon to precisely identify the location of a tumour for removal, or to monitor the progress of disease and of response to treatment. In particular, with glioma or other brain cancers it is important to be able to localize tumours. In cases for which surgery is needed, damage to external tissue must be minimized. For monitoring of treatment progression, accurate knowledge of a tumour’s extent is critical to properly inform further treatment options. A significant limitation in these cases would come from the need for fast acting dyes; many quenched markers are activated slowly, and full signal is not reached until many hours or days after the marker is first introduced. For this purpose, beacons which reach their target rapidly and are then quickly activated are ideal.

1.2 Applications of optical neural imaging

1.2.1 Animal models of disease

Optical imaging techniques have often been applied to study animal models of disease and other factors affecting hemodynamic response. A common functional imaging method involves stimulating the sensory cortex of an animal, by either stroking a whisker or electrically stimulating a paw. Through imaging the correct region of barrel cortex, the animal’s cortical response to stimulation can be observed [36]. This technique can be used to understand the nature of hemodynamic response in healthy subjects, as well as to monitor abnormal responses in damaged specimens [37].

In diseased states, research has shown that cortical hemodynamic responses can be
strongly altered. In stroke, for example, affected cortical regions will exhibit reduced response to stimulation. Reduced blood flow is also commonly seen in regions of stroke. LSCI and IOSI can be used to monitor the extent of stroke damage, as well as view progression of the injury and potential recovery. Many researchers apply these techniques in models of induced cortical ischemia for this purpose [38]. In humans, the progress of stroke recovery based on hemodynamics has been imaged using fMRI, showing strong correlation between brain activation and motor recovery [39].

In the case of epileptic seizures, the results of both LSCI and IOSI have been shown to be important in discerning seizure focii. Studies have shown that there is a clear increase in cerebral blood flow at the onset of a seizure, but in the region of an epileptic focus hypoxic conditions are produced as the increased flow cannot meet local metabolic needs [40, 41]. These coinciding phenomenon have been suggested as a way to both predict onset of seizures, and to localize the center of epileptic genesis in cases where surgical treatment is required.

1.2.2 Multi modality optical imaging systems

Several research groups have recently attempted to combine techniques for measuring oxygenation, blood flow or fluorescence simultaneously.

“Ultra-fast multispectral optical imaging of cortical oxygenation, blood flow, and intracellular calcium dynamics”, Fig. 1.8(a)

This system incorporates visible wavelength intrinsic signal imaging at high magnifications. LED sources at 530 nm and 470 nm are alternated using a microcontroller strobe triggered by camera outputs. Using magnification along with increased frame rates, this system is able to evaluate flow in small regions by tracking individual blood cells, using a technique referred to as line scan correlation. The system also attempts to incorporate fluorescence imaging of calcium sensitive dyes using a removable fluorescence emission
Figure 1.8: Multi modality imaging systems. (a) M. B. Bouchard, et al. (b) Z. Luo et al. (c) S. Sakadzic et al.
filter on the CCD. The use of lower wavelength sources in this work limits the depths of tissue which can be imaged, and the lack of full field real time flow data renders the line scan technique slow and computationally heavy [42].

“Simultaneous imaging of cortical hemodynamics and blood oxygenation change during cerebral ischemia using dual-wavelength laser speckle contrast imaging”, Fig. 1.8(b)

This system uses dual wavelength NIR laser diodes to image both blood flow and oxygenation changes, applying LSCI and IOSI. The frame rate of this system is limited by the use of a synchronized mechanical chopper to alternate sources, producing images at 2 FPS. Further, the use of coherent lasers for IOSI leads to limited sensitivity to intrinsic signals, and the system is only demonstrated in the case of strong ischemia [5].

“Simultaneous imaging of cerebral partial pressure of oxygen and blood flow during functional activation and cortical spreading depression”, Fig. 1.8(c)

This system uses phosphorescent dyes to determine partial pressure of oxygen, by measuring phosphorescent decay times. It combines this technique with LSCI to provide simultaneous oxygenation and flow data. With these two quantities, the metabolic rate of oxygen can be calculated. The system provides a full and quantitative look at hemodynamic changes, but requires many expensive components, as well as the use of an injected marker. These requirements limit the practicality of this system in many applications [6].

1.2.3 Our vision: Portable neural imaging using vertical cavity surface emitting lasers

With an understanding of the requirements involved, we intend to move the optical imaging techniques described here to a portable platform. By developing elegant solutions
which allow us to accomplish multi modality imaging using minimal components, we can begin to implement these techniques in a compact form, with implantable or wearable technology. The main advantage gained by moving to a portable platform, beyond cost, is the ability to image freely moving subjects over extended periods of time. All current modalities require some form of restraint of the subject, be it general anaesthesia or some physical form of restraint. This not only restricts the behaviour of the subject, but also restricts the time scale over which imaging can occur. Once an animal is released from restraint, imaging must end, to potentially be continued at a later time point. By allowing the imaging system to move along with the subject, we gain the ability to monitor a subject’s natural behaviour over whatever time course is desired. Some devices have already been developed to monitor fluorescent signal over extended periods of time in active animals through implantation [43]. If we do wish to implement the multi modality techniques outlined here in a portable form, we would ideally move to a single source, single detector scheme, or otherwise some scheme which would allow all required modalities to be compactly contained and controlled.

The work of this thesis comprises the preliminary steps in implementing neural imaging techniques portably. To accomplish this, we use vertical cavity surface emitting lasers (VCSELs) operated in optimized current schemes to produce illumination which can satisfy the needs of each imaging technique as needed. We demonstrate an implementation of combined IOSI and LSCI using a single VCSEL package, single CCD, and no further mechanical elements. By overcoming the difficulties in simultaneous flow and oxygenation imaging, we take an important first step for multiple modality portable imaging. Further studies with VCSEL-based fluorescence sensors demonstrate the potential of concurrent real time molecular imaging. By reducing the number and size of components needed to perform IOSI, LSCI, and fluorescence imaging, and by demonstrating simplified functionality, we set the stage for future studies in truly implementing portable neural imaging.
Chapter 2

Device Characterization

2.1 VCSELs in an imaging context

Vertical Cavity Surface Emitting Lasers (VCSELs) are semiconductor laser devices known for their ease of testability in fabrication and compact size. As opposed to more traditional semiconductor lasers, these devices are epitaxially fabricated and oriented vertically on a chip, with laser emission through the top surface [44]. The resulting devices can have very low thresholds and operate efficiently [45]. Although their small scale tends to limit the maximum output power available, VCSELs are commonly used in communication and fiber optic applications due to their ability to be modulated at high rates. The vertical orientation and narrow active region of a VCSEL also give the device thermal properties and wavelength tunability which we find useful in an optical imaging context. For our purposes, it is not the modulation bandwidth of VCSELs that we find as an advantage, but rather the subtle effects on laser output that occur during modulation. Wavelength tunability, along with small size, power efficiency and the associated reduced heat dissipation, make VCSELs well suited to portable imaging modalities. We will look at the details of some of these properties in this chapter.
2.1.1 Thermal and optical properties of VCSELs

A schematic of a VCSEL is shown in Figure 2.1. The main elements grown on the substrate are the upper and lower Distributed Bragg Reflector (DBR) layers, which act as laser cavity mirrors, and the active layers in the center. The figure is accurate insomuch as the active layers typically consist of 3 or so quantum wells, which are pumped with current applied through a top side contact[46, 47]. The oxide aperture is used to aid in confining the current through a small portion of the active region. This can be used to produce mode confinement leading to production a pure single mode beam, or to localize power in the active region to produce a low threshold current [44].

![Schematic diagram of a VCSEL device. Layers of doped semiconductor are epitaxially grown to produce a P-I-N junction, with the P and N regions each constituting a DBR stack, forming a cavity. The active region in the center is composed of several quantum wells, producing the gain required to achieve lasing. The vertical scale of the device is of the order of 10 \( \mu \text{m} \).](image)

The small scale of the active region leads to several properties that distinguish VCSELs from other lasers. VCSELs will support only a single longitudinal mode, since it is the cavity size itself rather than the gain profile which determines lasing wavelength. As a result of this, VCSELs experience substantial wavelength variability corresponding with temperature. Temperature increases caused by injected current will result in index of refraction changes in the active layers, increasing the effective cavity wavelength.
Figure 2.2(a) demonstrates this effect in a single mode device. Typically, \(d\lambda/dT \approx 0.07 \text{ nm/K} \) [44]. At the same time, as current is increased, spacial hole burning and thermally induced wave-guiding will cause higher order transverse modes to appear. These modes will have different spacial distributions than the fundamental mode, degrading the Gaussian beam profile. Figure 2.2(b) shows examples of some of these modes.

![Figure 2.2: Current effects on VCSELs. (a) As single mode VCSEL current is increased, a wavelength shift is seen. Eventually higher order modes begin to appear. (b) Low order Laguerre-Gaussian modes. The fundamental LP\(_{00}\) Gaussian mode is seen, along with higher “donut” modes. The asterisk denotes the superposition of two rotations, producing cylindrically symmetric modes.](image)

When using VCSELs for imaging, we are faced with different requirements depending of what imaging modality we choose. For Laser Speckle Contrast Imaging or other interference based techniques, we require highly coherent illumination, which can be provided with a single mode device. Depending on the result desired, a very pure single mode may not be needed. Strong side mode suppression is desireable, but not strictly necessary, as extra modes may reduce but not entirely eliminate temporal coherence. For Intrinsic Signal Imaging, we require strongly reduced coherence, as the noise produced from laser speckle will degrade the low amplitude signal changes associated with functional activity. For fluorescence imaging, a powerful uniform beam is needed to excite fluorophores. In essentially all cases, we require access to a range of output powers to ensure that we are
neither starved for photons in detection, nor saturating a sensor. By taking advantage of the properties specific to VCSELs, we attempt to alter the coherence and spectral characteristics of laser output, and satisfy the needs of the three indicated imaging modalities with a single device.

2.1.2 VCSEL operation schemes

To take advantage of VCSEL tunability, we have attempted to look at three main operation schemes for usage in imaging:

1. Single Transverse Mode (SM) operation, achieved near threshold, providing low power but highly coherent output.

2. Multi Transverse Mode (MM) operation, achieved at peak output, providing high power output with reduced coherence, due to wavelength variations between modes.

3. Current Sweep (SW) operation, achieved by rapidly modulating the laser current.

Of these three operation schemes, the current sweep is most unique to VCSELS, and is used to produce a more uniform beam shape while simultaneously producing a greatly broadened spectrum. The desired modulation amplitude is dependent on peak power of the device. Generally, the laser current is modulated from slightly above threshold to slightly above peak power using a sinusoidal waveform, to produce the widest spectrum available. Because of constantly improving device packaging capabilities, during the duration of this work several generations of devices were tested and used. Results shown here apply to generation 1 (G1) and generation 2 (G2) devices, as specified in each section. The main difference is that G1 devices had a high threshold current, near 5 mA, whereas G2 devices had a much lower threshold, near 2 mA. Further, G1 only applies to nominally 670 nm VCSELs, whereas G2 includes 670 nm as well as 680, 795 and 850 devices, each with their own characteristics. Most other properties are quite similar between devices
of similar wavelength. The change to 680 nm from 670 nm was made due to increased power availability, as technology in the 680 nm range is further developed. Coherence characterization was done on devices of both wavelength for this purpose, though in the final imaging system 680 nm VCSELs were used.

2.2 Beam Profiling

Near field and far field imaging were done on 670 nm G1 VCSELs to observe the effect of higher modes on spot shape and power, as well as to test the averaging properties of a sweep mode.

2.2.1 Near field imaging

In order to achieve near field imaging, a CCD camera with a f50 mm objective lens was used along with a f3 mm collimating lens and a 100 µm aperture, to image the VCSEL’s top surface directly. Using this setup, an effective 17x magnification was achieved, imaging the VCSEL mesa onto 80 pixels of the CCD.

G1 VCSEL Beam profiles were collected from below threshold (3 mA) to above peak power (19 mA), in steps of 2 mA (Fig. 2.3). Threshold is clearly noticeable by the appearance of a bright spot in the upper region of the surface, essentially an elongated Gaussian. As current is increased, higher order spatial modes appear giving the profile a characteristic donut shape. After peak power is reached near 15 mA, the modes are seen to degrade and intensity is decreased.

2.2.2 Far field imaging

Far field imaging was accomplished by removing the camera’s objective lens, and collimating the laser beam to be projected directly onto the CCD. ND filters were used to attenuate the beam intensity, in order to avoid detector saturation.
Figure 2.3: Near field imaging of VCSEL beam profile. (a) Below threshold, spontaneous emission is seen, scattering and illuminating the metallic contact ring. (b) Beginning with a single mode near threshold, multiple higher modes arise with increasing current, before the spot is degraded and output power decreases.

Far field images were taken at the same current points as with near field imaging (Fig. 2.4). The appearance of higher order modes is again clear here, with the added effect that higher modes show a much greater divergence angle than the fundamental mode.

Finally, a rapid sinusoidal sweep from 7 mA to 14.5 mA is shown. The averaging effects are clear, as the resultant profile lacks the annular shape of the individual MTM beam spots, and is altogether more uniform.

2.3 Coherence analysis

For the purpose of laser speckle imaging, it is the temporal coherence which has the most significant effect on the interference of scattered light. For illumination with high coherence time or, equivalently, high coherence length, any single photon scattered by tissue at a high depth can interfere with other photons scattered at shallow depths, resulting in a strong speckle pattern. On the other hand, when the coherence length is significantly less than the scattering depth, interference is much weaker and a reduced
Chapter 2. Device Characterization

Figure 2.4: Far field imaging. (a) Beam profiles obtained at various currents show an initial Gaussian beam, with higher order Laguerre Gaussian modes broadening the spot and degrading the shape as current is increased. The 6 nodes at 15 mA seem to indicate the presence of an LP$_{30}$ mode. (b) By applying a current sweep, the beam spot is smoothed somewhat by averaging the individual multimode shapes.

speckle pattern is seen. The theoretical basis of these interactions is discussed further in Chapter 4.

Interference strength can be quantified by a property called the fringe visibility,

$$Visibility = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$

(2.1)

where $I_{max}$ and $I_{min}$ are respectively the maximum and minimum intensity of a given fringe. We can define $l_c$ as the length in which interference fringe visibility drops to half. For a Gaussian spectrum, we can accurately measure the coherence length from the spectral bandwidth,

$$l_c = \frac{2ln2}{\pi} \frac{\lambda^2}{\Delta \lambda_{1/2}}$$

(2.2)

where $\Delta \lambda_{1/2}$ is the full width, half maximum (FWHM) of the spectrum [48]. This result comes directly from the observation that the interferogram of a beam can be obtained by taking a Fourier transform of the spectrum. For cases where the spectral shape is more complicated or uncertain, the direct interferogram can be used to more accurately determine the sources coherence profile. Both spectral analysis and interferometry were used in order to estimate the coherence lengths of the different operation schemes.
2.3.1 Optical spectrum analysis

The results in this section correspond to G2 devices at 670 nm, 680 nm, 795 nm and 850 nm. For each operation scheme, laser light was focused and coupled through an optical fiber for analysis using an Optical Spectrum Analyzer (OSA) (Ando AQ6317B). Fig. 2.5 shows the resulting spectra. Power levels of individual spectra have been normalized for presentation on a single axis. The current set-points for each operation scheme are indicated on their respective plots. In the case of SM operation peaks, as well as for the individual peaks within MM operation, spectral widths are resolution limited by the apparatus. The SM peaks are quite certainly more narrow than the 0.02 nm limit seen here, and the apparent widening seen in some MM peaks is most likely caused by the presence of several very close modes. The nominally SM scheme for 850 nm VCSELs in particular appears to in fact hide two modes in its main peak. The SW spectra are noticeably non-uniform due to the shifting gain profile along with the thermal shift. Some work was attempted in applying different sweep function forms to smooth this, but the benefits were found to be minimal, and the simple sine wave was selected as preferable. As an example, Fig. 2.6(a) shows a comparison between a sine wave and a triangle wave with the same amplitude and frequency. The main difference is seen in the spectral width: the triangle wave, spending less time at the high and low extremes of current, is noticeably more narrow. Similar peaks are still seen, although shifted slightly due to reduced heating effects. The effect of modulation frequency on spectral shape was also examined. Fig. 2.6(b) shows a comparison between modulation at 6 kHz, and modulation at 26 kHz. Higher frequency modulation causes a clear narrowing of the spectrum, most likely occurring due to the reduced settling time allowed at the high and low ends. The sinusoidal oscillations observed in the spectral shape, more pronounced in the 6 kHz case, are a result of temporal sampling limitations in the OSA, after lowpass filtering.

The results indicate that the application of swept current is an effective method for broadening VCSEL spectra. In all cases, the spectral widths exceeded 1 nm in SW mode.
Figure 2.5: Spectra of SM, MM, and SW operation modes with (a) 670 nm (b) 680 nm (c) 795 nm and (d) 850 nm VCSELs

While these values are still significantly narrower than an LED, which can typically have 20 nm or greater FWHM, they are orders of magnitude greater than the width of a typical single mode laser. For example, a 1 GHz line-width laser at 670 nm, which would already considered wide for a single mode source, has a FWHM wavelength of 0.0015 nm.

Table 2.1 shows the measured FWHM and estimated $l_c$ for SM and SW operation for each wavelength. The $l_c$ values for the MM cases were not included, as they are difficult to quantify due to discrete mode spacing. Power levels for the SW cases are
Table 2.1

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Operation scheme</th>
<th>Current</th>
<th>$\Delta \lambda_{1/2}$</th>
<th>$l_c$ (est.)</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>670 nm</td>
<td>SM</td>
<td>1.9 mA</td>
<td>0.022 nm</td>
<td>9.0 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW</td>
<td>2-10 mA</td>
<td>1.7 nm</td>
<td>0.116 mm</td>
<td>1.06 mW</td>
</tr>
<tr>
<td>680 nm</td>
<td>SM</td>
<td>1.2 mA</td>
<td>0.04 nm</td>
<td>5.1 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW</td>
<td>2-12 mA</td>
<td>2.3 nm</td>
<td>0.088 mm</td>
<td>2.49 mW</td>
</tr>
<tr>
<td>795 nm</td>
<td>SM</td>
<td>2.1 mA</td>
<td>0.026 nm</td>
<td>10.7 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW</td>
<td>2-10 mA</td>
<td>2.4 nm</td>
<td>0.116 mm</td>
<td>1.29 mW</td>
</tr>
<tr>
<td>850 nm</td>
<td>SM</td>
<td>2.9 mA</td>
<td>0.059 nm</td>
<td>5.4 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW</td>
<td>2-12 mA</td>
<td>1.4 nm</td>
<td>0.227 mm</td>
<td>2.57 mW</td>
</tr>
</tbody>
</table>

included for comparison. Again, the SM values are minimum estimates limited by the OSA resolution.

Figure 2.6: Comparison of different SW parameters (a) Triangle (ramp) function compared to sine sweep, each with the same amplitude and frequency. (b) Comparison of 6 kHz and 26 kHz modulation, each using a sine wave of the same amplitude.
2.3.2 Interferometry

Interferometry was conducted for SM, MM, and SW operation schemes on G1 670 nm devices, using a Michelson interferometer. A pinhole and photodetector were placed at the interferometer’s exit port to measure the fringe intensity. In order to obtain the interferogram estimates, the delay path was varied from the zero path position with consistent spacing, dependent on the resolution required. At each equally spaced point, the delay micrometer was adjusted gradually to find the maximum and minimum fringe intensity. These values were used to calculate the fringe visibility at each point.

Fig. 2.7 shows results for the 670 nm device. It is important to note that the path difference values used here are in fact twice the interferometer delay lengths, as the delayed beam must travel forward and backwards on the same path before meeting with the reference beam at the exit port. For the SW case, coherence length can be estimated immediately from the path difference at which the visibility drops below 0.5, giving a value of $l_c = 230\mu m$. For the SM case, the interferometer’s travel was not sufficiently long so as to reach the half max. A Gaussian fit was applied to the data to estimate the coherence length instead, giving a coherence length of $l_c = 56.4mm$. This is substantially greater than the estimate based on spectrum, and may in fact prove even greater, as visibility at large delays may have been underestimated due to slight misalignment. For the MM case, a simple metric of the coherence length based on the half max is not possible, due to multiple nodes caused from beating modes. Only the first few such nodes are shown. A numerical calculation of moments on the interferogram might instead be used, but this would require a far longer sampling of the pattern. We would nonetheless expect the coherence length to sit somewhere in between the SM and SW cases.

Interferometry was repeated for SW operation of 670, 795 and 850 nm G2 VCSELs, as shown in Fig. 2.8. Remarkable here is the similar $l_c$ in all three cases. At 670 nm we see $l_c = 130\mu m$, at 795 nm we have $l_c = 170\mu m$ and at 850 nm we have $l_c = 230\mu m$. Referring back to the estimates of Table 2.1, we note that the predictions based on spectral width
Chapter 2. Device Characterization

Figure 2.7: Interferometry results for G1 670 nm VCSELs. (a) Comparison of interferograms with SM, MM and SW operation. A Gaussian fit was applied to the SM result, for which interferometer travel was insufficient, giving a 56.4 mm half width. (b) Rescaled SW result, showing a 0.23 mm half width, and a single side lobe.

Figure 2.8: Comparison of interferograms of G2 devices at all 3 wavelengths. Similar coherence lengths are seen in the 3 cases.

were quite close. We may in fact expect the 795 nm and 850 nm VCSELs to show an even greater degree of speckle reduction, as these wavelengths penetrate substantially deeper into tissue.

There is not a precise relation for determining the reduction in speckle noise associated
with a decreased coherence length. It varies greatly based on the illuminated material, as well as image magnification and aperture size. An estimate based on tissue modeling, however, can be achieved, and will be presented along with a more theoretical look at the nature of speckle in chapter 4.
Chapter 3

VCSEL illumination noise analysis

Before we can apply VCSELs in LSC or IOS imaging schemes, it is important to carry out experiments with the purpose of evaluating the noise constraints of each operation scheme. To this end, we completed a group of experiments of VCSEL illumination on in three different imaging conditions: on a stationary opal glass surface, ex vivo on slices of mouse cortex, and in vivo on exposed rat cortex. In each case we look at both the temporal noise and spatial noise with each source. By comparing these scenarios, we can evaluate the effects of tissue absorption and movement on the noise values.

Much of the work in this chapter was completed in collaboration with Dene Ringuette. In particular, the ex vivo data were collected by Dene Ringuette, while the stationary and in vivo experiments were carried out by Dene and myself together. Dene performed the temporal analysis, while I performed the spatial analysis.

3.1 Sources of noise

For a typical illumination source, we on one hand expect to have noise intrinsic to the source, and on the other hand we have noise arising from the to the imaging system. In an incoherent source such as an LED, we see intrinsic noise coming from the power supply, in the form of capacitive coupling to external AC power and other source instabilities.
By selecting a well shielded and stable power source we can render this noise negligible.
In a laser system, intrinsic noise appears in the form of Relative Intensity Noise, or RIN, which arises due to a variety of sources, such as quantum noise, thermal fluctuations and gain changes [49]. Again, at low imaging frequencies RIN can be rendered negligible with a stable source.

What remains significant in an imaging context largely comes from the imaging device itself. One fundamental limit comes from the shot noise of the detector. Shot noise arises from uncertainties in the detected intensity as light is converted to photoelectrons. It can generally modeled as a Gaussian white noise, having relative intensity of $\frac{1}{\sqrt{N_e}}$, where $N_e$ is the average number of photoelectrons generated [50]. At high image intensity this value can be quite small, but still dominate compared to other noise sources.

For very low image intensity, the CCD read noise becomes important. This arises from a number of electronics-based sources, which are combined and estimated as a single number, usually specified as electrons per exposure. The RMS amplitude of this noise is constant and does not change with intensity, but may vary slightly between pixels. While it may be largely ignored for bright images, in dark images it can be significant.

Finally, for the specific case of laser illumination, a dominating source will be speckle noise. This is the main source which we are attempting to account for, and will be investigated in detail in the next section.

### 3.2 Theoretical basis of laser speckle patterns

Laser speckle arises from the interference of coherent reflected contributions from many different elements of a scattering surface. Since we cannot generally know details of the microstructure of the surface, and because such a large number of scattering events occur, we can view speckle from a statistical standpoint. To this end, we can model speckle as a random walk in phase space. Under the assumption of uniform illumination, each
Chapter 3. VCSEL illumination noise analysis

Figure 3.1: Generation of speckles from coherent illumination. (a) illumination with high spatial and temporal coherence is diffusely reflected by an uneven surface. The surface variations give each reflected component a random phase. (b) In the complex plane, individual components of equal magnitude sum in a “random walk”. For a large number of such random walks, the distribution of Eq. 3.1 is obtained.

Reflected contribution reaches the detector with the same amplitude and a random phase. This phase arises from micro scale variations on the scattering surface, which manifest as random delays in the path length from surface to detector. Taking a large number of such scattered elements and adding them in phase space gives a single net electric field phasor (Fig. 3.1). In the irradiance regime, we square this element to get a speckle intensity. If we take a large number of independent phasor intensities, we expect to get an irradiance probability distribution approximating an exponential decay:

\[ P(I) = \frac{1}{\bar{I}} e^{-I/\bar{I}} \]  

(3.1)

Proof of this result can be found in detail from [51], and a simulated example is shown in Fig. 3.2. From a distribution of this form, we can generate a fundamental result of speckle theory: for a fully developed speckle pattern, \( \sigma = \bar{I} \). That is to say, the standard deviation of speckle intensity is equal to the mean. In the context of signal to noise ratios, this result appears disastrous. If we had speckle noise at this level it would be
essentially impossible to determine small signal changes. We will see in the next section how this difficulty can be overcome.

Figure 3.2: (a) A simulated speckle pattern, produced in accordance with methods described in [52]. The smallest speckles here are approximately 12 pixels wide (b) Probability distribution of the simulation, as estimated from a histogram. An exponential with the same mean is plotted for comparison

3.3 Factors effecting speckle

3.3.1 Addition of independent patterns

Whereas the coherent elements producing speckle in the first place arise from addition in a complex amplitude basis, if we instead add uncorrelated elements on an intensity basis, we can obtain an effective reduction in speckle intensity. By adding uncorrelated speckle images of equal intensity, we get a contrast reduction corresponding to \(1/\sqrt{N}\), where \(N\) is the number of independent patterns used. Goodman [53] points to several ways in which we can accomplish this. One immediate benefit comes from polarization. If we are imaging a surface which depolarizes incident light, we can view the resulting speckle
pattern as emerging from the addition of two orthogonally polarized speckle patterns. Other independent patterns can be produced by illumination from different angles, or from illumination using different frequencies. The use of different frequencies in particular can be related to the coherence length of the illumination source.

### 3.3.2 Coherence length

If we broaden the spectrum of a current source significantly, we can effectively reach a point where, at least in the context of speckle, the spectrum can be considered to consist of multiple independent frequencies. The frequency separation which allows two elements to be considered independent is itself based largely on the surface which is being imaged. The theory behind multiple frequency speckle pattern illumination was originally developed in the context of a diffusely reflecting surface with some degree of surface variation. We can start with this and attempt to extend the theory to account for illumination penetrating into tissue.

For a surface illuminated with two frequencies, the surface roughness must be great enough such that the two frequencies can largely decorrelate. Mathematical models can be used to show that the separation condition is:

$$\Delta \nu = \frac{c}{2\sigma_z}$$  \hspace{1cm} (3.2)

where $\sigma_z$ is the standard deviation of the surface height, $c$ is the speed of light and $\delta \nu$ is the frequency separation of the two components [54]. We can extend this to multiple components. If we would like to combine $N$ different wavelengths each matching the minimum condition, we have the total separation bandwidth

$$\Delta \nu_N = (N - 1)\frac{c}{2\sigma_z}$$  \hspace{1cm} (3.3)

Fig. 3.3 shows a comparison between the interference pattern produced by a number of individual separated peaks, and the pattern produced by a Gaussian spectrum of
equivalent width. This demonstrates how we can approximate the effect of summing individual peaks with a single broadband source of width $\Delta \nu_N$. If we would like to transform this to the wavelength domain, the appropriate manipulation gives us the equivalent expression,

$$\frac{\Delta \lambda_N}{\lambda^2} = \frac{N - 1}{\sigma_z}$$

We then recall our expression for coherence length above (Eq. 2.2), $l_c = \frac{2\ln 2 \, \lambda^2}{\Delta \lambda_{1/2}}$, which leads us to the final relation,

$$N \approx \frac{4\ln 2 \, \sigma_Z}{\pi} \cdot \frac{1}{l_c} + 1 \approx \frac{\sigma_Z}{l_c} + 1$$

This is of course only an approximation, but we can expect it to be a reasonable guess in the case that $l_c$ is much less than $\sigma_Z$. If instead of a rough surface, we consider speckle patterns on tissue, optical path variation rather than surface deviation becomes the parameter we would like to use to calculate noise reduction. Monte Carlo simulations can be used to estimate this quantity, which should be similar to the mean optical path length itself.

Figure 3.3: (a) Spectrum of 20 evenly spaced individual peaks (comb), and a Gaussian beam of the same FWHM. (b) Calculated interferograms of the spectra in (a). Both results have a similar coherence length.
3.3.3 Imaging optics

Speckle is dependent not only on the light source itself, but also on the imaging optics used. This arises from the fact that, as an interference phenomenon, speckle spots have a characteristic size. We can view the individual smallest speckles as diffraction events produced by points at opposing ends of a circular aperture; many speckles may have smaller separation at the aperture, but none will be larger. If these are the electric field elements at the aperture, then we can apply the principles of Fourier optics, and take a Fourier transform to obtain the intensity at the imaging plane. Thus, using the Airy disk approximation, we expect the minimum speckle size to be

\[ d = \frac{2.44\lambda \times f/\#}{\#} \]

where \( \lambda \) is the wavelength of illumination and \( f/\# \) is the f-number of the imaging system. When a CCD pixel is much smaller than this minimum speckle size, we expect no speckle averaging, and would see the full speckle noise intensity. When CCD pixels are significantly greater than this number, we expect multiple speckles to be averaged within each pixel, producing a reduction in the apparent speckle noise. Research has shown that when pixels are larger than half the speckle size, contrast reduces rapidly by a factor roughly of the order of \( R \), the ratio of pixel width to speckle width [52]. Taking both the \( f/\# \) and magnification \( M \) of an imaging system into account, as well as the pixel size \( P \), we arrive at the relation

\[ R = \frac{P}{2.44\lambda M f/\#} \]  \hspace{1cm} (3.6)

In speckle modeling, these effects together appear as a scaling factor, \( \beta \). For fully developed speckle \( \beta = 1 \), and for reduced patterns this number scales as the square of the mean speckle contrast. Fig. 3.4 gives examples of speckles of different sizes as produced by simulation.

3.4 Methods

Imaging was carried out using a 12 bit Retiga 4000R CCD camera (QImaging. The CCD featured 7.4 \( \mu \)m wide pixels, with 40000 \( e^- \) full well capacity. The CCD size was
Chapter 3. VCSEL illumination noise analysis

Figure 3.4: Simulated speckle patterns with different aperture sizes, with R calculated from Eq. 3.6. 
(a) R=0.1 (b) R=0.5 (Nyquist sampled) (c) R=1 (below Nyquist). The intensity distribution begins to
narrow once speckles are under-sampled.

2048x2048 pixels, although smaller regions could be imaged to achieve higher frame rates. 
Nominal read noise was 18 $e^{-}$. For stationary and in vivo experiments, opposing f = 50 
mm lenses were used for the purpose of obtaining 1:1 magnification. The aperture was
kept at f/1.4 in all cases.

The stationary measurements were carried out by imaging illumination reflecting off 
of the surface of a 50 mm wide square of glass with a diffusing opal surface. In vivo 
measurements were carried out by imaging live adult rats. Male Sprague Dawley rats, 
200-350 g, were used. The animals were anaesthetized prior to imaging, and placed in 
a stereotaxic mount. The animals’ heads were shaved, and a region of the scalp was 
removed. Craniotomies were performed over one hemisphere of the brain, between the 
bregma and lambda ridges. The craniotomy regions were roughly 4 mm diameter. Once 
the craniotomy was completed, a petroleum wall was placed around the region, and the 
region was then filled with agarose maintained at 40° C. A cover glass was placed on 
top of the agarose to produce a level imaging window. After imaging, the animals were 
 euthanized.

Fig. 3.5 shows the setup used for illumination. A red LED (630 nm) and G1 VCSEL 
(670 nm) were each powered through independent current sources (Keithley 6221). The
LED source was maintained at a constant current of 80 mA, while the VCSEL current was applied in one of three schemes: Single mode (5 mA), Multi mode (14.5 mA) and Sweep mode (7-14.5 mA, 10 kHz sine wave). In each case, the VCSEL and LED were triggered in alternating frames using a fast switch (Keithley 7001). A trigger sent from the camera after each frame were used to signal the switch to gate each respective source for the frame duration. The sources were positioned to produce matching illumination in each case, and exposure times were adjusted in the 5-20 ms range to achieve an image mean of 70% of the maximum intensity (4096 gray levels). In each mode, sequences of 2000 total images (1000 LED + 1000 VCSEL) were taken. For in vivo measurements, for each mode n=7 specimens were used to obtain statistics. In the stationary case, n=5 sequences were used.

Ex-vivo studies were carried out in Dr. Peter Carlen’s lab in Toronto Western Hospital, with the assistance of Dr. Alex Tonkikh. Slices of 400 µm thickness were prepared from mouse brains using a vibrotome, and immersed in artificial cerebrospinal fluid (ACSF). During imaging the slices were maintained in a specially designed flow cham-
ber circulating ACSF. The Retiga 4000R camera was again used for imaging, this time mounted on a microscope with a NA=0.28, 4x objective. The illumination paradigm used in the in vivo and stationary cases was again applied, with n=5 specimens used in each case.

Image analysis was carried out in all cases to produce both spatial contrast and temporal noise values. Over the 1000 images of each set, the temporal standard deviation over mean intensity $\sigma/\bar{I} = (\sigma/\mu)$ ratio was calculated on a per pixel basis. For each set, the mean representative pixel value was recorded, and the variation in values was used to ensure statistical anomalies associated with outliers were mitigated. Spatial noise analysis was conducted on individual images in all illumination schemes. Within a single frame, $(\sigma/\mu)$ calculations were made over a moving 5x5 pixels region of interest (ROI), centered sequentially at each pixel of the image. The resulting spatial contrast map was then averaged over 40 images to obtain a short time scale statistical average. Contrast values were then averaged over the cortical imaging region to obtain the average spatial noise value. For ex vivo slices, in all cases the raw images were binned in 4x4 pixels, in order to compensate for the 4x magnification and the resulting increase in speckle size.

For one animal, in vivo trial averaging was done to measure the noise reduction effects that averaging could produce, and compare these directly between LED and SW. For this purpose, 64 sequences of 256 images were obtained for each of LED, SW, and SM illumination. The individual frames of each sequence were averaged between trials, producing 256 averaged supercells. Temporal noise analysis was again completed on the resulting averaged sequences, producing temporal noise maps.
Chapter 3. VCSEL illumination noise analysis

3.5 Results

3.5.1 Stationary opal glass noise

Fig. 3.6 shows the spatial and temporal noise on opal glass. These results are best described by the speckle theory as outlined above. The SM spatial values can be explained in accordance with Eq. 3.6. A quick calculation based on the imaging parameters gives $R = \frac{7.4}{2.44 \times 0.67 \times 1.4} = 3.23$, meaning that more than three speckle diameters fit in a single pixel. If on top of that we include a reduction factor of $\sqrt{2}$ to account for polarization, we observe that a total speckle noise reduction to 10% is reasonable in the SM case. The reduction found in MM and SW operation come from the surface roughness of the opal glass. We can infer that in the MM case, the equivalent of approximately 4 sufficiently separated modes are seen, giving a 2x reduction. In the SW case a greater
reduction is seen, the equivalent of 8 separated modes. We note that we expect even greater reductions in tissue, where the penetration depth is substantially greater, and the separation condition is weaker. Finally, in the LED case we see residual noise on the order of 0.7%. We assert that this is shot noise limited, as a quick calculation can confirm. Mean image intensity was maintained at 50-70% of the full well of 40,000 electrons. This gives approximately 20-30k electrons per pixel. The relative shot noise can thus be calculated as $\frac{1}{\sqrt{e}}$, giving approximately 0.6%-0.7%. When comparing temporal noise values, we note that these are in all cases substantially lower than spatial contrast, yet still increase with higher coherence illumination. We can intuit how this arises in a nominally stationary situation. Although the setup is shielded as much as possible from movement, there may still be slight perturbations in the position of the illumination source, or of the camera. Any change in individual pixel intensity due to these slight shifts will be related to the stationary noise pattern in the image. Even minute motion in a strongly speckled image can produce a strong change in intensity. Thus, while the temporal noise is largely reduced, we still see some residual noise in the presence of laser illumination. In the shot noise limited LED, we see no difference between the spatial and temporal analysis, further demonstrating the lack of any interference effects.

3.5.2 Ex vivo noise in cortical slices

The ex vivo results (Fig. 3.7) follow a trend very similar to the stationary case, with some key differences. If we look at spatial contrast, the trend between illumination sources is very similar to what was observed on opal glass. When we compare the spatial and temporal values for each source we note the effect of moving to living tissue. For laser illumination, spatial contrast values are decreased somewhat, and temporal values are increased. This two-fold effect is to be expected when we image live tissue, which is in a constant state of microscale movement. The movement will on one hand blur out speckles during each frame’s exposure, resulting in decreased contrast, and on the
other hand contribute to temporal shifts in the pattern, leading to increased temporal noise. The LED spatial and temporal values are again far lower than the values obtained with laser illumination, again at a level we expect to be shot noise limited. We observe that the temporal noise however appears to be lower than the spatial contrast, which is difficult to explain if the LED is in fact shot noise limited. We firstly note that the 4x4 binning here would decrease shot noise by a factor of 4. The temporal noise seems to validate this limit when compared to the opal glass result. The spatial noise, however, remains substantially higher. We propose that this is a result to actual slice contrast, due to physical differences seen in the slices. The presence of edges or gradients within the sampled region would produce such an increase, resulting in the values seen.
3.5.3 In vivo noise on exposed cortical tissue

The noise values in vivo are most important for the work that follows, as it determines the limits of our devices for actual physiological measurements. The results are shown in Fig. 3.8. The trend seen in transitioning from stationary to ex vivo measurement continues. The spatial contrast in all cases is even lower than Ex Vivo, and the temporal contrast is higher. The temporal and spatial values are in fact statistically equivalent in each operation mode. This can be explained as a result of the motion typically seen in vivo due to physiological artefacts such as breathing and heart rate, as well as blood flow itself. Slight movement on the micro scale produces sufficient shifts to the speckle pattern, making temporal samplings and spatial samplings essential equivalent. In statistical terms the sampling becomes ergodic.

The results are promising when one compares values between the SW operation VC-
SEL and the LED. The two differ by only about 40%, implying that we are within 40% of the shot noise limit when using SW in vivo. If we subtract the estimated shot noise (based on LED values, 0.8%) from the SW noise values \( n_{\text{speckle}}^2 = n_{SW}^2 - n_{LED}^2 \) we can estimate the speckle contribution to be 0.6%, which is less than the shot noise itself. By comparing this estimated SW speckle contribution to the SM case, we can quantify the effect coherence reduction has had on speckle noise in vivo. We note that, with shot noise removed, we obtain a noise reduction of \( K_{SW}/K_{SM} = 0.6%/3% = 0.2 \). The noise observed in SW operation is 1/5 that of SM operation. From this we can infer that we are effectively averaging approximately N=25 modes when imaging neural tissue. Using our previous relation based on coherence length, we would expect the standard deviation of optical path lengths in tissue to be \( \sigma_Z \approx (25 - 1)l_c = 24 \times 230 \, \mu m = 5.5 \, mm \). This number is fairly close to the number we obtain in simulation, specifically 8.7 mm (see Appendix D).

Fig. 3.9 shows the results of 64 trial averaging, in the form of noise maps. The top row shows maps for an individual trial with LED, SW and SM illumination. The bottom row shows the same maps after 64 trial averaging. The results consistently show the expected effect of averaging. For N independent samplings of approximately Gaussian noisy data, residual noise should scale as \( 1/\sqrt{N} \). In all cases here we see a factor of 8 reduction after 64 trials. This confirms that noise reduction through averaging is feasible for SW illumination.
Figure 3.9: Temporal noise maps for 3 illumination sources. The pixel-wise standard deviation over mean ($\sigma/\mu$) intensity values was calculated in each case on 256 frames. (a) LED illumination has low noise throughout. (b) Current sweep (SW) mode illumination has noise levels near that of the LED, particularly on the cortical surface. (c) Single mode (SM) illumination has noise levels an order of magnitude higher than the LED throughout. (d)-(f) show the same analysis repeated, on 256 images binned through 64 trials. While the qualitative characteristics remain similar, we see approximately 1/8 the noise values as seen in the initial case.
Chapter 4

Laser Speckle Contrast Imaging using VCSELS

The work in this chapter was accomplished in collaboration with Dene Ringuette. The mathematical model of speckle was researched by Dene and myself, and I developed the modifications to the model which we use. The in vivo experiments were carried out by Dene and myself, along with initial image processing. I performed the model fitting myself.

4.1 Basis of LSCI

As described in previous chapters, laser speckle is a coherence effect caused by the interference of scattered light from an uneven surface. By taking advantage of the effect that movement has on speckle patterns, LSCI allows us to discern detailed full field information on the movement of scatterers using any laser, and simple image processing algorithms. The technique was first described by Fercher and Briers in 1981, who suggested its use in imaging blood flow within the retina [27]. Since then it has been applied to a variety of scenarios such as perfusion in skin tissue, neural imaging, and image guided surgeries [56, 57]. The underlying theory shares much in common with the
Figure 4.1: Coherence based measurement techniques. (a) DLS measures the time varying speckle scattered off of moving particles, and applies correlation functions to derive information on particle velocities. (b) LDV uses two interfering beams, and measures the beating between the two caused Doppler shifts of their frequencies. Fourier analysis provides spectra corresponding to the speckle decorrelation. (c) LSCI uses broad illumination to measure the spatial variation of speckle intensity, in full field. The spatial correlation observed is the spatial analog of the temporal values found in DLS. [55, 3, 4]
related techniques of dynamic light scattering (DLS, also known as photon correlation spectroscopy) and laser Doppler velocimetry (LDV)[4, 58]. All three rely on the same principal, namely that movement of coherently illuminated scattering elements causes intensity fluctuations in the reflected interference pattern. DLS specifically looks at the temporal changes of this pattern using a fast detector, whereas LDV applies the same principal in the frequency domain, by monitoring shifts in reflected light relative to a reference beam [55, 3].

LSCI is distinct from the other two techniques in the fact that it is a full field technique, rather than a scanning technique [3]. Specifically, LSCI images the fluctuations of speckle as a time averaging effect over the duration of a frames exposure. Whereas static scatterers will maintain full speckle contrast, any moving elements will cause some degree of blurring in their vicinity, resulting in decrease in the apparent contrast. The degree to which the contrast itself is decreased is based on both the speed of the scatterers and on the frame exposure time. Fig. 4.2 shows an example of how the contrast seen in a raw image can be used to produce a flow map. This concept allows LSCI to be used to image relative velocities over large areas with a relatively simple optical setup, within single exposure. It however limits the technique from achieving accurate quantification, at least in its most basic form.

4.2 Multiexposure speckle velocity models

Fercher and Briers’ initial description of LSCI[27] relied on a result from Goodman[59] quantifying the variance of a dynamic speckle pattern averaged over time T:

\[ \sigma_s^2(T) = \frac{1}{T} \int_0^T C_t^{(2)}(\tau) d\tau \]  

(4.1)

\( C_t^{(2)}(\tau) \) is the auto-covariance function of speckle fluctuations. The authors then proceed to solve this expression in the case of a Lorentzian distribution of velocities, producing
Figure 4.2: Stages in producing a LSCI flow map. (a) Raw image of vessels, illuminated with a single mode laser. (b) After $\sigma/\mu$ is calculated in 5x5 bins about each pixel, we can see vessels as dark regions in the map. (c) Averaging several hundred contrast maps leads to noise reduction, clearly showing differences in speckle contrast levels. (d) By applying a long exposure limit model (Eq. 4.4), relative flow rates can be calculated from the contrast map.

the original LSCI model.

$$K = \frac{\sigma_s}{\bar{I}} = \left[1 - e^{-2x} \right]^{1/2}$$

(4.2)

To simplify writing of this expression, the exposure time $T$ and speckle correlation time $t_c$ have been combined into a single independent variable, $x = T/t_c$. For similar
moving scatterers, \( t_c \) is inversely proportional to the velocity of flow.

![Graph showing speckle contrast models and long exposure limit](image)

Figure 4.3: Comparison of original and modified speckle contrast models. The long exposure limit for the modified model is included, and is seen to match quite well for \( T/t_c > 10 \)

More recent developments with the speckle model have incorporated the \( \beta \) scaling factor, which was described in connection with speckle averaging, as well a correction to the weighting of the autocorrelation function, leading to a revised expression\(^5\):

\[
K = \left[ \beta \frac{e^{-2x} - 1 + 2x}{2x^2} \right]^{1/2}
\]  

This model and the previous one both reduce to the same trend in the long exposure limit, applicable when \( T \) is more than about 10 times as great as \( t_c \):

\[
K \propto \left( \frac{t_c}{T} \right)^{1/2} \text{ (Long Exposure Limit)}
\]

The proportionality here depends on \( \beta \). Fig. 4.3 shows a comparison of the two models along with the long exposure limit. Using this limit, it is possible to compute relative flow maps easily from one exposure: the velocity of vessel A relative to vessel B is simply \((K_B/K_A)^2\)\(^6\). This is the approximation used for single exposure speckle
imaging, and a large body of work has been completed studying it’s efficacy [60, 28, 61]. However, since $\beta$ itself is not typically known, if we would like to accurately calculate $t_c$ itself, we are required to make use of a wide range of exposures, fitting for both $\beta$ and $t_c$.

In practical applications, we rarely have a situation in which scattering is purely due to moving elements. For this purpose it is useful to attempt to account for the presence of static scattering elements in LSCI. Recent work by Parthasarathy has completed this model, by taking advantage of a heterodyne scattering model from DLS[58].

\[
K = \left[ \beta \rho^2 e^{-2x} - 1 + 2x^{2} + 4\beta \rho (1 - \rho) e^{-x} - 1 + x^{2} + \nu_s \right]^{1/2} \tag{4.5}
\]

A full derivation of this expression can be found in [4], but the essential concept revolves around the the addition of a term representing a static field in the time averaged speckle relation. The quantity $\rho$ represents the proportion of light that is dynamically scattered, i.e. $\rho = \frac{I_{\text{dynamic}}}{I_{\text{dynamic}} + I_{\text{static}}}$. Another additional term, $\nu_s$, is included to represent residual spatial variance not effected by moving scatterers, representing both camera noise and the non-ergodic effects of static speckle. A restriction resulting from the inclusion of this term is that in order for the model to be used accurately the image intensity must remain consistent between exposures. However, by slight modification of the model, this restriction too can be overcome.

### 4.2.1 Accounting for $\nu_s$

Our modifications to the model given in Eq. 4.5 stem from a need to account for the noise background, but give us further advantages. The $\nu_s$ term is troublesome even beyond the brightness restriction it produces. By designating $\nu_s$ a fitting parameter, it renders the problem somewhat intractable. As Fig. 4.4 shows, both $\rho$ and $\beta$ act essentially as scaling factors when fitting the multiexposure expression. If we could remove the $\nu_s$ factor we would have better hope in distinguishing between these effects.

We can attempt to remove $\nu_s$ by noting that it can be split into two terms, $\nu_s = \ldots$
Figure 4.4: Speckle model dependence on three parameters. (a) Varying $t_c$, with all other parameters fixed. Higher $t_c$ curves are shifted right. (b) Varying $\rho$, others fixed. Higher $\rho$ curves are taller. (c) Varying $\beta$, others fixed. Higher $\beta$ curves are taller.

$\nu_{ne} + \nu_{noise}$. These are mean square noise terms and thus can be added linearly. We can immediately identify $\nu_{noise}$ as arising from the combination of shot and read noise; these can be estimated and accounted for based on mean pixel intensity and known camera parameters. We are left to account for $\nu_{ne}$, the non-ergodic spatial contrast. There are two methods which we can use to accomplish this. One way is to go back to the heterodyne expression from DLS [4, 58], and replace the static component with a second dynamic component. A more straightforward method which leads to the same term follows from the recognition that all contrasts should converge to the same value in the short exposure limit, specifically $K = [\beta + \nu_{noise}]^{1/2}$. This is because at very
short exposure, the noise becomes equivalent to the static speckle pattern plus the added camera noise. Evaluating Eq. 4.5, this limit is straightforward:

\[
\lim_{T \to 0} K = \lim_{x \to 0} \left[ \beta \rho^2 e^{-2x} - 1 + 2x + 4\beta\rho(1 - \rho) \frac{e^{-x} - 1 + x}{x^2} + \nu_{ne} + \nu_{noise} \right]^{1/2}
\]

\[
= \left[ \beta \rho^2 + 2\beta\rho(1 - \rho) + \nu_{ne} + \nu_{noise} \right]^{1/2}
\]

\[
= \left[ \beta \rho(2 - \rho) + \nu_{ne} + \nu_{noise} \right]^{1/2}
\]

(4.6)

From the expected short exposure contrast, we should get

\[
[\beta + \nu_{noise}]^{1/2} = [\beta \rho(2 - \rho) + \nu_{ne} + \nu_{noise}]^{1/2}
\]

\[
\rightarrow \nu_{ne} = \beta(1 - \rho)^2
\]

\[
K = \left[ \beta \rho^2 e^{-2x} - 1 + 2x + 4\beta\rho(1 - \rho) \frac{e^{-x} - 1 + x}{x^2} + \beta(1 - \rho)^2 + \nu_{noise} \right]^{1/2}
\]

(4.7)

If we now attempt to draw curves based on the multiexposure model with this correction (Fig. 4.5), we see a clearer distinction between \( \beta \) and \( \rho \). \( \beta \) is the true scale parameter, as we may expect, and \( \rho \) (or rather, \( 1 - \rho \)) acts essentially as a long exposure additive offset. These parameters are much more easily fit, especially with the camera noise already accounted for.

### 4.2.2 Dynamic range of illumination

From the curves generated in figures 4.4 and 4.5, it is clear that, in order to accurately account for all fitting parameters, we require exposures ranging over at least 3 orders of magnitude. Previous studies by other groups have accomplished this with techniques such as acousto-optic modulation (AOM), using carefully specified fractions of a high power laser’s energy to maintain consistent illumination at all exposures [62]. When working with our low power VCSEL devices, this becomes impossible. The single mode properties of VCSELs quickly degenerate when powers above 1 mW are reached. We have the dual problem of needing to generate enough power to illuminate the CCD sufficiently
Figure 4.5: Effects of varying $\rho$ and $\beta$ in speckle model after $\nu$ correction. (a) Varying $\rho$ is seen to change the offset at long exposure. (b) $\beta$ remains a height parameter.

at very low exposures, and needing to be able to reduce illumination at high exposures while maintaining a stable lasing condition. The solution to these requirements is broken into two parts, namely what portion of the burden can we place on the CCD itself and what portion we can place on actual laser power.

The Retiga 4000R camera we began working with has a full well of 40000 electrons, and a read noise of approximately 18 electrons. This gives a maximum potential dynamic range of approximately 2000:1. We find that in practice, the useful range is substantially less when attempting quantitative speckle imaging. The first limit comes from saturation at the high end. Any saturated pixels would produce inaccuracies in the statistics of calculated speckles. In order to safely avoid this, mean image intensities are kept in the range of 50-75% of saturation, or about 20000-30000 electrons. The second limit comes from estimation of shot at read noise at the low end. For proper removal of the noise floor background, we must be relatively confident of our estimate of shot noise. We find that at the low intensity limit, it becomes difficult to be certain of our estimates. Practically, it is preferable to remain in the regime where the speckle contrast is substantially greater
that the camera noise itself. If we have a low exposure limit speckle contrast of 10%, similar to the values shown in stationary noise analysis, we would want shot and read noise to sum below 10%. This condition is met with approximately 200 e⁻ minimum intensity. Comparing the effective ceiling of 25000 e⁻ and the effective floor of 250 e⁻, we get a 100:1 dynamic range. Practically this means that without changing laser power, we can obtain speckle images with exposures varying within a factor of 100. For example, if the CCD was near saturation with an exposure of 1 ms, we could still maintain sufficient signal-to-noise with the same laser power if the exposure were dropped to 0.01 ms.

The CCD dynamic range leaves us with a factor of 10 needed to reach the desired 3 orders of magnitude for accurate multiexposure imaging. One of the simplest methods to achieve this factor is by varying the beam diameter at the specimen. This can be accomplished by using optics to vary the laser divergence angle, or by simply moving the divergent source along it’s z axis. While for proof of concept purposes this method is satisfactory, in actual application it serves only to make imaging more difficult and prone to error. Particularly for portable imaging applications, it would be necessary to control power entirely with electronics. It would be ideal to be able to vary laser power output just by current itself, while maintaining constant coherence parameters. In order to investigate the feasibility of this, we measure the power and static speckle pattern of the laser at different currents near threshold. Figure 4.6 demonstrates the outcome of such a measurement on a tissue phantom. If we maintain the laser near threshold, we can obtain a factor of 10 range of powers at which the speckle contrast is consistent within about 15%. At higher powers this contrast would be seen to rapidly decrease. We note that if we choose to start at low exposure with peak the power shown here (1.2 mA), then at higher exposures as we approach saturation we can gradually decrease current, and obtain contrast values in the long exposure limit which should remain accurate within 15%. These values should be accurate enough to allow us to obtain a reasonable fit.
Figure 4.6: Speckle contrast variation with laser power near threshold. If the laser power is kept in the range shown here, there is a factor of 10 change in the power output, over which speckle contrast values are consistent within 15%.

4.3 In Vivo application of VCSEL based LSCI

4.3.1 Methods

The optical setup for this experiment was similar to that described in the methods of Chapter 3. A Retiga 4000R camera was used with two opposing 50 mm lenses, to produce 1:1 magnification. For verification of the method’s robustness under varying illumination conditions, experiments were completed with apertures at f/1.4, f/2, f/2.8 and f/4. Exposures were taken at similar logarithmic intervals between 0.2 and 20 ms. For this first set of results, the laser power itself was maintained constant, but the beam was increasingly diverged at higher exposures to avoid saturation.

Camera triggering was carefully orchestrated to allow proper exposure time synchronization. The camera shutter itself allows exposures as low as 0.01 ms, but streaking effects from photon leakage during frame transfer begin to dominate at exposures even
Figure 4.7: Oscilloscope traces of camera triggers and laser response. (a) We see the laser pulse (orange), exposure trigger (purple) and readout trigger (cyan). The laser is set here to shut off 2 ms after the exposure begins. (b) Closer look at the laser pulse and exposure trigger. The laser is now set to turn on 300 µs before the exposure, and shut off 200 µs after the exposure begins. (c) Detail of the laser turn-on transient. Power levels off after 5-10 µs.

as high as 0.5 ms. For this purpose it was necessary to gate the laser itself, and ensure that the device was entirely off during the frame transfer phase. The Keithley 6221 power supply allowed for the remote programming of arbitrary waveforms which allowed us to achieve this. The camera frames could be maintained at a sufficiently long exposure time while the laser itself was gated on and off. One limitation which manifested particularly at low exposures was the settling time of the device, which was found to be on the order of 20 µs. For this reason, the laser could not be simply triggered at the start of each frame to power on and remain on for the desired exposure. Instead, the laser was be maintained in a baseline ON condition. When the exposure trigger was received, the power supply would wait for the desired exposure length, then shut off the laser entirely for the remainder of the camera exposure and transfer, before returning the laser to baseline ON 0.2 ms before the next expected trigger. Before in vivo work, the time-line of these events was monitored on an oscilloscope, with the laser power being monitored through a photodetector, to verify correct functionality (Fig. 4.7).

Male Sprague Dawley rats, 200-350 g, were used for imaging. Craniotomies were
performed between the bregma and lambda ridges, using the same procedure as outlined in Chapter 3. The camera was focused on the cortical surface. At each exposure and f/#, 100 images were taken. Contrast maps were taken from each image by calculating \((\sigma/\mu)\) within a moving 5x5 region about each pixel. The 100 images from each exposure were then averaged to reduce noise. The mean intensity in each 5x5 region was used to estimate shot noise. This value, along with the 20 e- read noise, was subtracted from the total noise. Within each f/#, the various exposures were co-registered to account for slight position changes. Fitting was done for each f/# over the various exposures with a program written in MATLAB. An initial least squares fit for each pixel used a simplified speckle model (Eq. 4.3) to estimate only \(\beta\), which relies largely on low exposure data. A second fit took this estimated \(\beta\) map and solved for the remaining \(\rho\) and \(t_c\) parameters using the full model (Eq. 4.5).

### 4.3.2 Results

The individual multiexposure frames for one aperture (f/2.0) are shown in Fig. 4.8. The decrease of speckle as exposure is increased is apparent, and we can get some idea of relative flow rates by noting which vessels remain visible at low exposures.

The resulting \(t_c\) maps are represented in Fig. 4.9. The values shown are \(1/t_c\), proportional to velocity. Very similar maps are produced with all 4 different f/#. This demonstrates that the technique is feasible under a wide range of illumination intensities. A factor of 8 change in the amount of collected light is seen between f/1.4 and f/4. There are, however, differences worth noting. A trend of increasing \(t_c\) is seen with increasing f/#. The change can be as great as 40% within large vessels. We posit that these differences come from changes in the speckle sizes themselves. Larger speckles are expected to have greater correlation time in the presence of moving scatterers. Thus the differences here reflect changing proportionality between correlation time and velocity, rather than actual velocity differences [63].
Figure 4.8: Series of multiexposure speckle contrast images, each averaged 200 times. Decrease of contrast is apparent as exposure time is increased.

Figure 4.9(b) shows a comparison between the contrast values obtained in a high flow region as compared to a low flow region, each taken f/2.0. In each case the fitted speckle model closely matches the experimental data. The strong difference in the $t_c$ values of the two regions is apparent from the horizontal displacement between the two sets of data. The low flow region is also seen to have a higher vertical offset. This is because, in a low flow region, the speckle reduction comes from a combination of Brownian movement in tissue and capillary flow deeper in the tissue. There are thus more static, or pseudo static, scatterers present, which leads to an increase in the $\rho$ parameter. Maps of $\beta$ and $\rho$ for f/2 are shown in Fig. 4.10. It is clear that the areas of low flow tend to have lower $\rho$ values as well, based on the static scatterer argument above. There is also a noticeable "donut" hole for $\beta$ values near the plot’s center. This is caused by higher transverse mode shapes beginning to appear in the VCSEL at 2.4 mA, giving reduced coherence in the beam’s center. Also, some of the vessels on the right side of the image have distinctly
Figure 4.9: (a) Flow speeds evaluated with multiexposure fits, in terms of inverse decorrelation time. Maps are similar at different apertures, with decorrelation times increasing at higher f/#. (b) Measured speckle values compared to the fitted model, for high flow region (black square) and low flow region (white square) in f/2.0 dataset.

lower fitted $\beta$ values. We note that these vessels are those found to have very low $t_c$. We can infer that the maximum speckle for these vessels was not reached at the lowest exposure used, leading to an error in the fit. This leads us to recognize that a purely single mode beam would be preferable, to provide an essentially uniform $\beta$ map and allow an easier fit, as well as a metric for determining potential errors.

4.3.3 Discussion

The results produced here demonstrate the potential of using VCSELS in a simple OEM scheme to produce multiexposure contrast maps. This result is of primary importance in implementing a portable multi-exposure LSCI scheme. Previous multi exposure speckle imaging studies have relied on the use of a high power laser, gated with an acousto-optic modulator to produce images of constant intensity regardless of exposure time [4]. This method does not lend well to integration in portable imaging. In this chapter, our mod-
Figure 4.10: (a) $\rho$ map for f/2.0 dataset. (b) $\beta$ map for f/2.0 dataset.

Modification of previous multiexposure models allows us to account for a noise background previously left as a fitting parameter. The key benefit this modification affords us is the ability to produce fits on images with varying grey level intensity. This greatly reduces the constraint on device power typically needed to obtain low exposure LSCI images. By taking advantage of this modification, we assert that multiexposure LSCI can be made more portable and applied with minimal components in the future. Applications of this are important. When imaging temporal changes in real time, a long exposure model can be used to evaluate concurrent flow changes, but a multi-exposure technique is greatly preferred in determining a baseline. We see this technique’s implementation alongside oxygenation imaging as an important step in portable monitoring of neural disorders. There are still some problems which remain to be solved in order to provide a more robust form of the technique. Primarily, the use of single mode devices which retain their purity over a wider range of powers would strengthen the technique, and avoid the spatial artefacts which present themselves as higher modes begin to appear.
Chapter 5

IOSI using current swept VCSELS

The application of current sweeps to VCSELS has been developed as a technique for reducing speckle noise, for the purpose of detecting the low amplitude signals associated with IOSI. This chapter will discuss the experiments completed to evaluate the SW technique’s performance in detecting intrinsic signal changes, as associated with ischemic hypoxia. The experiments in this chapter were completed in collaboration with Dene Ringuette. The in vivo experiments were completed by Dene and I together. Dene studied the ischemic model which we use to induce signal changes, and performed the surgical procedure allowing it. Dene and I each contributed to image processing and research on the HbR/HbO model.

5.1 In Vivo application of sweep mode imaging

The VCSEL sweep operation (SW) has been detailed extensively in Chapters 3 and 4. The benefits we achieve using this technique manifest when we attempt to image low intensity changes in reflectance. In order to produce these changes, we require a model that can be used to reliably produce hemoglobin concentration changes. To accomplish this, we have applied an ischemic stroke model, which proves useful for us for several reasons. It produces relatively strong signal changes which should be detectable with
even noisy illumination; as a first test of a new system, this is desirable. It is a reliable method and produces essentially global, rather than local changes. This ensures that the area that we choose to image will experience changes that we can observe. It also can be expected to produce measurably different effects between veins and arteries. We can use these differences to help us functionally map vessels, which is a helpful tool in understanding hemodynamic changes [5, 64]. While, strictly speaking, ischemic induction does not produce the same physiological state that leads to the intrinsic signals associated with neuronal activity, we assert that for the purpose of testing a new technique it is a useful and necessary first step. The intrinsic changes associated with functional activity can be expected to be an order of magnitude lower than those resulting from ischemia, and tend to be largely focused in the capillary bed rather than within larger vessels [28, 65, 24]. By quantifying sensitivity in an ischemic model, we validate the technique as effective in imaging hemodynamic changes, and evaluate its noise limits for future studies.

5.1.1 Linear HbR/HbO model

In order to go beyond a qualitative look at oxygenation levels and discern quantitative changes in hemoglobin concentration, single wavelength illumination is insufficient. As we have two species of Hb each contributing to reflectance changes, at least two wavelengths of illumination are required to produce a linear model. We in fact use 3 wavelengths, for reasons to be discussed below, and apply a least squares fit to accurately quantify blood oxygenation changes.

The linear model we use to determine oxygenation is based on the modified Beer Lambert law, which allows us to quantify differential concentration changes [36, 2]. The original Beer Lambert law is used to model light intensity in an absorptive medium:

\[ I_\lambda(z) = I(0)e^{-\epsilon\lambda Cz} \]  

(5.1)
where $\epsilon^\lambda$ is the wavelength dependent molar extinction, $C$ is the molar concentration of absorbing elements, and $z$ is the path length through the medium [66]. By comparing this version of the Beer Lambert law to 1.1, we identify $\epsilon^\lambda C$ with the absorption coefficient, $\mu_a$.

The modified Beer-Lambert law attempts to model changes in the presence of scattering and absorption, and can be written as [7]:

$$\frac{\delta I}{I_0} = e^{-\delta \mu_a L + G}$$

(5.2)

Here, $L$ is a path factor depending on absorption, scattering, and geometry, and can be modeled using simulations. $G$ is a further geometry dependent factor. For the case of IOSI, we presume that changes in the absorption or scattering coefficients during hemodynamic changes are all negligible except for the case of HbO and HbR. We now can look at the reflected light change over time as a function of temporal changes in [HbO] and [HbR]:

$$R(t)/R(0) = e^{-(\epsilon_o^\lambda \Delta [HbO](t)L_\lambda + \epsilon_r^\lambda \Delta [HbR](t)L_\lambda)}$$

(5.3)
where the subscripts \( r \) and \( o \) represent respectively HbR and HbO, \( L \) is now the mean optical path through cortical tissue, and \( R \) is the reflected intensity. By applying this model at several wavelengths, we obtain a linear system:

\[
\begin{bmatrix}
\ln\left(\frac{R_{\lambda 1}(0)}{R_{\lambda 1}(t)}\right) / L_{\lambda 1} \\
\ln\left(\frac{R_{\lambda 2}(0)}{R_{\lambda 2}(t)}\right) / L_{\lambda 2} \\
\vdots \\
\ln\left(\frac{R_{\lambda n}(0)}{R_{\lambda n}(t)}\right) / L_{\lambda n}
\end{bmatrix} =
\begin{bmatrix}
\epsilon_{\lambda 1}^o & \epsilon_{\lambda 1}^r \\
\epsilon_{\lambda 2}^o & \epsilon_{\lambda 2}^r \\
\vdots & \vdots \\
\epsilon_{\lambda n}^o & \epsilon_{\lambda n}^r
\end{bmatrix}
\begin{bmatrix}
\Delta[HbO](t) \\
\Delta[HbR](t)
\end{bmatrix}
\]  

(5.4)

If we wish to approximate this system for \( \Delta[HbR] \) and \( \Delta[HbO] \), we can apply the normal least squares equations, with \( r(t) \) representing the reflectance vector, and \( M \) representing the extinction coefficient matrix [5]:

\[
\begin{bmatrix}
\Delta[HbO](t) \\
\Delta[HbR](t)
\end{bmatrix} = (M^T M)^{-1} M^T r(t) = M^+ r(t)
\]  

(5.5)

Fig. 5.1 shows the NIR range absorption spectra of HbR and HbO [18]. The indicated wavelengths correspond to the VCSELs used for IOSI: 680 nm is near the minimum HbO absorption, and responds mostly to HbR changes. 795 nm, at the isobestic point between the two species, responds to each component equally, and can be used to represent total blood volume changes. 850 nm is near a minimum for HbR absorption, and responds mostly to HbO changes. By applying the least squares equation to the extinction coefficients at these wavelengths, we obtain a coefficient matrix \( M^+ \) which reflects these properties:

\[
M^+ = \begin{bmatrix}
0.1960 & -0.0083 & -0.0452 \\
-0.1519 & 0.1979 & 0.3017
\end{bmatrix} \times 10^{-3}
\]  

(5.6)
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5.2 Evaluating oxygenation using IOSI during cortical ischemia

The VCSEL based IOSI system was implemented in vivo using a cortical ischemia model. This model involves constricting the common carotid artery leading to one hemisphere of the brain, strongly reducing blood flow to the cortex. The resulting changes in oxygenation can be measured using the linear model described above, with SW operated VCSEL illumination.

5.2.1 Methods

The optical setup used was very similar to that described in Chapters 3 and 4. A 12 bit Retiga 4000R camera was used, with opposing f=50 mm lenses, producing 1:1 magnification. The aperture was maintained at f/1.4. The animals used were male Sprague-Dawley rats, 200-350 g. Before imaging, the animals were anaesthetized and placed on their backs. An incision was made in the throat, exposing the esophageal cavity. The right common carotid artery was found, and a single suture was looped around and left in a loose knot. The ends of the suture were long enough so as to be reached when the animal was reversed for imaging. The throat incision was closed with a single suture. After the ischemia preparations were made, the animals were placed upright in a stereotaxic frame, and a craniotomy was performed over the right hemisphere, between the bregma and lambda ridges, following the procedure outlined in Chapter 4.

The G2 lasers were driven with a Keithley 6221 current sources. The source was operated in a 2-11 mA sweep. The lasers were alternated using a Keithley 7001 switch, triggered by the camera exposure output. A 500x500 ROI was imaged, giving the camera a rate of 14 frames per second (FPS). Exposure times of 10 ms were used. The camera was significantly more responsive to the 680 nm, so the 680 nm VCSEL produced brighter images and, therefore, reduced shot noise. The 680 VCSEL images were maintained near
75-80% of image saturation, while the 795 and 850 nm images were at 20-30% of saturation. Image averaging was used to decrease noise in all cases. Fig. 5.2 shows examples of the raw images at each wavelength, normalized to peak image intensity. Before ischemic induction, a series of 200 images were taken using single mode illumination, in order to produce LSCI images for vasculature mapping. For ischemia imaging, the switching sequence was initiated, and a baseline series of 1000 frames was taken prior to induction. Near the 1000th frame, the suture loop was pulled tightly to constrict the artery, and held for 1000 frames, before release. 2000 more frames were taken for the observation of re-perfusion.

After imaging, the frames were sorted by their respective 3 wavelengths. Image alignment was done to ensure movement was kept to a minimum. Within each series, the first 500 images were averaged to produce a baseline reference, and subsequent images were calculated on an individual pixel basis as a ratio to this baseline. This was used to produce the $R(t)/R(0)$ values needed in Eq. 5.3. These images were then temporally binned in groups of 4 to further reduce residual noise. The linear model from Eqs. 5.4 and 5.5 was used to compute HbO and HbR differential concentration maps at each time point. The mean optical path through tissue was estimated from Monte Carlo simulations to be 11.5 mm (see Appendix D). The resulting HbO and HbR maps were summed at each time point to produce the volumetric HbT map.

5.2.2 Results

The experiment was attempted on several animals. The results shown here highlight data taken from a single experiment, but similar results are seen with other animals. Fig. 5.3 shows the vasculature as observed through LSCI, and for comparison the cortex as illuminated by a green LED. We note the distinct morphology already which allows us to make a first guess at which vessels are veins, and which are arteries: The veins include the thickest vessels in the center, and are seen to branch outwards and upwards
Figure 5.2: Raw images of VCSEL illuminated cortex at (a) 680 nm, (b) 795 nm, and (c) 850 nm. (d) Temporal noise $\sigma/\mu$ values for baseline 680 nm illumination. Values are typically below 1%, bringing noise close to shot limit. In some regions with more static scattering, greater noise is seen to the right, away from the midline. The arteries on the otherhand appear more slender and spidery, branching downwards to the right, towards the midline. This morphology is typical of neural vasculature [67].

Fig. 5.4 (a) shows the maximum reflectance changes in 680 nm during ischemia, averaged at the peak 4 frames. We can clearly see those vessels presumed to be veins
Figure 5.3: (a) Image obtained with green LED illumination, highlights vasculature where green light is highly absorbed. (b) LSCI image of the same ROI highlights vasculature obscured in green image, and indicates relative flow.

Figure 5.4: (a) Maximum reflectance changes seen with 680 nm illumination. The large veins are clearly highlighted. Change values are relative to baseline images. (b) 795 nm illumination. (c) 850 nm illumination.

darkening, indicative of general hypoxia. Images (b) and (c) show maximal changes at 795 nm and 850 nm. The changes seen here, however, remain essentially qualitative, as we cannot extract data on concentration changes using a single wavelength. By applying Eq. 5.6 along with the maps obtained at 795 nm and 850 nm, we can use these values to
produce temporal oxygenation maps. Fig. 5.5 shows time lapse montages of each of HbO, HbR and HbT. We can see that the ischemia caused an immediate decrease in HbO and corresponding increase in HbR globally, and preferentially greater changes in the veins. HbT experience a global decrease, with much smaller overall changes. After the
Figure 5.5: Time series montages of hemoglobin concentration changes. Labels are time points in seconds, with 0 s indicating onset of ischemia. (a) HbO data. (b) HbR data. (c) HbT data.
constricted artery is released, reperfusion is seen as HbR and HbO return to near-baseline values.

Time course plots of pixel ROIs within venal, arterial and capillary tissue are shown in Fig. 5.6. The results are consistent with the maps. We see near complete re-perfusion in all three regions, but total Hb concentrations remain somewhat decreased after ischemia. We can use our knowledge of the time dynamics of oxygenation to differentiate between veins and arteries [5]. The vessels which see a higher than average change during ischemia are presumed to be veins. In a speckle contrast image, we can see both arterial and venous vessels clearly. Thus, by comparing a baseline speckle image to an oxygenation image at the peak of ischemia, we can differentiate between which vessels are veins and which are arteries. Fig. 5.7 shows the results of such an analysis. The image showing veins was created by calculating the temporal standard deviation values of HbR concentration during ischemia. The image showing arteries was created by multiplying the vein image by a baseline speckle contrast image, and taking the reciprocal. In this way, the veins of the first image were “canceled out” from the LSCI image, leaving only the arteries with significant flow visible.

![Figure 5.6](image)

Figure 5.6: Time plot of Hb concentration changes in (a) veins, (b) arteries and (c) capillary tissue.
5.3 Discussion

With the results shown here, we have clearly demonstrated that applying SW current to VCSEL devices is an effective way to reduce noise, allowing unprecedented sensitivity in performing IOSI with laser illumination. Strong signal changes were seen in all three wavelengths, well above the noise limit of the devices. From the raw reflectance images, a linear model based on the Beer-Lambert law was used to calculate the corresponding hemoglobin concentration changes. The changes seen are similar to those found in other studies of ischemic models[68, 5]. The oxygenation changes are found to be most dominant in veins, and using baseline LSCI images as a reference allows us to identify veins and arteries clearly.

By demonstrating low noise measurement of oxygenation using VCSELs, we have made progress in portably implementing our devices. The next chapter will further demonstrate the capability of using our devices for compact multi modality imaging, by combining LSCI and IOSI for rapid real time measurement of flow and oxygenation during ischemia. While the results shown in this section show significant progress in the implementation of multi modality imaging with VCSELs, the true test of SW operation sensitivity would be to complete IOSI experiments during sensory stimulation. This is a key goal of future work. In these experiments, camera responsivity disparities between wavelengths were unaccounted for, and this led to excess shot noise in the 795 and 850 nm cases. In the next section, attempts will be made to rectify this issue to further reduce total noise levels and allow for higher sensitivity imaging.
Figure 5.7: Using LSCI along with knowledge of changes during ischemia allow us to highlight (a) veins and (b) arteries.
Chapter 6

Simultaneous multi modality neural imaging system

The benefits of using VCSELs for optical imaging come to light when we attempt to rapidly alternate operation modes, allowing us to image using different modalities in real time. The techniques of LSCI and IOSI can be combined to produce integrated simultaneous measurements of blood flow and oxygenation changes. This chapter will outline the methods and instrumentation used to implement rapid SW/SM switching, and demonstrate the systems functionality under a cortical ischemia model.

The experiments in this chapter were completed with Dene Ringuette. I focused on developing the device control algorithms and optimization. The image acquisition software was developed by previous summer students and modified to it’s latest functional form by Dene. The animal experiments and image analysis were done by Dene and myself.

6.1 Instrumentation

The instruments used for imaging have been touched on briefly in previous chapters. The details of the operation scheme and devices will be covered in more detail here, for the purpose of describing the system as a whole. A detailed schematic of the instrumentation
is given in Fig. 6.1. A single packaged VCSEL device is used for illumination. The VCSEL package contains several lasers at each wavelength (680 nm, 795 nm, 850 nm). Generally, only a single laser is powered at any given time, but the option is available to operate several lasers of one wavelength at once, increasing both linearity and maximum power. The 10 leads of the VCSEL are carried via a single cable to a break out box, where they are split into individual BNC connectors. Lead 1 is a universal n-terminal, and leads 2 through 9 are the p-terminals of the various individual VCSELS.

Each VCSELs n-terminal is connected to one of the Keithley 7001 switch’s outputs. The switch has two output cards, and thus the outputs may be powered through two supplies simultaneously. In many schemes we want to take advantage of the greater sweeping range of the 795 and 850 nm VCSELS, and so use one supply to power those lasers while the other supply powers the 680 nm devices. The switch settling time is less than 200 $\mu$s, and through proper triggering can be entirely bypassed. The device power is supplied by Keithley 6221 current source. These supplies are easily programmable and can be controlled by General Purpose Interface Bus (GPIB, IEEE-488) interface. Standard waveforms available include DC output, as well as sine, square, and ramp waves with variable duty cycle, frequency, offset and amplitude. Arbitrary waveforms can be programmed also through software. For the purpose of switching between SM and SW operation, we make use of the arbitrary waveform and camera triggers, in a manner which is detailed in a later section. To accomplish rapid simultaneous oxygenation and flow imaging, we alternate between the three wavelengths in SW operation, as well as one wavelength in SM operation. This allows us to determine Hb concentration changes using the methodology described in Chapter 5, as well as relative flow speed changes by implementing the single exposure LSCI approximation, from Eq. 4.4. The VCSEL illumination is not collimated, and has a divergence angle of approximately 10°, consistent for each wavelength. The illumination is generally not further focused onto the tissue. Beam spot size on the cortex is controlled by changing the distance between the device
and tissue.

Figure 6.1: Schematic of combined LSCI/IOSI system

6.1.1 Camera parameters

For the purpose of fast multi modal imaging, a Rolera EM-C² EMCCD camera (QImaging) was used in the place of the Retiga 4000R used in previous experiments. The main benefit of this camera was in speed increase, as well as sensitivity. Standard CCD sensors tend to have reduced quantum efficiency (QE) in the near IR, and the Rolera was chosen specifically for its higher QE. If we compare the Retiga camera to the Rolera camera, at 680 nm QE is boosted from 28% to 63%, at 795 nm QE is boosted from 13% to 43%, and at 850 nm QE is boosted from 8% to 39%. We see in particular that this setup allows us to equalize between the 3 light sources. The EM gain functionality of the camera is generally not used, due to the non linear response and amplified shot noise. The EMC2 has a CCD of 1004x1002 8 µm pixels. Each pixel has a full well of 32700 e⁻. When run
in 14 bit mode, pixels are digitized to $2^{14} = 16384$ grey levels, translating to $2 e^-$/grey level. This gives a full well shot noise level of 0.55%. As a comparison, the estimated read noise is $18 e^-$, or 0.055%. Thus, for well illuminated images, the camera is clearly shot noise limited. The camera frame rate is dependent on the ROI and binning selected. An arbitrary ROI within the 1 MPixel full frame can be selected and binned in regions of 1, 2, 3 or 8 pixels. The frame rate is dependent only on the number of resulting pixels read out. The full frame speed is 34 Hz (or frames per second). For a 500x500 ROI, this is increased to 62 Hz. For a 250x250 ROI, the speed surpasses 100 Hz. Two timing outputs are available from the camera. One output is the readout sync, which is active high while a frame is being read out from the camera. The other output has two modes. The first mode is the trigger link, which is active low when the camera is able to accept triggers through a handshake protocol. Once a return trigger is received, the next frame will be initiated and the process will repeat. The second mode is expose sync, which is active high for precisely the duration of a frames exposure. For most triggering purposes we use the expose sync trigger.

6.1.2 Software

In order to control the instruments for imaging, we have developed our own image capture and control software, using C++. Standard image libraries were used along with the API provided by QImaging for their cameras, as well as a GPIB communication library. This software allows us to have full control of camera function, as well as real time previewing. The current source and switch can be controlled and set in real time using the software’s GPIB interface. We have also implemented a preview mode featuring real time calculation of speckle contrast, allowing us to view blood flow in real time. Appendix A gives more details about the software.

For programming the arbitrary waveforms needed to perform SM/SW switching, we use a MATLAB script. We require a waveform that produces a sine pulse for a set period
Figure 6.2: Timing used for programmed SW/SM pulses. A single trigger begins a programmed waveform which produced the SW pulse for the next frame as well as the SM pulse for the second frame. The source then becomes dormant awaiting the next trigger of time, then drop current to 0 while awaiting the next frame, and finally raise current to a DC level slightly higher than the lasing threshold in time for the next frame. To perform this, we use triggering options which allow us to force the source to ignore triggers while a waveform is being output, and an option which sets the baseline current to a positive DC value. In order to implement this properly, the camera’s frame rate must be known. Fig. 6.2 demonstrates the timing used to produce the required waveform. The code used to accomplish this is shown along with several other operation modes in Appendix B.

### 6.2 Real time simultaneous blood flow and oxygenation imaging

#### 6.2.1 Methods

In order to test the system, we imaged live rats under the same ischemic model as presented in Chapter 5. Animals were prepared in the same method as described in Chapter 5. Imaging was done with the Rolera EMC2 camera as described in this chapter,
using a 500x500 pixel ROI, resulting in a frame rate of 62 FPS. Camera exposures of 10-12 ms were used. The lasers were triggered sequentially, in the order of 795 SW, 850 SW, 680 SW and 680 SM. As the camera is more sensitive to 680 nm illumination, the 680 SW illumination was gated to 3-4 ms. The resulting images can provide data for flow and oxygenation maps at a rate of 15 FPS.

Before ischemic induction, baseline flow maps were again produced using 200 SM illumination images. These were used to assist in determining best focus of the surface vasculature, and to localize the required ROI. During ischemia, relative flow speeds were calculated using the long exposure limit, from Eq 4.4. In order to reduce noise and obtain clearer maps, the maps were averaged in 15 frame temporal bins, providing flow maps at 1 FPS. Hemoglobin concentration changes were calculated in the same way as outlined in Chapter 5. The raw SW images were averaged in 5 frame temporal bins, providing oxygenation maps at 3 FPS.

6.2.2 Results

The results in this section focus on a single experiment, though similar results were seen in several animals. Fig. 6.3 shows time lapse montages for HbO, HbR, HbT and relative flow maps. The oxygenation images are very similar to those seen from Chapter 5. We can clearly see the veins highlighted as the oxygenation level changes. The addition of flow maps allows us to observe in more detail the physiological response to ischemia. We see that there is a global drop in flow across venous, arterial and capillary tissue. The larger vessels are distinctly highlighted, and the arteries (those vessels not highlighted in oxygenation maps) appear to have a somewhat more significant decrease than the veins. The flow decrease is almost instantaneous, and oxygenation changes seem to trail this abrupt change slightly.

Fig. 6.4 shows corresponding time series data taken from venous, arterial and capillary tissue. We again see similarities between this data and the data taken from Chapter 5.
Chapter 6. Simultaneous multi modality neural imaging system

Figure 6.3: Montages of processed ischemia results. (a) HbO changes (b) HbR changes (c) HbT changes and (d) Relative flow changes.
The reduction in flow values is as great as a 60% decrease in the case of larger vessels. Reduction in capillary tissue is still significant. We observe a strong re-perfusion on the time scale of a couple of minutes, though baseline values aren’t entirely recovered.

6.2.3 Discussion

The results of this section show the successful application of real time dual modality imaging. The speed and sensitivity we have achieved with minimal optical components.
is similar to other recent flow and oxygenation rapid imaging systems [42]. From this step of demonstration, we hope to be able to further investigate connections between blood flow and oxygenation in vivo. The imaging speed and field of view which our technique provides gives us an advantage in understanding the temporal dynamics of neurovascular coupling. The time series shown demonstrate that we can see clear differences in the responses of HbR, HbO and flow rates. We envision further applications in sensitive measurements of sensorimotor stimulation.

Further, the success of this technique is a strong demonstration of the practicality of VCSEL implementations for portable imaging. The small source, single detector, rapid imaging implementation of LSCI and IOSI demonstrates the basic layout for an integrated imaging device for monitoring fully mobile animals. The main limitation to further miniaturization lies in the electrical control components. We have already begun work on replacing the switches and source with microcontroller control, and once completed, this control system will help bring the combined technique to a truly portable format. The final step will be replacement of the CCD camera with a small scale CMOS sensor chip, and integrating all components into a portable device. Recent work by other researchers has demonstrated a portable LSCI scheme using CMOS sensors [69], and we see our system’s combination of multiple techniques as an important expansion in this direction. Further research will be needed to develop the methodology for imaging flow and oxygenation at close range, but we assert that the work shown in this chapter has already demonstrated the feasibility of our technique for portable dual modality imaging.
Chapter 7

Portable VCSEL-based fluorescence sensing

The devices used in this chapter were developed by Dr. Ofer Levi, Dr. Evan Thrush and Dr. Tom O’Sullivan with Dr. James Harris’s group, in the department of Electrical Engineering at Stanford University. The experiments were completed in collaboration with students from Dr. Gang Zheng and Dr. Brian Wilson’s group, in the Department of Medical Biophysics, University of Toronto. I maintained and operated the imaging system, designed experiments, and wrote the code used for both data acquisition and analysis. Particular recognition is given to Natalie Tam and Joseph Gold, for assistance in preparing samples and imaging with the IVIS and Maestro systems.

7.1 Detecting fluorescence with monolithically integrated sensors

Generally, in vivo fluorescence signals are imaged using large scale, full field imaging systems. These systems contain a closed anaesthesia chamber, in which an animal is held to eliminate movement, and illumination from a lamp with a selection of filters is
used to produce fluorescence. The resulting signal is imaged with a CCD sensor; dynamic range of the results are controlled by varying the image exposure time. This imaging scheme is useful for detecting slowly varying signal changes for brief periods of time. However, the size and cost of these systems limits their use in many other cases [70]. In particular, the requirement of keeping subjects anaesthetized and stationary limits the potential for long term studies, in which we may wish to continually monitor an animal over many hours or even several days. If we wish to be able to image the time dynamics of in vivo fluorescence in alert animals, a different platform must be used. To tackle this problem, we attempt to integrate all the main components of a fluorescence system in a portable package, which can be mounted on an alert animal. Similar small scale fluorescence sensors have been designed before, though generally in the context of a stationary sensor for lab-on-a-chip applications [71, 72]. An in vivo sensor would require high sensitivity, low noise, and robust performance allowing it to image real time, small signal changes in fluorescence.

Figure 7.1: Sensor package, with VCSEL and detector on chip, focusing lens and leads around outside of package.
7.1.1 Sensor Design

The integrated sensors we investigate in this chapter use the same VCSEL technology described in earlier sections. With modifications to the packaging and the addition of detectors and filters on-chip, we produce a compact device which can both provide illumination and detect the resulting fluorescence with high sensitivity. One important decision required in the design of such a device is wavelength specificity. Whereas many fluorescent dyes have somewhat wide excitation spectra, a laser only excites with a single peak. If laser/dye peak correspondence is not closely matched, excitation will be strongly reduced. On the other hand, the correct filters need to be selected to strongly reject the laser excitation while accepting the fluorescent emission. For the purpose of tissue imaging, the near infrared range is ideal because of the penetration depth afforded. There are several dyes in this range which are commonly used as markers. Our device has been optimized to match the peaks particular to Cy5.5.

![Schematic of fluorescence sensor components monolithically grown on chip. The VCSEL and detector are clearly marked. (b) Detail of the detector and fluorescence filter](image)

Figure 7.2: (a) Schematic of fluorescence sensor components monolithically grown on chip. The VCSEL and detector are clearly marked. (b) Detail of the detector and fluorescence filter

A schematic of the device is shown in Fig. 7.2. The main components which produce the desired functionality are all grown epitaxially on a monolithic GaAs substrate.
The VCSEL is composed of layers as described in Chapter 3. The active layers are composed of a mixture of GaInP and AlInP, together producing $Al_xGa(1 - x)InP$. The relative amounts of Ga and Al can be adjusted to tune the lasing wavelength. For excitation of Cy5.5, we use 675 nm devices.

2. P-I-N photodiode detector

The detector is grown directly on top of the VCSEL portion of the wafer. On top of the P-type DBR layers of the VCSEL, a P-type GaAs layer is grown, acting as the base of the detector. On top of this, an absorbing I-GaAs region is grown, where absorbed illumination causes carrier generation, and the resulting current. Finally, a thin top layer of N-type GaAs and electrical contacts are grown on top.

3. Filter

A two-layer filter structure is used to maximally isolate fluorescence signal to the detector. First, a dielectric filter is patterned on top of the detector. This layer is deposited as a thin film, essentially designed as a square notch filter from 730-770 nm. Due to fabrication inconsistencies, this layer is not reliably as strong as required, so a second filter layer is added. This second layer is a conventional fluorescence interference/absorption filter. With the addition of this filter, a 4-5 order of magnitude decrease in background signal is seen.

7.1.2 Markers

As mentioned above, the device was optimized for use with Cy5.5 dye. Cy5.5 is commonly used in biomedical applications as it is a sensitive marker with NIR emission, suiting it well for tissue penetration. The absorption and emission spectra of Cy5.5 are shown in fig. 7.3. In order to target specific proteins in tumour tissue, we are also interested in the use of a marker known as Pyropheophorbide-α, or Pyro. This marker has an emission spectrum similar to that of Cy5.5, as well as a secondary absorption peak at 664 nm. Based on the differing parameters between Cy5.5 and Pyro, we would expect Pyro to
produce substantially less signal with our sensors. Pyro has however been demonstrated as a fluorophore in beacons targeted to cancer specific proteins. Since the fluorescent peaks of Pyro are still near those of Cy5.5, we expect that these markers can provide a feasible method of sensing tumour specific signal in-vivo.

![Fluorescence excitation and emission spectra](image)

Figure 7.3: Fluorescence excitation and emission spectra for (a) Cy5.5 and (b) Pyro. Sensor laser emission and filter transmission curves are also plotted.

### 7.1.3 Modeling

In the specific case of direct illumination of a dye without significant scattering, we can develop a model to estimate the expected fluorescence signal detected by the device [73]:

\[
I_{\text{det}} = \lambda_{\text{em}} \frac{q}{hc} P_{\text{las}} Q E_{\text{dye}} Q E_{\text{det}} T_{\text{filter}} C_{\text{sensor}} (1 - 10^{-\epsilon d M})
\]

(7.1)

The final result we obtain with this, \(I_{\text{det}}\), is the detector current in amps. This model is largely based on the Beer-Lambert law applied to absorption of the excitation light. The various model parameters, explained in Table 8-1, are based on dye-specific quantities as well as device parameters. The most important parameters in matching the sensor to a dye are \(Q E_{\text{dye}}\) (dye quantum efficiency), \(T_{\text{filter}}\) (filter transmission based on the overlap with the dye emission spectrum) and \(\epsilon\) (the wavelength dependent excitation absorption...
Table 7.1: List of model parameters and their values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{em}$</td>
<td>Fluorescence emission wavelength</td>
<td>750 nm (Cy5.5)</td>
</tr>
<tr>
<td>$P_{las}$</td>
<td>Laser output power</td>
<td>1 mA</td>
</tr>
<tr>
<td>$QE_{dye}$</td>
<td>Dye fluorescence quantum yield</td>
<td>30% (Cy5.5)</td>
</tr>
<tr>
<td>$QE_{det}$</td>
<td>Detector quantum efficiency</td>
<td>77%</td>
</tr>
<tr>
<td>$T_{filter}$</td>
<td>Emission filter transmission</td>
<td>16% (Cy5.5)</td>
</tr>
<tr>
<td>$C_{sensor}$</td>
<td>Collection efficiency of sensor</td>
<td>0.06%</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>Molar extinction of dye</td>
<td>250,000 cm$^{-1}$M$^{-1}$ (Cy5.5)</td>
</tr>
<tr>
<td>$d$</td>
<td>Thickness of dye</td>
<td>Variable</td>
</tr>
<tr>
<td>$M$</td>
<td>Molar concentration of dye</td>
<td>Variable</td>
</tr>
</tbody>
</table>

CHAPTER 7. PORTABLE VCSEL-BASED FLUORESCENCE SENSING

Based on this model, we expect essentially linear behaviour for small amounts of fluorophore. For higher concentrations, saturation effects begin to appear as the majority of excitation light becomes absorbed by the dye. In order to obtain strong signal, it is important that the previously mentioned factors are well matched within the linear regime of dye concentrations.

7.2 Sensitivity: dye in solution

Initial tests were completed in order to quantify the device’s sensitivity to fluorescence produced by direct illumination of dye in solution. In order to grasp the limitations
associated with imaging dyes with sub-optimal excitation/emission peaks, we compared results with Cy5.5 dye and Pyro markers. A range of dilutions were used in each case, in order to both test the sensor signal model and determine the noise floor in terms of dye concentration.

### 7.2.1 Methods

The sensors were operated with the use of a lock-in amplified in order to eliminate excess noise. The devices were modulated at 23 Hz (selected as a low frequency avoiding any harmonics from 60 Hz electrical lines). A trigger from the source was used to match the lock-in with the modulation frequency. Illumination from 3 different lasers were used sequentially, each with 2 detectors, to obtain signal statistics. 5 measurements were averaged with each sensor/detector pair. In each case, the detector was allowed a settling time of 2 seconds.

Dye solutions were prepared in phosphate buffered saline (PBS). For each of Cy5.5 and Pyro, dilutions ranging from 1 nM to 100 µm were used. One well of PBS with no dye was used as a background. For the case of Cy5.5, two samples of each dilution were placed in a 96 black well plate. The plate was illuminated from below at a slight angle, to decrease back-reflection into the sensor. The plate was moved horizontally on a stand to allow sequential measurement of each well. Once the data were collected, the plate was taken to a wide field fluorescence imaging system (IVIS Spectrum, Xenogen) to obtain full field fluorescence images for comparison. A similar set of Cy5.5 filters was used. The results were quantified in terms of photon flux. For the case of Pyro, a single stationary well was used, to better ensure background did not change between dilutions, allowing observation of lower signals. The well was filled with solution sequentially starting with the lowest concentration, and then emptied and twice flushed with PBS between measurements. Again, one well of PBS alone was used to establish the background. After measurement, a separate plate of the same dilutions was prepared and again measured using the IVIS.
Chapter 7. Portable VCSEL-based fluorescence sensing

After measurements, expected fluorescent values were calculated using the sensitivity model from Eq 8-1. Values for Cy5.5 were well known, but the specific numbers of quantum yield, filter transmission and extinction coefficient were not precisely known for Pyro. Estimates were used where possible.

7.2.2 Results

Plots of the resulting signal for Cy5.5 with background subtracted are shown in Fig. 7.4 (a). Standard errors are included based on different laser/detector combinations. We can see a clear linear trend for concentrations in the range of 5 nM to 5 µM. At higher concentrations saturation effects are evident. At lower concentration, noise and background errors begin to dominate. The values obtained from the IVIS, normalized to the max of the Cy5.5 plot, are superimposed. We see a very similar trend in this case, with some deviations at low concentration. These differences could partially be caused by mis-estimation of the background in either case.

![Figure 7.4: Fluorescence concentration results for (a) Cy5.5 and (b) Pyro. Each plot includes the sensor’s detector signal, IVIS signal, and the model results for comparison.](image)

The background subtracted results for Pyro are shown in Fig. 7.4 (b). The curve is
found to be linear in the range of 50 nM to 10 µM, with saturation effects not occurring until the very highest concentration. The reason we don’t see saturation as quickly is due to the significantly lower absorption at 675 nm, as compared to Cy5.5. This also contributes to the generally lowered signal observed, along with the emission filter mismatch. The IVIS data for Pyro are superimposed. In each case, the sensor values appear to be more linear than the IVIS values. This may be a result of the automatic exposures in the IVIS; exposures optimized for the low concentration signals may be less effected by noise and produce more linear results.

Fig. 7.5 shows a direct comparison of Cy5.5 and Pyro, along with estimates based on the model of Eq. 7.1. The model parameters used are shown in Table 8-2. Pyro currents are seen to be approximately 15 times lower than the corresponding Cy5.5 values. A factor of 5 can be accounted for by the reduced extinction coefficient. The $T_{\text{filter}}$ value for Pyro was estimated based on the observation of a remaining factor of 2-3 to be accounted for. The quantum efficiency was estimated to be similar with each dye. The models seem to match experimental results quite well, especially in the Cy5.5 case. We note that while the model’s saturation seems to match the experimental results in the
Table 7.2: Comparison of Pyro and Cy5.5 parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pyro</th>
<th>Cy5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{em}$</td>
<td>750 nm</td>
<td>750 nm</td>
</tr>
<tr>
<td>$QE_{dye}$</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>$T_{filter}$</td>
<td>6%</td>
<td>16%</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>45,000 cm$^{-1}$M$^{-1}$</td>
<td>250,000 cm$^{-1}$M$^{-1}$</td>
</tr>
<tr>
<td>$d$</td>
<td>0.32 cm</td>
<td>0.32 cm</td>
</tr>
</tbody>
</table>

In the case of Cy5.5, it appears somewhat different in the Pyro case. This may be because we are in fact underestimating extinction at 675 nm, and the excitation peak is better aligned to the laser than predicted.

7.3 Sensitivity: Capillary submerged in Intralipid phantom

The sensitivity of the sensors was tested using Cy5.5 dye submerged in a tissue simulating phantom. These experiments were done to help establish an understanding of how the signal will be effected by sensor placement. Since the tissues we would like to study are generally somewhat below the dermal surface, it is important to understand how the presence of skin tissue, for example, will attenuate and spread fluorescent signal.

7.3.1 Methods

The experimental setup is outlined in Fig. 7.6. A thin glass capillary ($\varnothing$ 1.15 mm) was filled with 50 uM Cy5.5 in PBS. The dye was submerged in a small container filled with 0.6% Intralipid in water (Fresenius Kabi, Germany). Intralipid is a lipid based fluid commonly used as a tissue phantom because of its scattering properties. At this dilution
it has a reduced scattering coefficient, $\mu'_s$, of approximately $6 \text{ cm}^{-1}$, similar to that of living tissue. The container and dye were placed on a X-Y-Z translation stage. The sensor was submerged in the Intralipid, facing downwards towards the dye tube. The position of the sensor vertically and laterally from the capillary were adjusted using the translation stage micrometers. At each position, the sensor sequentially measured signal using 2 detectors, each time averaging 4 measurements. In this way, a 2-D map of fluorescence signal was produced. Fits were applied to the resulting data to help evaluate resolution and sensitivity at depth.

![Figure 7.6: Setup for sensor spatial sensitivity experiments. The sensor was kept stationary, while the dye capillary position was scanned in the z (depth) and y (lateral) axes.](image)

### 7.3.2 Results

Plots of the fluorescence signal are shown in Fig. 7.7. We can see the shape of the sensitivity curves along both z (depth) and y (axial) axes. The axial width of the result shows that signal can still be clearly discerned as far as 2 mm aside from the target. The
decay of the depth curve indicates that signal is reduced to 10% of its maximum value when the target is 2 mm deep. While the signal itself is dependent on the strength of fluorescence emission, these results suggest that we could obtain significant sensitivity through thicknesses of several millimeters.

Figure 7.7: Several views of a surface plot of sensor current based on depth and lateral displacement of fluorophore. (a) Current vs. lateral distance, giving a Lorentzian-like shape. (b) Current vs. depth, showing exponential decay. (c) Current (colour) as a function of depth and displacement (Logarithmic mapping, to show full dynamic range).

7.4 Ex vivo study of homogenized tissue

7.4.1 Methods

As a final study of sensor functionality, we measured sensitivity to Pyro dye mixed in ex vivo tissue samples. To accomplish this, we produced homogenized samples of chicken breast. Dye was mixed with the ex vivo tissue, producing total concentrations ranging from 10 nM to 100 µM, and placed in a 96 well plate. The measurements were completed in a manner similar to what was done for the dye concentration measurements described above. Two wells with each concentration were measured and averaged. A measurement of tissue with no dye was taken to estimate background. After sensor imaging, the 96
well plate was taken to the IVIS imaging system for measurement.

7.4.2 Results

The ex vivo fluorescent values are shown in Fig. 7.8, alongside the IVIS values. We note that in each case, a large background is seen relative to the dye based signal. This background is probably due to autofluorescence of the tissue. This result indicates a strong limitation to in vivo measurements. In all cases, we must ensure that dye based signals are measurable on top of autofluorescence background. In particular, if the background level is seen to have deviations in a range comparable to the dye signal level, it will be difficult to produce accurate results. This seems to be the case for dye concentrations below 0.3 µM. In both the IVIS image and the sensor data, the lowest concentrations have no discernible trend.

Curve fits of the same form as the model, Eq. 7.1, are applied to both datasets, with an additional offset term accounting for autofluorescence. Rather than using prior knowledge of parameters, the fits were necessary because of tissue absorption and scattering factors. The total fluorescence collected by the sensor will be reduced somewhat compared to dye in solution, due to absorption of the emitted light in tissue. Further, the excitation path length through tissue is difficult to estimate. The resulting exponential absorbance from the Beer Lambert law is dependent on both tissue scattering, as well as the geometry of the imaging set-up. Fitted path lengths are in the range of 1.5 cm, which, when compared to the well depth of 0.5 cm suggests significant scattering effects.

On the whole, the sensor signal appears to deviate from the fit more than the IVIS data. This is most likely due to fluctuations based on the sensors position relative to the tissue samples. The individual sensor measurements themselves show extremely low noise, on the order of 2 pA or less for a signal strength of 500 pA. We can infer that the variances arise from differences in light collection efficiencies. Although we attempted to maintain consistent geometry, we would expect significant variability with even slight
changes in the sensor’s position relative to the sample, as shown in the previous section. Particularly in the case of tissue, this becomes difficult when attempting to measure signal on top of the autofluorescence background. With highly scattering tissue, a further contribution of noise could come from reflected excitation light entering the sensor at wide angles. The emission filters are highly rated only near normal incidence. When light enters the device at angles greater than 30°, the filter is less effective at blocking the excitation light, and thus a higher background noise level will be seen. These excess noise factors are most likely to be problematic in evaluating in vivo signals.

![Figure 7.8](image)

Figure 7.8: Signal obtained from Pyro dye in ex vivo tissue. (a) Sensor current values. (b) IVIS signal. Fitted models are included for comparison.

### 7.5 Initial in vivo results

With the characterization of device sensitivity complete, we have moved on to initial in vivo tests of dye uptake in vivo. For an initial pilot study, subcutaneous tumours were grown over the flank of nude mice. Injections were conducted through the tail vein, of either Prosense 680 (Perkin-Elmer, NEV10003), a Cy5.5 based commercial molecular
imaging agent, or of Pyro based markers. We then attempted to sense the time dynamics of dye entering the tumours, and compared these values to a reference signal in the opposite flank. After sensor measurements, the animals were taken to the Maestro animal imaging system for full field signal imaging. The expectation was that the tumour tissue would metabolically uptake dye at a faster rate than healthy tissue, resulting in heightened signal.

The results up until the present have revealed further difficulties in in vivo imaging. Fig. 7.9 shows a typical time course of signal produced from conjugated pyro peptide (CPP) dye accumulating in a tumour, and in the reference flank. An image taken with the IVIS after sensor measurement is shown for comparison. It is clear that, while strong signal can be seen using the IVIS, the relative changes observed with the sensor do not show clear accumulation. The causes of this lack of signal are most likely traced back to the concerns detailed in the previous sections. Slight movement of the animal during imaging is likely to overwhelm any signal changes due to fluorescent dye. The combination of autofluorescence background and back scattered light provide a much higher signal than the Pyro dye itself. The back scattered signal itself could be changing based on hemoglobin concentration changes, on top of any movement. If we wish to image dye based changes, we must be able to strongly isolate these other contributions.

A similar experiment using Prosense is shown in Fig. 7.10. Since Prosense uses Cy5.5 as a fluorophore, we expect stronger than in the Pyro case. We do in fact see gradual accumulation of signal in both the tumour and the reference. The IVIS image seems to confirm that the tumour has not preferentially produced signal several hours after injection. This may result because Prosense is a quenched probe, and signal increases occur only at the rate at which the beacon can be cleaved. At later times, the signal in the tumour and several internal organs are seen to be substantially increased.

The Prosense results indicate a further need for fast-acting dyes in applications of our device. If signal differences between tumours and healthy tissue develop within a few
Figure 7.9: (a) Time course of sensor signal for CPP injected through tail vein showing accumulation in both tumour and healthy tissue. Strong background variations make signal difficult to discern. (b) Maestro image of same animal, after sensor measurements, show strong accumulation in tumour.

Figure 7.10: (a) Time course of sensor signal for Prosense680 injected through tail vein showing accumulation in both tumour and healthy tissue. Gradual increase of fluorescence is seen in both cases. (b) Maestro image of same animal, 1.5 hr after injection. (c) Maestro image of same animal, 18.5 hr after injection, showing accumulation in tumour and other organs.

hours, we can easily measure dye uptake continuously. If a slower agent is used, it becomes difficult to continuously measure signal while maintaining anaesthetic conditions. Any movement or replacement of the device would lead to a change in background signal, leading to further difficulties in discerning the relevant signal.
7.6 Discussion

An initial goal of my work was to implement fluorescence sensing in tandem with the other neural imaging modalities in vivo. The difficulties experienced in accounting for background noise in vivo limited progress along this line. We have obtained a greatly increased understanding of the needs and limitations of implementing the current generation sensors in vivo, and intend to use this knowledge to carry forward in exploring the sensors’ applications. In particular, the in vivo background fluctuations must be accounted for, by either increased sensitivity to the selected dye, or by decreased movement, which could be assisted by fixing the sensor directly to the animal tissue. Proposed future experiments will include adhering a sensor directly on an animal, allowing longer term sensing while keeping the sensor stationary. In the case of glioma, attachment directly to the skull could be used as a way of keeping the sensor tightly anchored. Other work will be done to develop devices which use VCSELs more closely aligned to the Pyro peak, as well as investigating potential dye variants which exhibit stronger sensitivity to NIR illumination.
Chapter 8

Conclusions and Future Work

The work of this thesis has described the development of a system which combines the techniques of IOSI and LSCI using VCSEL devices. We have also demonstrated the use of the same devices for portable fluorescence sensing. The advantages of our system comes from its low cost and simplicity. By taking advantage of VCSEL wavelength tuning, we are able to reduce the coherence length significantly, producing low-speckle laser illumination allowing us to perform sensitive, low noise imaging. The results show that sweeping VCSEL current allows us achieve noise levels approaching the shot noise limit typical of scientific cameras. The use of SW operation allows us to implement IOSI and LSCI simultaneously, giving us information on real time changes in oxygenation and blood flow speeds. We have further demonstrated a modified model for LSCI measurements, which allows us to quantify blood flow correlation times using low power, single mode VCSELs. Further experiments are being planned to test this method in a controlled environment using microfluidics, to verify the accuracy of the technique. We have also characterized VCSEL fluorescence sensor responses with two different dyes. We have evaluated the signal reduction associated with sub-optimal dye excitation and emission with dye both in solution and ex vivo in tissue samples. This has helped us determine limitations when moving to in vivo imaging with Pyro based targeted probes. We have
yet to demonstrate repeatable measurements in animal subjects. To accomplish this, we intend on looking at models which allow us to adhere the sensor to the animal, reducing signal changes associated with movement. We are currently looking at other solutions which will assist us in accounting for background variations unrelated to the fluorescent signal.

We envision future applications which exhibit the full range of capabilities of our system. In particular, we wish to look at disease models which can benefit from the use of the three techniques in tandem. We have yet to demonstrate application of all three together, simultaneously. We are currently investigating potential applications with a glioma model. By imaging blood flow and oxygenation changes along with fluorescent signal in brain cancer, we hope to to gain a better understanding of tumour vascularization, metabolization, and damage to the functionality of surrounding tissue. Other groups have already applied LSCI and IOS to image vessel perfusion and vascularization in tumours, and combining the two along with fluorescence would provide further understanding of the progress of angiogenesis and treatment in cancer [75]. We are also investigating applications to epilepsy and stroke models, working with researchers in Sunnybrook and in Toronto Western hospitals. We are currently working with the Stefanovic group at the Sunnybrook Health Sciences center, focusing on looking at neurovascular coupling by using our system in tandem with two-photon imaging. We hope to obtain a greater understanding of how flow and oxygenation are correlated with neural activity at different cortical depths. These studies are also being used as a platform with which we can develop a further understanding of how LSCI can be implemented at depth. Generally, LSCI is used to evaluate velocities in surface vasculature, and contrasts resulting from flow in lower layers is ignored or considered negligible. We have already observed that flow in lower layers can be evaluated by varying the cameras focus (Fig. 8.1). Proper quantification at depth is difficult, as it involves accounting for both static scattering from above, as well as contributions from flow in higher vessels. Early results seem to indicate
that relative flow speeds can still be established within groups of vessels at the same depth.

![Figure 8.1](image1.png) ![Figure 8.1](image2.png)

Figure 8.1: Speckle imaging at depth. (a) LSCI inverse decorrelation times with single depth focus. Some vessels are in focus but others cannot be seen clearly, especially narrow capillaries. (b) With multi depth focus, maximum decorrelation time is matched at best focus. By projecting maximum values, all layers can be brought into best focus.

Our system describes a paradigm for optical imaging which, if further developed, could lead to significant advances for portable imaging techniques. This has been a main goal of our group’s research direction. There are still a number of important steps which need to be carried through before the system described here can be used portably. Future steps include miniaturizing the control electronics, replacing the camera with a small scale sensor, and modifying image analysis algorithms for close-range imaging. Initial work has begun on several of these steps already, and other studies are being planned in the near future to complete this further. Our group has begun to look into the components needed to replace the current sources and switch. A schematic of the proposed system using off-the-shelf components is shown in Fig. 8.2. Lasers of all three wavelengths are controlled with modulated VCSEL driver ICs, such as Analog Devices ADN2530. A custom (or
IC) function generator is used to provide the SW modulation voltage, which is converted into current modulation by the laser drivers. One driver is operated in DC mode to produce SM current. Signal to the individual drivers are gated with the use of solid state switches. A microcontroller such as the Arduino Uno is used to read camera triggers, and provide the necessary gating and exposure times for each VCSEL. A control unit like this could easily be programmed for custom trigger sequences and exposure times. The entire control system could be packaged on a PCB several inches in size. Initial studies for replacing CCD components with CMOS detection have also begun. Our group has started to work with portable CMOS imagers supplied by DALSA. These cameras have small pixel sizes and frame rates of up to 1000 fps, while retaining noise levels similar to CCD cameras. Their small scale and low power consumption makes them ideal for implementation in portable imaging [76].

The applications and further developments described here are not exhaustive. We see portable functional optical imaging as an important modality for investigating behaviours in awake subjects, both in healthy and diseased states. Beyond neural imaging,
quantification of fluorescence, oxygenation and flow speeds are important in many biological systems, and our group will continue to investigate applications to a wide range of problems.
Bibliography


Appendices
Appendix A

Simulating speckle patterns

The methods used for speckle simulation in figures such as 1.4 are outlined here. The technique is adapted from [52]. The technique used for simulating realistic speckle patterns is based on the principals of Fourier optics. The speckle pattern can be interpreted as the image resulting from summing equal amplitude random phase elements at the aperture of an imaging system. To produce the simulation, we simply produce a circular aperture of diameter D, and fill it with phase elements. We place the aperture on a LxL grid which is inversely proportional to the desired pixel size. Fig. A.1 shows an example of such a grid. Squaring the 2-D Fourier transform of the resulting grid will give a speckle pattern with the desired distribution. The ratio 2L/D is the sampling rate of the pattern. If D=L, the Nyquist criterion is exactly matched, and the minimum speckle will span 2 pixels. The MATLAB function used to generate speckle patterns is shown here. This function will display the aperture grid, the speckle pattern, and the probability distribution of the pattern intensity.

```matlab
function [field,spec,center]=genfield(L,D,pix)
close all

%Generate speckle pattern of size L. Speckle size will be 2L/D.
```
Appendix A. Simulating speckle patterns

```matlab
field=zeros(L,L);  
%generate empty grid

downsamp=1;
if(D>L-2) %scale down for undersampled patterns
    D2=D;
    D=L-2;
    downsamp=D2/D;
end

center=randi([D/2+1 L-D/2],2,1);  
%Select random center for aperture

%Generate aperture with random phase elements
for i=(-D/2:pix:D/2)+center(1)
    ang=asin((i-center(1))/(D+2)*2);
    start=int16(D/2*cos(pi-ang));
    fin=int16(D/2*cos(ang));
    for j=(start:pix:fin)+center(2)
        phase=2*pi*(rand(1)-0.5);
        field(i:i+pix-1,j:j+pix-1)=exp(1i*phase);
    end
end

figure
imshow(angle(field),pi*[−1 1]);  
%display grid

%Calculate speckle pattern. Interpolate if pattern is undersampled.
spec=fftsift(fft2(field));
spec=spec.*conj(spec)./L^2;
[XI,YI]=meshgrid(1:downsamp:L);
spec=interp2(spec,XI,YI);
[XI,YI]=meshgrid(1:1/downsamp:L);
spec=interp2(spec,XI,YI);
```
spec=spec(1:L,1:L)/max(spec(:));

figure
imshow(spec,[0 max(max(spec))]) %display speckle pattern

%Calculate and display probability distribution
[N,X]=hist(spec(:),1000);
figure
N=N/length(spec(:))/(X(2)-X(1));
semilogy(X,N);
xlabel('Intensity','FontSize',16)
ylabel('Probability Density','FontSize',16)

%Plot exponential distribution with same mean
Dist=exp(-X/mean(spec(:)));
Dist=Dist/sum(Dist(:))/(X(2)-X(1));
hold on;
plot(X,Dist,'r');
sum(N*(X(2)-X(1)))
sum(Dist.*(X(2)-X(1)))
Figure A.1: Process of simulating speckle pattern. (a) Generate circular field of random phase elements. (b) Fourier transform gives speckle pattern, with speckle sampling rate $2L/D$. 
Appendix B

Fitting multiexposure speckle models

This section contains the MATLAB functions used to produce several speckle model functions, as well as the fitting program used on multiexposure images. The original speckle model as described by Fercher and Briers in not included as more recent corrections show it to be invalid.

Listing B.1: Corrected weighting speckle model, from Bandyopadhyay [55]

```matlab
function yhat = multispec1(beta,x)
%non–static scattering speckle model, from Bandyopadhyay, including noise term

tc=beta(1);
B=beta(2);
%vs=beta(3);
t=x;
yhat=sqrt(B.*(exp(-2.*t./tc)-1+2.*t./tc)./(2.*t./tc).^2));
```
Listing B.2: Speckle model with static scatterers, from Parthasarathy [4]

```matlab
function yhat = multispec3(beta, t)

tc = beta(1);
p = beta(2);
B = beta(3);
vs = beta(4);

yhat = sqrt(B*p^2.*(exp(-2.*t./tc)-1+2.*t./tc)./(2*(t./tc).^2)+4*B*p*(1-p)*
        .*(exp(-t./tc)-1+t./tc)./(t/tc).^2+vs);
```

Listing B.3: Modified static scatterer model, with noise removed

```matlab
function yhat = multispec2(beta, t)

%non-static scattering speckle model, from Parthasarathy, including noise
%term

tc = beta(1);
p = beta(2);
B = beta(3);

yhat = sqrt(B*p^2.*(exp(-2.*t./tc)-1+2.*t./tc)./(2*(t./tc).^2)+4*B*p*(1-p)*
        .*(exp(-t./tc)-1+t./tc)./(t/tc).^2+B*(1-p)^2);
```

Listing B.4: Script used for fitting speckle model

```matlab
% Exposure times in us

times = [20 50 70 100 200 300 500 700 1000 2000 3000 5000 7000 10000 15000
         20000];
```
IM=0;
fone=[1];  \%starting frame for fit

close all

for k=1:1
    IM=0;
    t=times;
    cd(path);

    \%Read images into array
    for(i=1:length(t))
        temp=imread(sprintf('%dus.tif',t(i)));
        a=size(temp);
        IM(i,1:a(1),1:a(2))=temp;
    end

    \%Time values in seconds
    t=t*1e-6;

    \%options for fits
    options=optimset('FunValCheck','off','Diagnostics','off','MaxIter'=>70,'LargeScale','on','Display','off');
    options2=statset('FunValCheck','off','MaxIter',70);
    warning off;

    \%Set first frame
    init=fone(k);

    \%Run initial fit on each pixel, omit 10 pixel boundary
    for i=10:a(1)
        [i 1]
for j=10:a(2)
    fits=nlinfit(t(init:end),IM(init:end,i,j)',@multispec1←[0.0001 0.2^2],options2);
    beta(i,j)=fits(2);
    tc(i,j)=fits(1);
end
imshow(beta,[0 0.05])
drawnow;
end
close
%bmean=mean(mean(beta(15:end,15:end)));

figure
figure
figure

figure
figure
figure

%Run second fit, omit boundary
for i=15:a(1)-5
    [i 2]
    for j=15:a(2)-5
        bmean=mean(mean(beta(i-5:i+5,j-5:j+5)));
        fun=@(beta,t)multispec2([beta bmean],t);
        fits=lsqcurvefit(fun,[0.0001 0.5],t(init:end),IM(init:end,i,j←)
                          ',[0 0], [Inf 1],options);
        beta2(i,j)=bmean;
        tc2(i,j)=fits(1);
        rho(i,j)=fits(2);
    end
figure(1)
imshow(beta2,[0 0.03])
Appendix B. Fitting multiexposure speckle models

Listing B.5: Function displaying experimental values and fit when pixel is clicked

```
function [a]=touchplot(tc2,rho2,beta2,IM,t)
close all

%Display plot of speckle values and model fit for clicked pixel in image

%Make beta matrix, if uniform beta is used
if(size(beta2)==1)
    beta2=ones(size(tc2)).*beta2;
end

y=0;
x=0;

while (1)
    yl=y;  %previous click coordinates
```
Appendix B. Fitting multiexposure speckle models

\[
x_l = x;
\]

\textbf{figure (1)}

\texttt{imshow(1./tc2,[0 40000]);} \%redisplay image

\texttt{[y x]=ginput(1);} \%copy coordinates of click

\texttt{x=int16(x);}

\texttt{y=int16(y);}

\textbf{figure (2)}

\%delete previous plot if double clicked

\texttt{if ((y==y1 && x==x1)||y<5||x<5)}

\hspace{1cm} \texttt{try}

\hspace{2cm} \texttt{delete(hL1);} \\
\hspace{2cm} \texttt{delete(hL2);} \\

\hspace{1cm} \texttt{catch}

\hspace{2cm} \texttt{delete(get(gca,'Children'))} \%delete all plots on triple click

\texttt{end}

\textbf{else}

\hspace{1cm} \%find average values for all times, in 7x7 roi near clicked pixel

\texttt{for (i=1:length(t))}

\hspace{2cm} \texttt{mid(i)=mean(mean(IM(i,x-3:x+3,y-3:y+3)));}

\hspace{2cm} \texttt{dev(i)=mean(std(IM(i,x-3:x+3,y-3:y+3)));}

\texttt{end}

\hspace{1cm} \%hL1=semilogx(t,mid,'-');

\hspace{1cm} \texttt{hL1=errorbar(t,mid,dev,'-');} \%plot experimental values

\hspace{1cm} \texttt{errorbarlogx(0.03);}

\hspace{1cm} \texttt{hold on}

\hspace{1cm} \texttt{hL2=semilogx(t,multispec2([tc2(x,y) rho2(x,y) beta2(x,y)],t←/1000000),[r']);} \%plot fitted model

\hspace{1cm} \texttt{legend(‘Raw Data’,’Model Fit’)
set(get(gcf,'Children'),'FontSize',14);
xlabel('Exposure time (\mu s)','FontSize',14)
ylabel('Contrast','FontSize',14);
end

hold off

real([1/tc2(x,y) rho2(x,y) (beta2(x,y))^-0.5]) \list parameters
end
a=1;
Appendix C

VCSEL control script

The following MATLAB code was used to control the VCSEL using one of 4 operation schemes. SMpulse was used to pulse the VCSEL in single mode, SMoff was used to trigger the SM VCSEL and include a pre-exposure settling time, SWpulse was used to pulse the VCSEL in SW operation, and SWswitch was used to program the alternating SM/SW operation.

Listing C.1: Function displaying experimental values and fit when pixel is clicked

```matlab
k6221 = initialize_gpiib(12);
k7001 = initialize_gpiib(7);
%k6487 = i n i t i a l i z e
\texttt{g p i i b(22)} ;
fopen (k6221);
fprintf(k6221,':\texttt{output}\ \texttt{off}') ; \% Turns the output off.
fprintf(k6221,':\texttt{CLS}') ; \% Turns the output off.
fprintf(k6221,':\texttt{current}\texttt{:range }20\text{e}\texttt{\text{\text{-3}}}') ; \%max output 20 mA
fprintf(k6221,':\texttt{current}\texttt{:compliance }10') ; \%Sets voltage compliance to 10V.
fprintf(k6221,':\texttt{system}\texttt{:preset}') ;
fprintf(k6221,':\texttt{status}\texttt{:preset}') ;
```
fopen (k7001);
fprintf (k7001, ':abort;:open all'); %open all switches
fprintf (k7001, '*CLS');
fprintf (k7001, ':system:reset');
fprintf (k7001, ':status:reset');

def

freq=62; %frequency of camera frame rate, for 500x500 roi

%Switching scheme options. Each described below
SMpuls=0;
SMoff=1;
SWpuls=0;
SWswitch=0;

%SM pulsing. Camera trigger turns on laser for set duration
if (SMpuls)
    offset=0e-3;
    ampl=1.1e-3 %single mode amplitude, in amps
    fwav=100e3; %wave period lasts 10 us
    nwav=500; %pulse time in 10 us
    ival=0;
    wav=ones(1,100);
    wavstr=sprintf('%d,',wav);
    fprintf(k6221, strcat('SOUR:WAVE:ARB:DATA ',wavstr(1:end-1)));
end

%SM off trigger mode. In this mode, laser default is on. Trigger will ↔ turn
%laser off after set delay, keep off until 200 us before next frame. This
%allows laser warmup to occur before frame begins
if (SMoff)
Appendix C. VCSEL control script

%camera frame rate
freq = 61.8;
dt = le5/freq; %camera period, times 10 us

%waveform period ends 200 us before next frame

%delay before shut off, times 10 us
ontime = 100;

offset = 0e-3;
ampl = 1.20e-3; %single mode amplitude

fwav = le5/dt; %wave frequency based on above period
ival = 1;

wav = zeros(1, dt); %each point of waveform lasts 10 us
wav(1:ontime) = 1;

fprintf(k6221, sprintf('SOUR:WAVE:ARB:DATA %d', int16(wav(1))));
for i = 1:(dt-1)/100+1
    wavstr = sprintf('%d', wav((i-1)*100+1:min(i*100+1, dt)));
    fprintf(k6221, strcat('SOUR:WAVE:ARB:APPEND ', wavstr(1:end-1)));
end

%Camera trigger is used to pulse sweep mode, for set number of ↔
%oscillations

if(SWpulse)
    offset = 5e-3; %sweep offset
    ampl = 5e-3 %sweep amplitude
    fwav = 10e3; %each SW cycle lasts 100 us
    ival = -1; %return to 0 current after sweeps
    x = 0:49;
    wav = double(int16(sin(x*2*pi/50)*20))/20*0.8;
    wavstr = sprintf('%1f', wav);
end
Appendix C. VCSEL control script

```matlab
fprintf(k6221,strcat('SOUR:WAVE:ARB:DATA ',wavstr(1:end-1)));
nwav=4;  \%total pulse time, times 100 us
end

\%trigger starts SW/SM switching. On trigger, current source emits SW
\%pulse for set time, the switches to SM while the next trigger is ignored←
\%SW680 pulses shorter than exposure time, due to higher camera ←
    sensitivity
if(SWswitch)
    offset=5e-3;
    ampl=5e-3;
    twav=double(int16(1000/(freq*0.9)));
    f wav=1000/twav;
    nwav=1;
    ival=-0.7
    t=4; \%SW pulse time in ms
exp=10; \%exposure time

x=0:49;
wav=double(int16(sin(x*2*pi/50)*20)/20*0.8);
wavstr=sprintf(' \%.1f ,',wav);

fprintf(k6221,strcat('SOUR:WAVE:ARB:DATA ',wavstr(1:end-1)));
for(i=2:t)
    fprintf(k6221,strcat('SOUR:WAVE:ARB:APPEND ',wavstr(1:end-1)));
end

wavstr=sprintf(' \%.1f ,0*wav-1);
for(i=t+1:exp+2)
    fprintf(k6221,strcat('SOUR:WAVE:ARB:APPEND ',wavstr(1:end-1)));
end
```
end

wavstr=sprintf('%.1f',0*wav-0.8);
for(i=exp+3:twav)
    fprintf(k6221, strcat('SOUR:WAVE:ARB:APPEND ',wavstr(1:end-1)));
end
plot(wav,'."
end

fprintf(k6221,'SOUR:WAVE:ARB:COPY 1'); %program waveform
fprintf(k6221,'SOUR:WAVE:FUNC ARB1'); %select waveform
fprintf(k6221,sprintf('SOUR:WAVE:FREQ %f',fwav));
fprintf(k6221,sprintf('SOUR:WAVE:OFFS %f',offset));
fprintf(k6221,sprintf('SOUR:WAVE:AMPL %f',ampl));
fprintf(k6221,sprintf('SOUR:WAVE:DUR:CYCL %d',nwav));
fprintf(k6221,'SOUR:WAVE:EXTRIG on'); %allow external trigger
fprintf(k6221,'SOUR:WAVE:PMAR:OLIN 1'); %set phase marker line
fprintf(k6221,'SOUR:WAVE:EXTRIG:ILINE 3'); %line 3 to listen for trigger
fprintf(k6221,'SOUR:WAVE:EXTRIG:IGNORE ON'); %ignore triggers while on
fprintf(k6221,sprintf('SOUR:WAVE:EXTRIG:IVAL %f',ival)); %default value
fprintf(k6221,'SOUR:WAVE:ARM');
fprintf(k6221,'SOUR:WAVE:init');

% single laser only
fprintf(k7001,':close (@ 2!5)');

%%alternating, use for SWswitch with 3 wavelengths
fprintf(k7001,':trigger:source ext');
fprintf(k7001,':scan (@ 2!5,2!5,1!1,1!2,1!5)'); % last is dummy
%fprintf(k7001, ':initiate ');
%fclose(k6221);
fclose(k7001);
Appendix D

ASAP simulation

ASAP is a software package used to model illumination and imaging. In order to estimate optical path lengths in neural tissue, we ran a Monte Carlo simulation in ASAP. The results were used to calculate the mean and the standard deviation of the optical path length. The mean was used for the path length correction factor in IOS Hb concentration calculations, and the standard deviation was used to estimate the reduction of speckle contrast associated with SW operation.

The simulation results give a mean path length of 11.5 mm, and a standard deviation of 8.7 mm.

Listing D.1: ASAP Monte Carlo simulation code

******************************************************************************

SYSTEM NEW
RESET
UNITS MM
WAVELENGTH 675.50 NM
PIXELS 501
DEFINE RAY SPLIT AND SCATTER CHARACTERISTICS

SPLIT 1500 MONTECARLO
LEVEL 1000
FRESNEL AVE

!!Tissue properties: g, u_s', u_a (both in cm⁻¹)

MODEL
VOLUME 0.898 10⁻0.1 1

MEDIA
1.4 SCATTER 1 'TISSUE'
2.5 'GLASS'
1 'AIR'
2.5 'DETECTOR'

COATING PROPERTIES
0 0 'ABSORB'

************************************************************GEOMETRY←

**********************************************SWID=30
SKIN=10
THICK=25

************************************************************TISSUE INTERFACE*************************************

!!TISSUE BOUNDARIES

SURFACE
PLANE Z (SKIN) ELLIPSE (SWID) (SWID)
OBJECT 'TISSUE.Z1'
INTERFACE COATING BARE AIR TISSUE
APPENDIX D. ASAP SIMULATION

REDEFINE COLOR 14

SURFACE

PLANE −Z (SKIN)+(THICK) ELLIPSE (SWID) (SWID)
OBJECT 'TISSUE.Z2'
INTERFACE COATING BARE AIR TISSUE
REDEFINE COLOR 14

SURFACE

TUBE Z (SKIN) (SWID) (SWID) (SKIN)+(THICK) (SWID) (SWID)
OBJECT 'TISSUE.EDGE'
INTERFACE COATING BARE AIR TISSUE
REDEFINE COLOR 14

!!Detector surface

SURFACE

PLANE Z 54 ELLIPSE 15 15
OBJECT 'DETECTOR'
INTERFACE COATING ABSORB AIR AIR
REDEFINE COLOR 12

!!Light source

GRID ELLIPTIC Z 54 −24 −8 −8 8 100 100
SOURCE DIR 0.8 0 −1
FLUX TOTAL 50

BEAMS INCOHERENT GEOMETRIC

WINDOW X Z

PLOT FACETS OVERLAY
Listing D.2: MATLAB analysis of path length data

detector=54; %detector z position, in mm
source=54; %source z position in mm
surf=35;  \textit{tissue surface z position in mm}

a=find(raydata(:,2)==detector);  \textit{only include rays which end on detector}
ray2=raydata(a,:);

len=ray2(:,1);  \textit{path lengths}
zcos=ray2(:,3);  \textit{z direction cosine on detector}
amp=ray2(:,4);  \textit{flux amplitude}
inlen=sqrt(1^2+0.8^2)*(source-surf);  \textit{path component before tissue}
outlen=(source-surf)./zcos;  \textit{path component after tissue}
len=len-inlen-outlen;  \textit{path length only in tissue}

nums=zeros(1,1000);

for(i=1:1000)  \textit{sort and bin path lengths}
  nums(i)=0;
  binnew=min(len)+(i)/1000*(max(len)-min(len));
  binold=min(len)+(i-1)/1000*(max(len)-min(len));
  binmean(i)=(binnew+binold)/2;
  for(j=1:length(len))
    if(len(j)>=binold && len(j) < binnew)
      nums(i)=nums(i)+amp(j);
    end
  end
end

plot(binmean,nums);

nums=nums./sum(nums);  \textit{normalize flux}
avg=sum(nums.*binmean);  \textit{calculate mean path}
S=sqrt(sum(nums.*(binmean-avg).^2));  \textit{calculate standard deviation}
Appendix D. ASAP simulation

Figure D.1: Ray trace of Monte Carlo simulation of tissue illumination

Figure D.2: Optical path length distribution of ray trace simulation