Photoacoustic Imaging for Characterizing Blood Clots and Demyelinated Structures in the Brain

by

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Abstract

Photoacoustic imaging (PAI) is a relatively new imaging modality with high spatio-temporal resolution, suitable for functional imaging. This work examines the potential of PAI for studying diseases within the brain such as stroke and multiple sclerosis. Blood samples imaged on two different photoacoustic systems were analysed though statistical measures in gray level co-occurrence matrix (GLCM), allowing for the classification of clotted and unclotted blood with high sensitivity and specificity (>95%). Using chromophore spectral demixing, lipid-rich myelin was mapped and examined in rodent models of demyelination. We demonstrated focal oxygen deprivation associated with a rodent model of photothrombotic stroke. GLCM statistics independently showed characteristics of blood clotting in these hypoxic regions. Our results support PAI as an investigative tool for studying rodent models of stroke and MS.
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Chapter 1

Background

1.1 Photoacoustic Imaging Principles

1.1.1 Photoacoustic Effect

The photoacoustic effect is the emission of sound waves from a substance following the absorption of light. Several different mechanisms that produce the photoacoustic effect, but the primary mechanism of concern is the photothermal mechanism (other mechanisms include change in molecular organization and change in material phase). In the photothermal mechanism there are three principle steps: (1) the pulsed radiation is converted into thermal energy, (2) the intermittent laser pulses cause sequential heating and cooling of the tissue, resulting in expansion and contraction of the tissue, (3) the alternating thermoelastic expansion and contraction results in the emission of acoustic waves. These three principal steps are shown in Fig. 1.1, as well as a forth panel showing the signal transduction and image reconstruction. The volumetric expansion following laser excitation can be expressed in

![Figure 1.1: Depiction of PA principal.](image)
the following equation (1.1) [15]

\[
\frac{dV}{V} = -\kappa p(\vec{r}) + \beta T(\vec{r})
\]  

(1.1)

Where \(\frac{dV}{V}\) is the local fractional volume expansion, \(\kappa\) is the isothermal compressibility, \(\beta\) is the thermal coefficient of volume expansion, \(p(\vec{r})\) is the change in pressure and \(T(\vec{r})\) is the change in temperature.

The thermal relaxation time, which represents the time required for heat to conduct away from a directly-heated tissue region, can be described by the following equation: [15]

\[
\tau_{th} = \frac{d_c^2}{4D_T}
\]  

(1.2)

Where \(\tau_{th}\) is the thermal relaxation time, \(d_c^2\) is the desired spatial resolution and \(D_T\) is the thermal diffusivity, (in soft tissue, \(D_T = 0.14\text{mm}^2/\text{s}\)). [16]

Due to the short duration of laser pulses \(\tau\) (several nanoseconds) compared to the thermal relaxation time, thermal diffusion away from the illuminated tissue can be neglected. This condition is represented in the following equation:

\[
\tau \ll \tau_{th} = \frac{d_c^2}{4D_T}
\]  

(1.3)

Similarly, due to the short laser pulse duration relative to the stress confinement, volume expansion within the pulse duration can be neglected so long as the following condition is met:

\[
\tau \ll \tau_{st} = \frac{d_c}{v_s}
\]  

(1.4)

Where \(\tau_{st}\) is the \(d_c\) is the desired spatial resolution, \(v_s\) is the speed of sound (approximately 1540 \(\text{m}/\text{s}\) in soft tissue). [15]

Using a spatial resolution of 100\(\mu\text{m}\), the thermal relaxation time can be calculated to be 17.9\(\mu\text{s}\), and the stress relaxation time can be calculated to be 65\(\text{ns}\). Since both of these values are significantly larger than the pulse time for the excitation laser (10\(\text{ns}\)), both thermal and stress confinement can be assumed, meaning
there is no significant diffusion of heat to surrounding tissues, and volume expansion is negligible. Thus Eq.(1.1) can be rewritten as:

\[ p_0(\vec{r}) = \frac{\beta T(\vec{r})}{\kappa} \]  

(1.5)

Using values for \( \kappa \) of \( 5 \times 10^{-10} \text{ Pa}^{-1} \) and \( \beta \) of \( 4 \times 10^{-4} \text{ K}^{-1} \) within soft tissue, the relationship from change in temperature to change in pressure can be calculated as an 800 kPa increase per degree K. The temperature rise (T) can be expressed in terms of optical absorption:

\[ T = \frac{A_e}{\rho C_V} \]  

(1.6)

Where \( A_e \) is the absorbed energy density, \( \rho \) is the mass density, and \( C_V \) is the specific heat capacity at constant volume. [15] Combining Eq.(1.5) and Eq.(1.6), we obtain:

\[ p_0(\vec{r}) = \frac{\beta A_e}{\rho C_V \kappa} \]  

(1.7)

The absorbed energy density can be written as the product of the absorption coefficient \( \mu_a \) and the local optical fluence \( F(\vec{r}) \) \([\text{J} / \text{cm}^2]\).

\[ A_e = \mu_a F(\vec{r}) \]  

(1.8)

Using the Grueneisen parameter \( \Gamma(\vec{r}) = \frac{\beta}{\rho C_V \kappa} \) [15] and substituting into Eq.(1.7) we obtain:

\[ p_0(\vec{r}) = \frac{\beta A_e}{\rho C_V \kappa} = \Gamma \mu_a F(\vec{r}) \]  

(1.9)

Thus, if the local fluence is known, the absorption coefficient of the tissue can be determined based on the resulting pressure change. The generated pressure creates a wavefront with a bipolar shape, compressed on the inner side of the sample, and expanded on the outward side. The photoacoustic pressure is ultimately detected by an ultrasound transducer array. Using the signal timing and strength recorded in the transducer array, the pressure signal, and ultimately the tissue absorption coefficients, can be determined.
1.1.2 Image Reconstruction and Formation

There are two main methods of photoacoustic acquisition and image formation: (1) direct image formation and (2) reconstruction image formation. Direct image formation is done using a single ultrasound transducer moved mechanically along a path; this is mainly used in photoacoustic microscopy (PAM). Reconstruction image formation is done using an array of ultrasound transducers, and is used in photoacoustic computed tomography (PACT). An example schematic for a whole body, ring shaped PACT system, aimed for imaging of small animals is shown in Fig. 1.2. While exclusively PACT systems were used within this work, PAM is discussed briefly to provide a comparison to PACT.

Figure 1.2: Schematic of cylindrical PACT system designed for small animal imaging. Taken from: [1].
In PAM, the image is constructed pixel by pixel using the signal individually from each spatial step of the transducer. There are two main subdivisions of PAM: optical resolution PAM (OR-PAM) and acoustic-resolution PAM (AR-PAM), which are differentiated based on the focusing of the laser. In optical resolution-PAM, the laser is tightly focused, and the lateral resolution of the image is determined by the optical focal spot size. In acoustic resolution-PAM, the laser light is more diffuse and the lateral resolution is mainly determined by the ultrasound focusing. [15] A comparison of OR-PAM and AR-PAM can be seen in Fig. 1.3 where OR-PAM demonstrates superior resolution, while AR-PAM show superior penetration depth (shown by arrows).

![OR-PAM and AR-PAM images of vasculature within a mouse ear](image)

Figure 1.3: OR-PAM and AR-PAM images of vasculature within a mouse ear. Taken from: [2].

In PACT, the acoustic focus is wider (larger acceptance angle), and the data is reconstructed through merging data from all elements of the array from each point on the image. The photoacoustic signal for an ideal transducer can be represented in the following equation:

\[
p_d(\vec{r}, t) = \frac{\partial}{\partial t} \left[ \frac{t}{4\pi} \int_{|\vec{r}_d - \vec{r}| = v_s t} p_0(\vec{r}) d\Omega \right]
\]

(1.10)

Where: \( p_d(\vec{r}, t) \) is the pressure at the detector, \( t \) is time (seconds), \( \vec{r}_d \) is the position of the detector, the source is at point \( \vec{r} \), \( d\Omega \) is the solid-angle element of \( \vec{r} \) with respect to the point at \( \vec{r}_d \), and \( v_s \) is the speed of sound. The universal back-
projection (UBP) formula can be obtained through inverting the previous equations to solve for pressure \( p_0(\vec{r}) \). [15]

\[
p_0(\vec{r}) = \frac{1}{\Omega_0} \int_S \left[ 2p_d(\vec{r}_d, t) - 2t \frac{\partial p_d(\vec{r}_d, t)}{\partial t} \right] \bigg|_{t=|\vec{r}_d-\vec{r}|/C} \]

(1.11)

Where: \( S \) is the detection surface, and \( \Omega_0 \) is the solid angle of the whole surface \( S \) with respect to a given source at point \( \vec{r} \). The UBP formula integrates along each detector with respect to the source, and uses the weighting factor \( d\Omega_0/\Omega_0 \) for each back projection. The back-projection method can be used for spherical, cylindrical or planar geometries. For other arbitrary geometries, other back-projection methods can be used such as the time-reversal (TR) method. [15]

As well as geometric limitations for the UBP formula, there are other modifications and limitations to consider. The formula makes three main assumptions: (1) transducers obtain signal at a single point, (2) the acceptance angle of the transducer is wide, (3) the transducers have an infinite acoustic bandwidth. Since none of these assumptions are perfectly true, determining and correcting for the system characteristics can enable a more accurate back projection method. Significant research had been done in model-based photoacoustic imaging inversions, such as work done by Ntziachristos et al. [17]. They observed that regular reconstruction methods resulted in image artifacts and loss of quantification accuracy in PA images that used partial projection data from limited-view systems (where the physical layout of the system prevented data from being acquired at certain angles). Using an interpolated-matrix-model with direct and iterative regularization methods, the accuracy of image reconstructions was enhanced and artifacts were eliminated.

Additionally, in many scenarios the entire acoustic wave can not easily be recorded. In breast tissue imaging, only a hemisphere around the tissue is easily accessible for recording. This limited access to the signal can produce blurred boundaries, or streaking in the case of insufficient sampling. A method of correcting for this is to use acoustic reflectors to redirect signal back towards the detector array.
1.1.3 Resolution of Photoacoustic Images

Resolution is another key consideration. Axial resolution is primarily determined by the bandwidth of the ultrasound transducer, while lateral resolution is determined both by the bandwidth of the transducers as well as their acceptance angles. The axial resolution is constant across the image, and can be approximated in the following equation [15]:

\[ a = 0.6\lambda_c \] (1.12)

Where \( a \) is the axial resolution, and \( \lambda_c \) is wavelength at the high frequency cutoff of the ultrasound transducers. This approximation holds true for axial, lateral, and cylindrical geometries. The lateral resolution for spherical and cylindrical geometries can be represented in the following equation [18]:

\[ l = \sqrt{a^2 + \left(\frac{r}{r_0}d\right)^2} \] (1.13)

Where \( l \) is the lateral resolution, \( a \) is the axial resolution, \( r \) is the distance between the imaging point and the scanning center, \( r_0 \) is the radius of the scan circle, and \( d \) is the width of each transducer element. In a planar geometry, the equation simplifies to [18]:

\[ l = \sqrt{a^2 + d^2} \] (1.14)

1.1.4 Depth-Resolution Trade-Off and Multi-Scale PAT

Within most optical imaging modalities, there is a trade-off between imaging depth and image resolution. In photoacoustics, this trade-off also exists, but one distinct potential advantage of PA imaging is the ability to adjust the trade-off between the spatial resolution and imaging depth, and switch between high-depth imaging and high-resolution imaging using the same system setup. This adjustment can be done both on the transmit optical side and on the receive ultrasound side. On the ultrasound side, targeting higher frequencies will result in a higher resolution, but
lower penetration depth. Typically, the readout ultrasound is tuned between 30 MHz to 1 MHz, which allows for an axial resolution between 50 µm to 1500 µm. In cases where the resolution is determined by the optical side, as in OR-PAM, the numerical aperture (NA) of the laser’s objective lens can be used to adjust the resolution-imaging depth trade-off. For example, a system with a numerical aperture of 0.1, would have a 2.6 µm lateral resolution with a 1.2mm imaging depth; increasing the NA to 1.0 would result in a ten-fold improvement to the lateral resolution: 0.26 µm and a ten-fold decrease to imaging depth: 120µm. [19]

A study performed on a mouse with a subcutaneous tumor, using LacZ as a reporter gene demonstrated the versatility of PAT. In the study, the system was able to image and visualize LacZ at a depth of 5cm (placing chicken breast tissue over the tumor). Removing the tissue, and imaging at a lower imaging depth, permitted a spatial resolution of 0.4 µm, such that the LacZ cell structure could be resolved. [3] This study proved that both high resolution and high imaging depth images could be obtained using the same system. In Fig. 1.4, we can observe images of the same mouse specimen demonstrating the capability for either high imaging depth or high imaging resolution.

![Figure 1.4: Image of LacZ cells (green) in a mouse shown at: (a) high imaging depth overlaying chicken breast tissue. (b) high imaging resolution- 3D depiction of blood vessels and tumor. Taken from: [3].](image)

Currently, the maximum imaging depth demonstrated using a photoacoustic system is 8.4cm through chicken breast tissue. [20] [21] The imaging depth of PAT
is expected to increase to tens of centimeters with advances in wave-front engineering to minimize optical scattering (and hence signal attenuation). [22]

1.1.5 Super-resolution PAT

The optical diffraction limit of 250nm makes obtaining images of cellular and subcellular structures challenging. A variety of different methods have been used to obtain super resolution images using photoacoustic systems. One approach uses fluorescing molecules and the photobleaching effect. Photobleaching occurs when molecules at the center of the target region are exposed to higher intensity illumination will be photochemically altered and unable to fluoresce, leaving signal from only the molecules around the periphery. Examining the differential image from before and after the photobleaching allows for the center targets to be isolated, which effectively creates a smaller spot size and hence higher resolution. Images obtained of gold nanoparticles have been obtained with pixel resolutions of approximately 80nm, close to three times the diffraction limit. [23]

1.2 Motivation: Imaging and Treatment of Neurological Disease

Neurological disorders as a whole represent a significant cost, both in quality of life and monetarily, to Canadians. The versatility of photoacoustic imaging and its ability to pick up, isolate and analyse signal from biologically relevant chromophores, such as oxygenated hemoglobin, deoxygenated-hemoglobin, water and lipid (a surrogate for myelin), makes it broadly applicable to many different neurological diseases. The principal neurological diseases examined in this thesis will be stroke, epilepsy, and multiple sclerosis.

1.2.1 Burden of Stroke

Stroke is characterized by abnormal blood flow within a region of the brain and the sequential death of cells. Strokes can be classified under two main sub-classifications:
hemorrhagic (20% of cases in Canada) - where a ruptured blood vessel results in blood leaking into brain tissue, and ischemic (80% of cases) - where blockages to blood vessels result in reduced flow or no flow to a region of the brain. A diagram of hemorrhagic and ischemic strokes can be seen in Fig. 1.5.

![Hemorrhagic and Ischemic Stroke](image)

Figure 1.5: Hemorrhagic and Ischemic Stroke. Taken from: [4].

Globally, strokes account for the 6.3 million deaths each year, second only to coronary artery disease (2015). While the annual global incidence of ischemic stroke is far higher (6.9 million) compared to hemorrhagic stroke (3.4 million), due to the higher mortality of hemorrhagic stroke the resultant number of deaths from ischemic (3.3 million) and hemorrhagic (3.0 million) stroke are comparable.[24]

In Canada, the incidence of stroke is approximately 50,000, and the mortality rate associated with stroke is close to 14,000. While the Canadian healthcare system is much better than the global average, the one-year survival rate of stroke is still only 78%. [25] In addition to the deaths caused by stroke, many survivors will be left with lasting disability. The annual direct costs of stroke in Canada totals $664.9 million; this includes hospital care, physician care and treatment costs. The indirect costs total $2.1 billion which includes lost wages and decreased productivity. This gives a combined cost for strokes of $2.8 billion per year in Canada.[26].

Treatments for ischemic stroke that aid in thrombolysis (breaking up the clot), such as tissue plasminogen activator (tPA), aid in restoring blood flow and saving brain tissue in affected areas. However, thrombolysis treatments tend to be time sensitive; if given within three hours of the stroke onset, it is shown to result in a
significant decrease (10%) to the rate of post-stroke disability. However, if given between three and four and a half hours, the effect are less apparent. Drugs such as tPA, which aid in the break down of clots can also cause bleeding elsewhere in the brain- which increases mortality from intracranial haemorrhaging. Beyond four and a half hours, treatment with tPA is not recommended. [27]

Decisive therapeutic action, which requires efficient and accurate imaging, is of vital importance to treating stroke and reducing risk of permanent disability. Photoacoustic imaging presents a promising alternative to currently used brain imaging techniques - magnetic resonance imaging (MRI) or computed tomography (CT)- for diagnosis, therapeutic planning and treatment guidance.

1.2.2 Stroke and Epilepsy in Neonates

The risk factors, pathophysiology, and treatment for strokes within neonates are fundamentally different than that of adults. Many of the imaging and treatment options developed for adults are unsuitable for premature neonates, making it an under-serviced vulnerable group. Traditionally, stroke within neonates has been viewed as difficult or impossible to treat, so it has received comparatively little attention. While risk factors for stroke in adults are well established such as smoking, age, and diabetes, these risk factors are generally inapplicable to neonates. Adults are more likely to suffer from stoke due to chronic events such as the slow build up of plaque and thrombotic or plaque emboli. Premature infants are also much more likely to suffer from certain types of cardiovascular disease such as: periventricular leukomalacia, and germinal matrix hemorrhage. [28] Periventricular leukomalacia is necrosis and coagulation of white matter near the lateral ventricles, due to hypoxia often from thin walled fetal blood vessels that are unable to provide sufficient blood flow, or damage in the blood brain barrier (BBB). Germinal matrix hemorrhage is the hemorrhage of cerebral blood flow into the germinal matrix- the site of proliferating neuronal and glial precursors. Within premature neonates, blood vessels walls within the germinal matrix are very thin and cerebral blood flow is poorly regulated, this area is at high risk to have a higher blood pressure which is a risk factor for
hemorrhaging. Consequentially, the percentage of hemorrhagic stroke compared to ischemic stroke is far higher within the neonate population (45% hemorrhagic) than in the adult population (20% hemorrhagic) [29].

The relatively large number of different etiologies in paediatric populations complicates stroke diagnosis. This decreases the frequency at which a final diagnosis is made within the first critical three hours where thrombolysis treatments are viable and beneficial. Among survivors of the initial stroke, the rate of recurrence is significant, ranging between 6% and 30% depending on the etiology. Recurrence will happen within the first six months in the vast majority of cases. Reducing this risk can be done through antiplatelet agents, such as aspirin and dipyridamole; a daily dose (3 to 5 mg/kg/day) is suggested to have minimal side effects and reduce early recurrence of ischemic stroke.

Beyond the risk of recurrence, among children surviving strokes there is still a huge cost to quality of life; approximate 60% will face long-term neurological deficits such as epilepsy, cerebral palsy, or forms of cognitive and sensory impairment [30].

Due to the current limitations of photoacoustic imaging—limited imaging depth, attenuation though thicker adults skulls, it is not ideally suited for diagnosis of adult strokes and associated therapeutic planing. However, neonates have soft-spots in their unfused cranial sutures, which make for ideal imaging windows. Additionally, neonates also have much smaller skull circumferences 35cm, giving a radius of 5.5cm, well within current recorded maximum imaging depth of photoacoustics of 8.4cm, and certainly within the theoretical maximum [15].

1.2.3 Multiple Sclerosis and Other Forms of Demyelination

Multiple sclerosis (MS) is the most common of the demyelinating disease, where damage occurs to the myelin sheaths around axons or the cells that form the myelin. Demyelination can occur for several different reasons: viral infections, acquired metabolic derangements, hypoxic-ischaemia, and focal compressions. Within multiple sclerosis, the underlying mechanisms can include the destruction of myelin sheaths by the body’s immune system (inflammatory demyelination), or the failure
of myelin producing cells. These processes are often attributed to genetics, while a viral infection can act as a trigger. In other types of demyelination, such as progressive multifocal leukoencephalopathy (PML), a viral infection is the primary cause: the papovavirus, JC virus. Other types of viral-associated demyelination include: acute-disseminated encephalomyelitis (ADEM) - a T cell hypersensitivity reaction to an infection (usually viral), most common in children, and acute haemorrhagic leukoencephalitis - a hyperacute variant of ADEM: rare and usually fatal. Examples of acquired metabolic demyelination central pontine myelinolysis (CPM) and extrapontine myelinolysis, which are a demyelination of the pons and lower midbrain, commonly associated with an alcoholic liver. In cases where the brain cells are not fully killed due to hypoxia, often the oligodendrocytes (responsible for myelination) are killed, eventually resulting in the local brain tissue becoming demyelinated. Finally, while very rare, demyelination can also occur due to physical compression, often of the trigeminal nerve root fibres due to an overlying artery or vein [31].

Regardless of origins, demyelination results in the loss of signal transduction along the axon of the nerve cell, as seen in Fig. 1.6. Common symptoms resulting from this are: double vision, blindness in one eye, muscular weakness, difficulty with coordination or impaired physical sensation. These symptoms can occur as isolated attacks (relapsing forms) or as a build up over time (regressive forms); the life expectancy for patients affected by MS is 5 to 10 years lower than the unaffected population [32].

Multiple sclerosis affects approximately 2.3 million people world-wide. The prevalence of MS varies dramatically geographically from below 5/100,000 in areas of Africa, South America and Asia to over 100/100,000 in areas of Scotland, Scandinavia and Canada. This variance is likely due to environmental factors present in different regions. Canada has the highest rate of MS in the world with an estimated 100,000 patients in Canada with MS; a prevalence of over 275/100,000. This makes the study of MS within Canada a particularly applicable and important issue. [26]

Currently MS along and most other types of demyelation are currently imaged using MRI. However, work done in the body of this thesis suggests that photoacous-
Figure 1.6: Normal functioning of healthy nerve fibre (top) and damaged signal transduction due to disrupted connection from demyelination. Taken from: [5].

Photoacoustic imaging can measure fat content. Since myelinated tissue has a much higher fat content compared to demyelinated tissue, through this, PAI can be used to measure demyelination. This suggests that PAI could be an excellent, inexpensive alternative for research involving MS and other forms of demyelination.

1.2.4 Comparison of Imaging Modalities

In order to fully examine the validity and advantages of pursuing photoacoustics as an imaging modality, it is important to examine what advantages and disadvantages classical imaging modalities possess. This comparison can act to inform where photoacoustic imaging is applicable and what capabilities it possess that could lead to novel advances.

As discussed previously, photoacoustic imaging has a maximum inherent spatial resolution of approximately 250nm based on the optical diffraction limit. While this limit can be exceeded using super-resolution methods, these methods only increase the resolution by a factor of two or three, to a resolution of 80nm. Additionally, on a typical photoacoustic system, the maximum spatial resolution will have to be sacrificed for imaging depth, giving a more realistic resolution on the order of $100\mu$m.
Methods with higher resolution exist, such as two photon microscopy, which can expect spatial resolution on the order of tens of nanometres, but tend to have extremely low penetration depths. A comparison of different optical imaging modalities and their corresponding spatial and temporal resolution at different imaging depths can be seen in 1.7.

![Figure 1.7: Spatial and temporal resolution of optical imaging modalities. This figure was reprinted from [6].](image)

Other high resolution imaging modalities typically used for imaging of the brain include magnetic resonance imaging (MRI), X-ray computed tomography and ultrasound. Both MRI and CT scanners are prohibitively expensive and remain inaccessible for many hospitals, particularly in rural locations. MRI requires the patient to remain immobile for long periods of time, which is especially problematic in pediatric patients as this can only be achieved through the use of sedation, which is a high risk for neonates. With CT scans, ionizing radiation is used, which is harmful to the infant. Both MRI and CT also lack the capability of being used within a handheld scanner for point of care access, which is problematic for imaging of premature neonates within incubators. Ultrasound is excellent for viewing the gross anatomy of the brain and evaluating soft tissues, but is limited in its ability perceive functional
changes to the brain, essential in characterizing stroke and post-stroke effects, such as
epileptogenesis (the development of epilepsy from a normal brain).

A distinct advantage of photoacoustic imaging is its ability to perform functional
brain imaging, tracking concentrations of chromophores, such as oxy-hemoglobin,
deoxy-hemoglobin, water and lipid. Other functional imaging techniques include:
functional MRI (fMRI), electroencephalography (EEG), magnetoencephalography
(MEG), positron emission tomography (PET), single photon emission computed
tomography (SPECT). Functional MRI (fMRI) which is a widely used technique,
suffers from low spatial resolution (5mm) and temporal resolution (1Hz) as well as
long acquisition times. It is unable to characterize tissue on a cellular level so would
be more limited in its ability to determine blood clot characteristics- an important
part of stroke treatment. MRI systems and hence the ability to perform functional
MRI is also expensive and often not available for certain hospitals. Out of the listed
techniques, only PET and SPECT are able to provide high resolution, and both
of these techniques use radioactive contrast agents, which are more dangerous to
neonates [34, 6].

Unlike most coherent imaging modalities such as pulse-echo ultrasound imaging,
or optical coherence tomography, photoacoustic imaging can produce images free
of speckle noise. Classic speckle theory indicates that speckle is present in signal
where there is interference of coherent waves with randomized phases. While the
central structure within photoacoustic images, much like ultrasound images, will
receive acoustic waves with randomized phases, particles close to the boundaries
will send out waves with approximately equal phase delays. In cases where the
particle density is sufficiently large, the correlated power within the photoacoustic
signal will be much stronger than the uncorrelated power. The strong boundary
buildups tend to be the dominate signal and will suppress the speckle artifacts [35].

The main drawback of photoacoustic imaging (in its current form) is its difficulty
in imaging through dense skull tissue. This limitation means it is best suited for
early pediatric brain imaging (before closure of the fontanel). Within a study of
stroke and demyelinating diseases within neonates, photoacoustics is an excellent
tool when considering all important factors: price, point of care access, spatial resolution, temporal resolution, imaging depth, radioactive exposure, and ability for functional imaging.

### 1.2.5 High Intensity Focused Ultrasound (HIFU)

High Intensity Focused Ultrasound is a method of using ultrasound to thermally lyse/remove tissue in a minimally invasive measure [36]. One of its applications is to lyse clots within blood vessels to restore blood flow after a stroke. [37] The relatively low frequency and high focusing power allows for precise lysis of deep structures with minimal damage to surrounding tissue. An image of the basic HIFU setup can be seen in Fig. 1.8.

![Figure 1.8: Basic principal of HIFU: acoustic pressure resulting in tissue heating and lysis. Taken from: http://hifumx.org/](image)

HIFU thrombolysis has been found to be a feasible method of restoring arterial blood flow (in vivo clots within rabbits), achieving up to a 99.2% clot erosion [37]. The procedure of HIFU clot lysis has been done through MRI guidance, however, imaging artifacts inhibit real time tracking of the clot lysis, which results in poorly optimized treatments where the clot is either under or over treated. The use of PA imaging has shown to be an effective method of localizing clots within a piglet model of intraventricular hemorrhage, which closely simulates the physiology of a human
neonate [38]. Ultimately the goal would be to combine PA imaging technology with HIFU treatments to characterize and treat blood clots in the brain.

1.3 Blood Clot and Stroke Induction

1.3.1 Blood Clot Formation and Composition

It is important to understand what signals should be visible within the photoacoustic signal of clotted, unclotted and lysed blood and how this signal should change over time when comparing acute and chronic clots. An understanding of the biological processes and molecules involved in creating blood clots, and the physical composition of blood clots aids in understanding, interpreting and predicting changes to photoacoustic signals from blood clots. A blood clot or a thrombus is generally formed through hemostasis- a healthy response which prevents bleeding, but can also be formed thrombosis- a harmful response where a blood clot forms within a blood vessel, constricting blood flow. Both hemostasis and thrombosis will be discussed.

Hemostasis is the process of the body aggregating blood cells together with the intentions of preventing further blood loss from a damaged blood vessel. There are three major steps within hemostasis: (1) vasoconstriction and platelet adhesion, (2) platelet plug temporary blockage, (3) formation of a fibrin clot - blood coagulation. In the vasoconstriction step, the blood vessel walls react to reduce the blood loss. Smooth muscle within the vascular endothelium receive signal from sympathetic pain receptors, which causes a contractile response reducing the size of the blood vessel and hence blood flow and blood loss within it. In the platelet plug formation phase, platelets encountering exposed collagen from the site of injury, change shape, adhere to the site and release granules such as: adenosine diphosphate (ADP)- which acts to attack more platelets; serotonin - which acts as a vasoconstrictor; and thromboxan A2 - which assists in both platelet aggregation and vasoconstriction. The positive feedback loop within this chemical cascade ensures a fast reaction, forming a platelet plug over the site of injury. This phase is referred to as primary
homoeostasis. Notably, platelets are not cells, but circulating fragments of cells, containing both lipid and protein components. Platelets are comprised of three subcellular components: granules, membranes and a soluble fraction. A study of the lipid composition of human blood platelets found the ratio of lipid to protein within the whole platelet to be 0.28 (28%), with a greater fraction within the platelet membrane (0.58) and platelet granules (0.56), and a lower lipid fraction within the soluble fraction (0.11). [39] The formation of the fibrin clot occurs after the platelet plug whose chemical cascade results in activated clotting factors and ultimately fibrin forming from inactivated fibrinogen plasma protein. A fibrin mesh is formed around the platelet plug, which traps red and white blood cells during its formation. This results in a thicker and harder plug being formed. This phase is called secondary hemostasis [40]. A depiction of the three stages of blood clot formation can be seen in Fig. 1.9

![Blood Clot Formation Diagram](image)

Figure 1.9: Blood clot formation. Taken from: [7].

Thrombosis is similar to hemostasis in the mechanism of formation of the thrombus, but occurs within blood vessel, causing an occlusion or total obstruction of the vessel. A piece of the thrombus may break off, travel as an embolus and become logged in a different blood vessel as an embolism. Most frequently, the thrombus will form around fatty, atherosclerotic plaques.

While the primary components of clots will always be fibrin, platelets, red blood cells, and white blood cells, the ratios of these components may vary between clots, and may be accompanied by fatty plaque as well. Blood clots can be classified by age
as fresh (<1day), lytic (1–5 days), or organized (>5 day). In a study of thrombus aspirated during percutaneous coronary interventions (PCI), findings indicated that plaque was found in 395 out of 1009 patients (39%). They also found that only 60% of blood clots were fresh (<1day), indicating that in 40% of patients, plaque disruption and blood clot formation happened more than 24 hours before symptoms were apparent to the patient. This emphasized the need to study blood clots of significantly different ages. [41] In the scope of photoacoustic use for identifying blood clots in neonates, the presence of fatty plaque is extremely unlikely (given the nature of blood clots that form in neonate compared to adults). However, if photoacoustic imaging is extended to image blood clots in different populations, the presence of a high fat concentration could act as a distinct signal and aid in localization and characterization of the clot.

In a study of 50 thrombi specimens retrieved from patients with acute ischemic stroke, the components of fibrin, white blood cells, and red blood cells were quantified using histopathology. [42] A long established system of belief indicated that slow blood flow tended to form ”red” (erythrocyte) clots and high flow forms ”white” (fibrin) clots. This study found the correlation between blood flow and clot type to be weak and instead examined occurrences of Hyperdense middle cerebral artery sign (HMCAS) (increased attenuation of the proximal cerebral artery in CT evaluation) and blooming artefact (BA)(a susceptibility artifact in MRI often exploited to reveal certain pathologies). They found the occurrence of HMCAS and BA to be linked to red blood cell dominated clots, whereas fibrin clots lacked these features. The composition of the clots is considered important since it determines the efficacy of different treatment types. For example, tissue plasminogen activator (tPA) has been shown to be much more effective in fibrin rich clots than red blood cell rich clots. [43]

Additionally, the age of the clot is thought to have an impact on the effectiveness of tPA; chronic features such as endothelization and calcification are thought to decrease the penetration and hence efficiency of tPA. Ultimately, the blood clot composition and age are important aspects in informing treatment. Signals within
both CT and MRI are able to aid in characterizing clots. This provides motivation to examine changes in the photoacoustic signal which, could indicate the composition of the clots, and help inform clinical treatment.

1.3.2 Stroke Induction Methods

There are several different models for inducing stroke within animal specimens. Many of these methods will be briefly listed and discussed in their advantages, disadvantages and applicability to our study. Ultimately the Rose Bengal method was selected; it will be discussed in greater detail.

One approach to creating a stroke is though using an intraluminal suture in the middle cerebral artery. this approach is highlighted in study using a mouse model to examine blood flow, behaviour, and survival. [44] This model was ideal for the study’s criteria; blood flow was measured using indocyanine green to verify the blockage (using near infra-red radiation), and behaviour and survival were monitored over 28 sequential days. Two main problems exist with this study in its applicability to work done in this thesis. Primarily, the use of an intraluminal suture will block off the blood vessels and cause a stroke, but will not result in a blood clot. Since blood clots are the the principle target for imaging, that makes this technique less than ideal. Additionally the delicate surgery required for isolating the middle cerebra artery, placing temporary ligations in connecting vessels and guiding the nylon suture along the lumen of the vessel to the desire location, represented a difficult procedure for members less experience with animal surgery.

[44]

Another approach used within rodent models of stroke is endothelin-1 (ET-1), a vasoconstrictor which is applied directly to the surface of the middle cerebral artery after craniotomy. More recently, this model was modified using an ET-1 injection through minimally invasive surgery to target smaller vessels and produce focal cerebral ischemia. A distinct advantage of this technique is that it allows excellent control over the location and extent of the artery constriction and hence stroke (though adjusting dosing of ET-1). However, once again, this technique would
not result in the typical blood clot desired for photoacoustic imaging. [45]

The use of different emboli (microspheres, black bead, silicone rubber cylinders and performed clots) into the internal carotid artery has also been shown to produce stroke. However, the locations and extent of strokes are unpredictable and variable from trial to trial. Similarly, approaches that use transgenic or spontaneously hypertensive stroke-prone animals result in highly variable stroke locations. Additionally, while the use of transgenic animals would result in a much more accurate model of the blood clot, the timing of the stroke is uncontrolled, making temporal imaging of the stroke impracticable to impossible. [46]

Ultimately, the selected method was a photothrombotic stroke model which used Rose Bengal and excitation light from an LED.

1.3.3 Rose Bengal Induced Photothrombotic Stroke

Rose Bengal is a photoactive dye that can be used to generate a singlet oxygen from triplet oxygen. The singlet oxygen can then undergo a variety of reactions, such as cycloadditions with alkenes. The structure of Rose Bengal can be seen in 1.10. In a photothrombotic stroke, Rose Bengal is injected into the target vessel, or into the blood stream. The target location is then illuminated using light, often from a laser. The maximum absorbance of Rose Bengal is at approximately 561nm, as seen in 1.10. This means the emission spectrum of the light source should have the majority of its power centered around or close to 561nm. Once excited, Rose Bengal creates singlet oxygen - a high energy form of \( O_2 \) with a different quantum state with a total quantum spin of 0 compared to triplet (regular) oxygen, which has a total quantum spin of 1. The free radicals (singlet oxygen) generated by the photo-activated Rose Bengal attack components on the epithelial cells, resulting in damage. The damaged epithelial cells result in platelet aggregation and the formation of a clot in the same process as regular thrombosis. [47, 48, 49]
1.3.4 Aspirin and Tissue Plasminogen Activator Treatment after Rose Bengal Induced Stroke

In addition to imaging of the blood clot and stroke dynamics, it is important to image effect of treatment and demonstrate and ability to assess efficacy of treatments.

A study by Kuroiwa et al. examined the effect of Aspirin and tissue plasminogen activator (tPA) on recovery from a Rose Bengal induced clot within a rodent model. [9] Aspirin or acetylsalicylic acid (ASA), is an anti-inflammatory athat suppresses the normal functioning of platelets; it is often used in long term prevention of heart attacks, strokes and blood clots. The protein tPA promotes the breakdown of blood clots. It catalyzes the conversion of plasminogen to plasmin- which is in turn responsible for clot breakdown.

The study examined lesion volumes for rats in four different groups (all of n=6; total n=24): (1) saline injection (1.0 ml); (2) given aspirin (30 mg/kg, dissolved in 1 ml distilled water, i.p. injection) 2 hours before photothrombosis; (3) given tPA (10 mg/kg, dissolved in 1 ml saline, intravenous injection) 0.5 hours after photothrombosis; (4) a control group where no Rose Bengal was injected, and photothrombosis did not occur.

Pathology was used to determine lesion volume- though staining with hema-
toxylin and eosin. In addition, one day before and one day after the photothrombosis, behavioural tests were performed to assess the effects of the stroke.

The largest lesion volume was observed in the saline injection group (5mm³). Administering Aspirin prior to Rose Bengal resulted in a decrease of approximately 30% (3.5mm³). The tPA group showed a much more dramatic decrease of close to 60% (2.0mm³). As expected, the control group with no Rose Bengal had no significant lesion volume.

![Figure 1.11: Effect of tPA, and Asp on stroke recovery in rodent model (rat). Both tPA and Asp results in observed decrease to lesion; tPA has a significantly greater effect than Asp. Taken from: [9].](image)

Significant neurological deficits were noted after photothrombosis using forelimb placing test, forelimb use asymmetry and corner turn test. These severity and duration of these deficits were closely correlated to the size of the lesion (an hence treatment group). [9]
Chapter 2

Blood Clot Phantom
Classification Within a Planar Illumination and Detection Scheme

2.1 Visual Sonics Vevo LAZR System

The Vevo LAZR is a combined photoacoustic (PA) and ultrasonic imaging system developed by VisualSonics. While the approach of creating a combined system results in increased complexity and expense, it allows for registration and combined visualization of anatomical and functional information. Certain features, such as non-perpendicular interfaces, are more easily visualized in ultrasound than in PA images. The registration of the photoacoustic images to B-mode ultrasound images allows for physiological parameters determined from the PA image to be localized within structures visualized in the ultrasound image. The system has an image acquisition rate of 5-20 Hz, which is determined by the laser repetition rate and the desired field of view. The laser used in the system is a Nd:YAG laser with an optical parametric oscillator. The laser produces wavelengths between 680 and 970nm with
A minimum step size of 2nm. The laser fires pulses of 4-6ns at a frequency of 20Hz. Since an image can be acquired for each laser pulse, the maximum acquisition rate is also 20Hz. However, for a full field of view [14-23mm], PA signals are captured on quarter-segments of the 256 element ultrasound transducer array (elements: 1-64, 65-128, 129-192, 193-256). Thus, for a full field of view, 4 sequential laser pulses are required per scan, giving an image acquisition rate of 5Hz. For smaller fields of view in faster acquisition modes, fewer transducer receiving elements are used, and the full 20Hz image rate can be achieved. The system allows for high resolution imaging as well as multispectral photoacoustic analysis. If at least 2 wavelengths are acquired with a differing optical absorption between oxygenated and deoxygenated hemoglobin (Hb, HbO₂), the system is able to predict vascular oxygen saturation (sO₂). A single photoacoustic slice (2D) has an acquisition time of 0.2 seconds, and a 10mm 3D volume can be constructed in 74 seconds. The signal to noise ratio for the photoacoustic signal is 20dB +/- 10dB. Two different transducer probes exist for this system. The first transducer has an axial resolution of 44µm with a broadband frequency of 32MHz-55MHz; the second transducer has an axial resolution of 75µm with a broadband frequency of 13MHz-24MHz. [50]
2.2 Blood Phantom Creation

Blood clots were created through the following steps: pig’s blood was obtained from a piglet study and preserved using citrate. Degassed water was mixed with calcium chloride dihydrate–\(CaCl_2(H_2O)_2\)–at a ratio of 25mL degassed water to 1.7 grams \(CaCl_2(H_2O)_2\) to create a 0.5 molar solution. The calcium chloride dihydrate solution was added to the blood using 0.1 mL of solution per 0.9 mL of blood. This counteracts the effects of the citrate and re-initiates the clotting process. The resulting solution was poured into a grid to allow for discrete samples. The solution was put into a vacuum degasser at -22mmHg for fifteen minutes. This removed microbubbles induced during mixing that would create artifacts in the PA images. After samples were removed from the degasser, they were allowed to sit for at least thirty minutes to allow for complete clotting.

Lysed blood was created in the following manner. A gel pad containing clotted blood was placed within 3 tesla MRI machine to identify and locate the blood clot. Using an MR compatible HIFU system, sonification was conducted. The following parameters were used: duty cycle 0.1%; power: 350 watts; frequency 1.2 MHz; number of pulses: 1200; pulse duration 1ms; pulse repetition 1s; total time 25s. Using a honeycomb shape, this pulse train was repeated 8 times in different locations for sonification of adjacent regions. This allowed for a large region of the clot to be precisely lysed step by step.

Initially clotted, unclotted and lysed blood were inserted into manually created indents in an optically transparent ultrasound gel standoff pad. The samples were covered with a plastic sheet and liquid ultrasound gel was placed over the samples. While this allowed for preliminary imaging to be obtained, this method meant the sample existed within an unspecified ROI. Additionally, images obtained through this method contained large signal artifacts from air bubbles present between the plastic sheet and the gel pad despite efforts to minimize their formation.

To increase the repeatability of the experiment, a more sophisticated phantom set up was created. AutoCAD designed and 3D printed custom parts were used to
create a mold for the phantom model. The phantom was created by first melting an ultrasonic gel pad. The melted gel pad was poured into the mold base Fig. 2.2a. The upper segment contained indents that were fit with 3 cylindrical, smooth surfaced rods Fig.2.2c. The upper segment was placed on top of base such that the rods were indented into the gel as it solidified, creating three cylindrical pockets for blood samples to be poured into (diameter 5mm and depth of up to 5cm). Spacer rings of 0.5 cm thickness were used to adjust the depth of the cylindrical pockets Fig.2.2b.

A representation of the resultant phantom set up can be seen in Fig.2.3.

2.3 Experimental Overview

A series of experiments were conducted at the STTARR facility, using a combined ultrasound and photoacoustic machine developed by VisualSonics. Initial experiments were conducted on single samples of clotted, unclotted and lysed blood. Images were
taken both at a wave length of 750nm and over a spectrum from 680nm to 970nm with a step size of 5nm. An investigation of the clotting process was conducted though a one-hour time series taken of unclotted blood turning to clotted blood, after adding the clotting agent (calcium chloride dehydrate). An image was taken of the unclotted blood (t=0) as well as every five minutes for the duration of the experiment; (16 images). These images were taken using spectral imaging between 680nm to 970nm. A larger data set with 6 clotted samples and 6 unclotted samples was taken to be used for developing a classifier. This data set was collected both using spectral imaging (680-970nm) as well as temporal imaging at a single wave-length of 700nm over 25 seconds (approximately 250 frames per sample at 10 frames per second).

2.4 Mean and Spectral Signal Intensity Analysis

In work done by Kariouk et al. the intensity of photoacoustic images were observed to change with the progression from acute clots to chronic clots. A decrease in signal intensity was observed with the age of the clot associated with the loss of red blood cells (RBC), which represent a significant source of signal. [51] A comparison of the mean signal intensity from photoacoustic images of three clotted and three unclo
blood samples taken using the Vevo LAZR, can be observed in Fig. 2.4. Notably, in our acquired data there is no significant difference between the intensity from clotted and unclotted blood (clotting initiated approximately 24 hours before imaging). The simulated blood clots created within the phantom do not fully mimic the blood clots observed in Kariouk’s study. Particularly when the blood clot is contained within a gel phantom, instead of within a physiological environment, there will be no loss of RBCs and hence, no observable intensity difference with age. Consistent and reliable trends do appear in the spectrum of the data, with an upward trend towards the longer wavelength (970nm) corresponding to the combined effect of the spectrum of water and oxy-hemoglobin.

To examine the visibility of the blood sample compared to the surrounding ultrasound gel, signal intensity was calculated for ROIs containing pure blood samples and ROIs containing pure ultrasound gel. The ultrasound images were used to help define these ROIs since the sample location was often difficult to localize using only the photoacoustic image at any one wavelength. As will be later discussed, examining the image across a series of wavelengths can allow for better photoacoustic localization of the sample. An example of a single frame of an image obtained from

Figure 2.4: Mean signal from clotted and unclotted blood show similar signal intensity levels and share the same approximate spectra.
Figure 2.5: Combined ultrasound (left) and photoacoustic (right) output of a blood sample, easily localized in the US domain, allowing for an accurate ROI selection in the PA domain.

The Vevo LAZR system can be seen in Fig. 2.5. The ultrasound can be seen in the greyscale image on the left side of the figure. The yellow square outlines the region over which the photoacoustic image was taken. The photoacoustic image can be seen in redscale on the right side of the figure. The sample, in this case clotted blood, is only easily visible in the ultrasound image. In many of the other cases, the blood clot was possible locate on the photoacoustic image, but to maintain consistency the ultrasound image was used for registration in all of the cases. In addition to the ROI selected for the blood sample, a second ROI was taken of an equal size and depth, adjacent to the clot to act as a control as seen in Fig. 2.6.

Figure 2.6: ROI selection for blood samples and reference gel samples.

Examining the optical spectrum of the signal intensity showed that areas contain-
ing the blood sample displayed a much higher mean intensity at lower wavelengths (where oxyhemoglobin is the dominant chromaphore), while at longer wavelengths (where water has a higher absorbency) the gel pad regions caught up to or surpassed the blood sample intensity. The most striking difference between spectra of blood samples and gel pad can be seen in blood clot sample 5 (Fig. 2.7a), whereas a more typical spectral difference can been seen in sample 6 (Fig. 2.7b).

The most notable trend between clotted and unclotted blood when solely examining the intensity images is clotted blood tended to have weaker signal at interfaces, whereas unclotted blood tended to have stronger signal. This is likely due to a larger mismatch in the index of refraction between the gel pad and liquid blood compared to the gel pad and clotted blood. This is a well known interface-effect often observed in organ or blood vessel boundaries, which produce well-defined backscattered RF signal, as noted in work done by the Kolios group [52].

As seen in Fig. 2.8a, the interface signal corresponding to the area of clotted blood (4-6mm) is the same or weaker than the interface seen along the rest of the phantom (gel pad - gel pad interfaces). Whereas in Fig. 2.8b, a much stronger signal is seen at the gel pad-sample interface. This result has a somewhat limited application to analysis of clotted and unclotted blood within physiological environments where this technique is ultimately valuable. The exact interface difference would be a unique feature of our particular set up with gel pads and blood samples. However,
Figure 2.8: Difference in interface intensity observed between clotted and uncotted blood.

similar differences in interface visibility could be observed between the blood vessel walls and clotted or uncotted blood within them.

2.5 First Order Statistical Analysis

2.5.1 Speckle Contrast Theory

Within methods of laser imaging, random scattering through the medium results in a detector receiving signal from difference positions with a distribution of distances travelled resulting in constructive and destructing interference. The random interference patterns result in stronger and weaker signal, which appears as speckle. Moving particles create both spatial and temporal fluctuation in the speckle patterns which can be analysed, allowing for the motion of the particle to be quantified. This is often done in mapping blood flow, where the blurring of the speckles indicated the speed of blood flow. This can be done either using spatial contrast, which allows for better temporal resolution, or using temporal contrast, which allows for better spatial resolution. [53] [54] Speckle contrast K can be calculated as the ratio of standard deviation \( \sigma \) to mean intensity \( < I > \). This relationship is shown in Eq. 2.2.

\[
K = \frac{\sigma}{< I >} \tag{2.1}
\]
Figure 2.9: (a) Raw image from the thin skull of a rat; speckle pattern is visible but features are hard to discern. (b) Spatial speckle contrast map of the raw image applied using 7x7 window size. Image taken from [10].

A map of spatial speckle contrast can be created for an image, where each pixel within the new image is calculated based on a window of pixels surrounding the location such as a 3x3 or 11x11. The selection of the exact window size is of some significant importance. Changing the size of the window results in the following trade off: the smaller the window size, the higher the spatial resolution, but the less accurate the estimation of speckle contrast is. Within an overview of literature, a window size of 7x7 pixels is most frequently selected as a reasonable trade off between spatial resolution and contrast. However, for difference imaging systems, and specimens with unique resolutions, speckle size and required contrast resolution, the optimal window size could be smaller or large than the suggested 7x7. [10]. An example of speckle contrast being used to visualize blood vessels within a rat’s brain, is shown in Fig. 2.9. Another important consideration is the visible speckle size. To satisfy the Nyquist sampling criteria, the speckle size must be atleast two times larger than the imaging system’s pixel size. For speckle imaged using a camera, the minimum speckle size is given by the following equation:

$$\rho_{\text{speckle}} = 2.44 \lambda \left(1 + M\right) f/#$$  \hspace{1cm} (2.2)

Where $\rho_{\text{speckle}}$ is the speckle size, $\lambda$ is the wavelength of light, $M$ is the magnification of the imaging system, and $f/#$ is the f-number of the optical system (the ratio of the system’s focal length to the diameter of the entrance pupil). [55].
2.5.2 Speckle Contrast Maps

Using the linear-scale images, speckle contrast maps were created. Maps were created for all six clotted and six un-clotted blood samples. An example of the original intensity image (log-scale) can be seen in Fig. 2.10a and the corresponding speckle contrast map (linear-scale) can be seen in Fig. 2.10b.

Notably, signal artifacts caused by air bubbles apparent in the original images are amplified within the contrast images. Blood-gel pad interfaces are also highly visible in the images.

Different window sizes for the speckle contrast were examined at window lengths from a 3x3, 5x5, 7x7 up to 21x21. To illustrate the visual effect of window size, the extremes: 3x3 and 21x21 are show in Fig. 2.11. Ultimately, a window size of 7x7 was chosen based on best visibility of features and compliance with literature.

Contrast was examined at different wavelengths within the range of 680nm to 970nm. To examine spectral behavior of contrast, images at three wavelengths where different chromophores are dominant are shown: 680nm - where deoxyhemoglobin is dominant; 970nm where oxyhemoglobin is dominant; and 800nm, where the absorption coefficients for oxyhemoglobin and deoxyhemoglobin are approximately equal. This can be seen in Fig. 2.13.

Visually, the difference between the contrast maps at different wavelengths is fairly subtle. Because of this, the average contrast value within the ROI was graphed
Figure 2.11: Effect of window size on speckle contrast images.
Figure 2.12: Comparison speckle contrast map for wavelengths (680, 800, 970) in for sample 4, clotted blood.
with respect to wavelength for both clotted and unclotted blood, shown in Fig. 2.13.

2.6 Analysis and Conclusions

Notably, the unclotted blood presented a very uniform contrast spectrum across the 6 samples. The clotted blood however, show a bimodal distribution with 3 of the samples displaying much higher contrast values than the other three samples. Clotted samples 1-3 displayed average contrast values close to 0.100 at the low end of the spectrum (680nm), decreasing to 0.070 at the high end of the spectrum (970nm). Clotted samples 4-6 showed spectra with contrast values close to 0.070 at the low end of the spectrum (680nm), and decrease down to approximately 0.055 at the high end of the spectra (970nm). Resultantly, clotted samples 1-3 demonstrated a spectra with a large difference from that of unclotted blood, while clotted samples 4-6 show a spectra with a much more narrow separation between them and the unclotted blood spectra. This trend is likely indicative of the preparation of clotted specimens 4-6; either that these blood samples exhibited incomplete clotting or that small amounts of unclotted blood occurred at the surface of these clotted blood specimens. If unclotted blood pooled on the surface of clotted specimens 4-6 it would cause their surface interface to behave like unclotted blood. Since the surface interface is the source of the majority of signal, this would explain why their contrast spectra appeared more similar to unclotted blood.

2.6.1 Classifier

Based on the speckle contrast spectrum, a classifier was developed to identify clotted and unclotted specimens. Initially leave-one-out testing, was performed on the 12 sample data set using the average speckle contrast value at three points on the spectra: 690nm, 800nm and 970nm. Using the root-mean-squared distance between the tested point and all other points for clotted and unclotted blood, each sample was classified as either clotted or unclotted based on the minimum distance. This initially lead to correct classification of 6 out of 6 unclotted blood specimens and 3
Figure 2.13: Contrast Spectrum of Clotted and Uncotted Blood.
out of 6 clotted blood specimens. This result reiterates the possibility of unclotted blood appearing on the surface of clotted samples.

The classification method was refined, and adjusted in three different ways: (1) using only the K-nearest neighbours (from 3 to 9) for classification; (2) through increasing the number of points on the spectra used (3 point to 59 points); (3) adjusting the size and location of the selected ROI (broader image ROI and smaller surface focused ROI). Based on these adjustments, a classifier was created that could correctly identify 6/6 clotted and 6/6 unclotted specimens. However, this result should be taken with scepticism as sufficient adjustment of parameters with the relatively small data set could likely yield any desired result.

2.6.2 Conclusions and System Suitability

Using the higher speckle contrast values observed within clotted blood could be an indication of higher signal variability within clotted regions. However another possibility is that in the preparation of the clotted blood sample, higher levels of microbubbles/air pockets were present, which in turn skews the speckle contrast values.

Extensive use of the Vevo LAZR system revealed many limitation of the system. The system set-up was highly susceptible to artifacts from air pockets/microbubble. Even if air bubbles were totally eliminated within the sample, small air pockets could be introduced between the probe and the liquid ultrasound gel, or between the ultrasound gel and the sample, or microbubbles could occur within the ultrasound gel. Even if the air pocket or bubble occurred significantly above the region of interest often an echo of the artifact would be present over top of lower data, obscuring the information from the sample’s signal. While best efforts were made to minimize the presence of air pockets in the imaged region, totally eliminating air proved to be difficult. Additionally, the system’s imaging rate of 10 Hz for PA signals was insufficient to obtain significant results from temporal spectral analysis. Finally, while the system provided strong signals from interfaces, the system lacked information about the interior of the specimen.
Ultimately, the lack of interior signal information and high susceptibility to artifacts directed the project towards examining different options for PA imaging systems. The different PA scanner that was selected was the iThera MSOT scanner available St. Michael’s Hospital.
Chapter 3

Blood Clot Phantom

Classification Within a

Cylindrical Illumination and

Detection Scheme

Signal originating primarily from the surface interface severely limited the available image processing techniques, and ability to classify samples. Obtaining richer textural information for blood phantom imaging, gave motivation to using a cylindrical imaging system optimized for deep tissue imaging. Obtaining information from the interior of tissue allowed for higher order spatial statistics to be successfully applied, which in turn increased the specificity of the developed classifier.

3.0.1 iThera Multispectral Optoacoustic Tomography System (MSOT)

Buehler et al. [56] developed and published details on the first custom-made system comparable to the commercial iThera MSOT system used for experiments in this thesis. The system provides real time acquisition of three dimensional (3D) structure in animals, as well as physiological features and molecular activity in tissue. Advantages of the system include its nonionizing radiation, ultrasonic spatial resolution,
real-time operation and versatile sensing of chromophoric molecules. The systems ability to provide real-time full body imaging represents a significant advance over previous photoacoustic systems, which had either been unable to provide full body imaging or required long acquisition times rendering them unsuitable for real time purposes. The design of the system was based on a 64-element curved transducer array (Imasonic SaS, Voray, France), which had covered an angle of 172°. This system makes use of 630 fibers partitioned into 10 arms to allow for cylindrical focusing and a full 360° view of the sample; as seen in the figure below. The system uses a tunable (680-980nm) oscillator laser (InnoLas Laser GmbH, Germany), to provide pulses of excitation light (duration 10ns, repetition frequency 10Hz). The signal is detected in a curved array of ultrasound transducers allowing for parallel data acquisition. The effective spatial resolution of the array is 150µm in plane and 800µm elevational. A linear stage allows translation of the animal holder along the z-axis for acquisition of 3D data sets with a minimum step size of 0.5mm. Image data from the ultrasound transducers is ultimately reconstructed using a back projection method creating a 200x200 grid with a pixel size of 110 µm. [56]
3.1 Blood Phantom at Saint Michael’s Hospital

In experiments conducted using the iThera MSOT system, clotted and unclotted blood samples were used. The procedures for the preparation of the clotted sample were identical to methods for the Vevo LAZR system described in previous section.

The imaging phantom was created out of a (1.5%) agar solution, using 0.75g of solute powder agar within 50mL of water. The combined solution was microwaved for approximately 40 seconds in 10 second intervals with mechanical agitation between each interval until the agar powder was completely dissolved. A heated solution is required for the supersaturation of agar within water. While hot, the solution was poured into a syringe; a rod was used to create a void for the sample. The phantom was allowed to solidify for 30 minutes.

The blood and blood clot samples were placed into an optically clear straw (3mm diameter). The ends of the straw were sealed using a thermal adhesive glue. Excess straw was cut and removed. The section samples contained within the straw were placed in the void of the agar phantom. The phantom was placed within a water filled Ziploc bag, which was submerged in the imaging chamber. This set up can be seen in Fig. 3.2

![Figure 3.2: Schematic Blood Phantom Imaging](image_url)
Spectral images were taken between 680nm and 980nm with a step size of 5nm. Six separate samples were used (three of clotted and three of unclotted blood); spectral series were obtained at 54 different locations (32 locations for clotted blood and 22 locations for unclotted blood).

3.2 Image Segmentation

In order to properly classify a portion of the image as either clotted or unclotted, a region of interest (ROI) must be selected. Using photoacoustic imaging, this region of interest can be selected manually, or through image co-registration using ultrasound. However, other methods exist to perform computerized segmentation of images dispensing with the need for manually selected ROIs.

3.2.1 Theory

In Koprowski et al. a method of tissue segmentation and surface detection is described.

In the described method, analysable areas the tissue are isolated: removing the substance (air or fluid) above the sample, as well as the deep portions of tissue in the sample that are below the noise floor. The original image is first passed through a Gaussian filter. After Gaussian filtering a binary map is constructed setting all pixels above a certain threshold to 1 and pixels below the threshold to 0. This threshold can be calculated in a several different ways: as a percentage of the maximum pixel value, as a set number of standard deviations above the mean value, or as an arbitrary top fraction of the data range. After the binary map has been constructed, due to noise, a large amount of unwanted holes within the tissue sample exist as well as dots outside of the tissue sample. Using standard methods of image connection and hole filling, the section of image corresponding to the tissue is identified. After the hole filling section of the algorithm, random regions of high noise are still visible outside of analysable areas the tissue. This noise is removed using a dot removing algorithm.
3.2.2 Automated Segmentation for ROI Selection in Blood Phantom Images

A method of isolating the blood sample within the image such that surrounding gel or water was not analyzed was developed. This method was based on the method described in Kropowski et al. The method used sequential Gaussian filtering, construction of a binary map, hole filling and dot removal. This method was successfully able to isolate the set of pixels corresponding to the sample, a task which had previously been done manually. Various methods of calculating threshold for the initial binary map were examined (percentage of maximum, mean plus n standard deviations, top fraction of the data range). Ultimately, the threshold was set to 10% of the maximum pixel value. Image connection and hole filling was done using a 5x5 pixel map. Dot removing was done using a maximum dot size of 200 pixels.

While ROIs within the images could have been selected manually given the relatively small size of the data set analysed (54 samples) and the uniformity of the samples (approximately circular), a more robust method is advantageous. If a series of volumes of blood vessels were to be analysed using the developed methods, ROI
Figure 3.4: Blood clot image before and after image segmentation applied.

selection could be performed on thousands of images almost instantaneously. Additionally, the algorithm performs a more constant and 'fair' ROI selection through removing human inconstancy in ROI selection as a source of error.

The developed algorithm was constructed with additional complexity to maintain functioning in images with lower signal to noise ratio (SNR), or images with less clearly defined samples. For the presented blood clot images, the intensity threshold step was sufficient for ROI selection. In the analysed images, the Gaussian filtering, image connection, hole filling and dot removing portions of the algorithm contributed very little. This is largely due to the nature of the phantom images: a single cylinder containing high signal intensity blood, surrounded by an extremely low intensity agar phantom and water. In more complex physiological images, blood vessels will not occur in isolation; they will be surrounded by other chromophores of significant intensity. In these scenarios, the full sequence of image segmentation steps would likely become necessary for accurate ROI selection.

3.3 First Order Statistical Analysis

3.3.1 Speckle Contrast Statistics

While speckles is suppressed within the interior of photoacoustic imaging and was not truly observed within the collected images, speckle-like statistics were ob-
served. This is likely due to surface roughness creating speckle-like features.[57] This anomaly is more thoroughly discussed in section 3.6.

Texture based contrast images were created for clotted and unclotted blood. Given that the pixel resolution of obtained images was 75μm and the straw width containing the blood clot was only 3mm, the pixel diameter of the ROI (as calculated and manually verified) is only 40 pixels. Because of this relatively low pixel count, contrast images were created using a window size of 5x5 instead of the standard 7x7.

![Image](image.png)

(a) Original blood clot image. (b) Corresponding contrast map.

In Fig. 3.5 the selected ROI for the intensity image and the corresponding contrast image calculated using a 5x5 window are displayed. Notably the outer pixels (within 3 pixels of the circumference of the ROI) will use a window of pixels that extends beyond the selected ROI. This means these pixels would be calculating contrast using some number of zero values. Due to this phenomenon, the contrast values are much greater in the outer rim of the ROI. While these contrast values do bear significant meaning (this high contrast region emphasises the interface between blood and gel), these extremely high values of contrast inhibit visualization and statistical calculation of contrast in the interior of the sample. To accommodate for this, displayed contrast graphs were adjusted to only display contrast for pixels that calculate contrast values using a window fully within the blood specimen’s ROI. This process essentially creates interior contrast maps.
The interior contrast maps for a sample of clotted and unclotted blood are displayed in Fig. 3.6.

![Interior Contrast Maps](image)

(a) Interior Contrast Map - Clotted Blood, Sample 1, (680nm)
(b) Interior Contrast Map - Unclotted Blood, Sample 1, (680nm)

Figure 3.6: Interior contrast images for (a) clotted blood samples and (b) unclotted blood samples.

From the ROI selected texture contrast maps, contrast can be graphed as a function of wavelength. This was done through taking the average value of all pixels on the texture contrast graph within the selected ROI for each blood sample. While the selected ROI was different for each sample, this difference was mainly positional to account for its position in the imaging window, the shape and total pixel size of all ROIs were nearly identical. All ROIs were approximately circular in shape with a radius of 20 pixels and a resultant area of approximately 1256 pixels.

A possibly concerning observation could be that the intensity of each pixel is dependant on its depth within the sample due to signal attenuation. Thus by using the mean of the entire ROI, pixels from different depth and signal intensities are being compared. However, due to the similar shape and size of the selected ROI for each sample, all analysed mean texture contrast values were calculated using nearly identical numbers of pixels at each depth. Since the majority of attenuation occurs within the sample, the majority of the variation is signal intensity will be based on the pixel depth relative to the surface of the sample. However, to a lesser extent, intensity will vary with depth relative to the imaging plane of the system. While samples were roughly centred within the imaging plane, as later discussed,
imprecise centring of the sample represents a source of error. The full set of contrast-wavelength plots for the full set of 32 clotted blood samples and 22 unclotted blood samples can be seen in Fig. 3.7.

Figure 3.7: Mean spectral contrast plots for (a) clotted blood samples and (b) unclotted blood samples.

Notably there is much more variation within the clotted blood compared to the unclotted blood. The group of clotted blood can be divided into three separate clusters: (1) samples with contrast spectra similar to unclotted blood, (2) samples with a flatter spectra (average contrast = 0.38) with two humps around 740nm and 920nm, (3) samples with a similar spectra to group 2, but with average contrast = 0.33. These grouping can be seen in Fig. 3.8.

Figure 3.8: Mean spectral contrast plots for clotted blood samples: (a) group 1) spectra similar to unclotted spectra and (b) group 2) and 3) - unique spectra with two humps around 740nm and 920nm.
Notably in the grouping of clotted blood groups 2) and 3) the spectrum appears very similar in shape, differing only by an offset and a scaling factor. Theses groups of data were taken on separate days. This is likely due to a combination of three different factors (1) power of laser, (2) phantom alignment (3) phantom set-up. The power of the iThera laser was seen to be variable between maintenance periods when it was tuned. While the change in power was never a significant problem when acquiring data on a single day, variations of a few percent (1-5%) were to be expected when using the system a week apart. Additionally, the amplitude of the signal received in the system is location dependant, with the most signal in the center location. While the phantoms were attempted to be aligned in the center of the system, there was no mechanism to ensure precise alignment of the center of the specimen with the center of the imaging plane. The last and likely less significant factor was phantom set-up; the agar phantom was recreated for each experiment so its consistency and hence attenuation were slightly variable.

To account for the change in laser power and difference in phantom attenuation, the data was scaled and an offset was applied. The allowed for the blood clot groupings 2) and 3) to be more easily related and compared. The normalization of this segment of data can be seen in Fig. 3.9. The figure shows that after normalization, two data sets indeed display the same approximate spectral shape.

![Figure 3.9: Mean contrast of unique spectra group normalized.](image)

Based on points on the contrast spectrum a classifier was created. The classifier identified 29 out of 32 of the clotted blood samples as clotted and 3 out of 32 as
unclotted. It identified and 17 out of 22 of the unclotted blood samples as unclotted and 5 out of 22 as clotted. For a confusion matrix which shows the number of true positives, false negative, true negatives, and false positives, different measures of accuracy, such as sensitivity and specificity can be reported. Sensitivity or true positive rate (TPR) can be calculated as the ratio of true positive(TP) over number of positive samples (P): TPR=TP/P. Specificity (SPC) or true negative rate can be calculated as the ratio of true negatives(TN) over number of negative samples (P): SPC=TN/N.

This classifier had a sensitivity to clotted blood of 91%, and a specificity of 77%. While this result appears promising, it should be viewed sceptically. Some of the spectral differences observed in clotted and unclotted blood phantoms could be due differences in oxygenation of the specimens resulting from the inherent difference in sample preparation (adding and mixing of clotting agent) rather than inherent difference in sample composition. Later analysis using gray level co-occurrence matrices resulted in much more reliable differentiation independent of sample preparation.

3.4 Gray Level Co-occurrence Matrix Texture Analysis

3.4.1 GLCM Theory

Gray level co-occurrence matrixes (GLCM) are a tool used in assessing the texture of images. The matrix measures the number of times within an image each pixel value is adjacent (in a particular direction) to each other pixel value. The GLCM can then be used to evaluate a number of different texture measures while maintaining spatial information, such as contrast, dissimilarity, energy, entropy, correlation, autocorrelation, angular second moment (ASM) and homogeneity. [58]

Since images are often recorded in 16-bit or 32-bit values, a scaled image can be defined to avoid evaluating $2^{32}$ different values. Based on the range of values present in the original image and the desired number of levels, a scaled image will be created. Most frequently, 8 levels will be used for intensity images, or 2 levels will be used for binary images. For the purpose of illustrating the theory, 3 levels
will be used in the example Fig. 3.10.

**Figure 3.10:** Scaling of original image (3 levels) in preparation for GLCM creation.

From the scaled matrix, a co-occurrence matrix C will be created of size NxN, where N is the number of levels. Each entry $C(i,j)$ is a count of the number of times in the scaled image I that a reference pixel with value $i$ is located at a pixel offset of $\Delta x, \Delta y$ to a pixel with value $j$. This is defined by the following equation:

$$
C_{\Delta x, \Delta y}(i,j) = \sum_{x=1}^{n} \sum_{y=1}^{m} \begin{cases} 
1, & \text{if } I(x,y) = i, \text{ and } I(x + \Delta x, y + \Delta y) = j \\
0, & \text{otherwise}
\end{cases}
$$  \hspace{1cm} (3.1)

Typically an offset will be selected based on the 8 cardinal directions: [0,1],[1,1],[1,0],[1,-1],[0,-1], [-1,-1],[-1,1] corresponding to angles 0, 45, 90, 135, 180, 225, 270, 315. The GLCM is created based on this pixel offset. In the following example, Fig. 3.11 the direction selected was [0,1]. This means each reference pixel was compared to a pixel at an a vertical offset of 0 pixel and a horizontal offset of 1 pixel; each pixel was compared to the pixel to its immediate right.

**Figure 3.11:** Scaling of original image (3 levels) in preparation for GLCM creation.

An example of calculations for particular cells is shown in Fig. 3.12. Since there are two occurrences of a scaled value of 1 being found at an offset of [0,1] from a value of 1, the first cell [1,1] in the GLCM is filled with a value of 2. Since there is
one occurrence of a scaled value of 3 being found at an offset of [0,1] from a value of 3, the last cell [3,3] in the GLCM is filled with a value of 1.

![Figure 3.12: Example calculation for cells [1,1] and [3,3].](image)

Symmetrical GLCMs can be created where each entry counts both instances of value \(i\) being located \(\Delta x, \Delta y\) from \(j\) as well as \(j\) being located \(\Delta x, \Delta y\) from \(i\). Essentially a symmetrical GLCM is the summation of \(C(\Delta x, \Delta y)\) and \(C(-\Delta x, -\Delta y)\). This creates symmetry along the diagonal of the matrix. Symmetrical GLCMs provide a more accurate representation when texture should be assessed along the axis, not solely in a particular direction. For example, an image of moving object or particles (such as blood flow within a vessel) would ideally be assessed in the direction of motion, whereas images with layers, but no clear direction, are best assessed symmetrically (such as a scan of tissue with layers corresponding to tissue depth). The symmetric GLCM for the illustrated example is shown in Fig. 3.13.

![Figure 3.13: Symmetric GLCM created with offset of [0,1].](image)

If texture analysis is concerned with detection of rotationally invariant features, this can be approximated by calculating and summing symmetric GLCMs calculated with angularly rotated offsets (ex: 0, 45, 90, 135).

### 3.4.2 GLCM derived properties

Once the GLCM has been calculated with an appropriate selection of number of levels and offset, statistical properties derived from the GLCM can be used to measure
texture. Three broad groups of texture analysis will be discussed: (1) measures related to contrast, (2) measures related to orderliness, and (3) descriptives statistics of GLCM textures measures. [58]

### 3.4.3 Contrast related measures

Contrast is a weighted indication of intensity differences between spatially adjacent pixels as shown in Eq. 3.2. For a constant image, the contrast is equal to zero. The weighting is proportional to the squared difference between pixel values.

\[
\text{Contrast} = \sum_{i,j=1}^{N} |i - j|^2 p(i, j) 
\]

(3.2)

Where \(p(i,j)\) is the GLCM matrix value at row \(i\) and column \(j\). Dissimilarity is similar to contrast, but uses linearly instead of exponentially increasing weights. Its equation is shown as Eq. 3.3.

\[
\text{Dissimilarity} = \sum_{i,j=1}^{N} |i - j| p(i, j) 
\]

(3.3)

Homogeneity is also calculated similarly to contrast and dissimilarity, based on a weight distribution of pixel value difference. However, homogeneity emphasises sections of similarly valued pixels and uses exponentially decreasing weights away from the diagonal (\(i=j\)) of the GLCM. Its equation is shown in 3.4. For a diagonal GLCM (all pixels are exclusively adjacent to pixels of equal value in the defined direction), homogeneity is 1; this would be an image consisting of strips in the defined direct or and image consisting of a single solid colour. [58]

\[
\text{Homogeneity} = \sum_{i,j=1}^{N} \frac{p(i, j)}{1 + |i - j|^2} 
\]

(3.4)

### 3.4.4 Orderliness related measures

Orderliness, how predictably sets of pixel value pairs appear. Two different images could have the same degrees of directional contrast, but could have very different
values for orderliness. Both energy and entropy are measures of orderliness. Angular second moment (ASM) is defined as the summed square of GLCM entries, given in Eq.3.6. [58]

$$\text{ASM} = \sum_{i,j=1}^{N} p(i,j)^2$$  \hspace{1cm} (3.5)

Energy is defined as the square root of the ASM.

$$\text{Energy} = \sqrt{\text{ASM}} \hspace{1cm} (3.6)$$

The equation for entropy is defined below:

$$\text{Entropy} = \sum_{i,j=1}^{N} p(i,j)(-\ln(p(i,j))) \hspace{1cm} (3.7)$$

Higher energy regions of an image indicate constant values or constant ordered variability (Ex: a value close to 15 is always proceeded by a value close to 200 to its right). Higher entropy regions indicate complex variability and can be used to isolate complex areas of the image from less complex areas.

### 3.4.5 Other descriptive statistics

Descriptive statistics for GLCMs use the same names as first order statistics, (mean, variance, correlation), but instead of using the pixel values within the image, they are performed based on the co-occurrence of pixel pairs. [58]

A 'regular' (first order) mean averages the values for each pixel within an image, or equivalently, weights the values of pixels by their frequency of occurrence. If in a binary image, a pixel value of '0' appears 40 times and pixel value of 1 appears 60 times, the mean of the image would be 0.6. In a GLCM mean, the pixel value is weighted by the frequency of its occurrence spatially adjacent to another pixel value. The formula for this is given in Eq. 3.8. [58]

$$\mu_i = \sum_{i,j=1}^{N} ip(i,j) \hspace{1cm} (3.8)$$
Variance in a GLCM, similarly to mean, is redefined to be weighted not by the frequency of pixels, but the frequency of pixel combinations. The formula for variance is given in Eq. 3.9.

\[
\sigma_i^2 = \sum_{i,j=1}^{N} p(i,j)(i - \mu_i)^2
\]  

(3.9)

Similarly to how the first order variance defines the dispersion of value around the mean, GLCM variance defines the dispersion of differences between reference and neighbour pixels.

Finally correlation measures the linear dependency of pixels to their neighbouring pixels. It is defined in the Eq. 4.4.

\[
\text{Correlation} = \sum_{i,j=1}^{N} p(i,j) \left[ \frac{(i - \mu_i)(j - \mu_j)}{\sqrt{(\sigma_i^2)(\sigma_j^2)}} \right]
\]  

(3.10)

3.4.6 GLCM Maps

GLCM maps were created for four selected features: contrast, correlation, energy homogeneity. The maps were used with the automated ROI selection to isolated the clotted or unclotted blood sample. The GLCMs were created using a 5x5 window size and a directionality of [1,1]. A sample GLCM map for both clotted and uncotted blood for each of the four feature are shown in Fig. 3.14 through 3.17.

![GLCM Maps](image)

Figure 3.14: GLCM contrast map for clotted and uncotted blood.
Figure 3.15: GLCM correlation map for clotted and unclotted blood.

Figure 3.16: GLCM energy map for clotted and unclotted blood.

Histograms for the average value of contrast, correlation, energy and homogeneity were constructed to visualize the differences between the two data sets in Fig.3.18.

3.5 Classifiers and Analysis

Classifiers were created based on GLCM calculated values of contrast, correlation, energy and homogeneity. It is important to differentiate texture contrast from GLCM contrast. Contrast values of an image essentially show the standard deviation of a window of data, normalized by the mean value. The pixel arrangement within a particular window does not affect the contrast for that window. In GLCM
contrast, the intensity difference is only considered based on pairs of spatially adjacent pixels, and is highly variable based on pixel arrangements within the window. GLCM contrast is high when adjacent pixels have high intensity differences.

Contrast was selected to show the pixel variability while accounting for precise spatial information. Homogeneity was selected as another measure similar to contrast which is weighted towards emphasising large regions of similar valued pixels. While contrast and homogeneity will always inversely correlated, the two measures will emphasis different aspects of spatial variability. Energy was selected as a basic measures of orderliness or how predictably pixel pairs will occur (regardless of how close in value the pixels are). Correlation was selected as a measure of a pixel’s linear dependency on adjacent pixels.

Within the histogram plots, the further the separation of the clotted and unclotted data sets, the better the predictive value of the GLCM feature was. Both homogeneity and energy showed no clear trends or separation between data sets. The histogram for correlation showed that clotted blood tended to have higher values, where as unclotted blood tended to have lower values, but the data sets had significant overlap. By far the best feature was GLCM contrast where the unclotted blood had values between 0.32 to 0.45, where as clotted blood had values between 0.00 to 0.08. One unclotted blood specimen did have a significantly lower contrast value, but this was the only outlier.
Classifiers created based on GLCM features were constructed. Predictably, the classifier based solely on GLCM contrast far out preformed the other classifiers. The classifier identified 32 out of 32 of the clotted blood samples as clotted and 0 out of 32 as unclotted. It identified 21 out of 22 of the unclotted blood samples as unclotted and 1 out of 22 as clotted. This classifier had a sensitivity to clotted blood of 100%, and a specificity of 95%.

3.6 Physical Explanation

3.6.1 Brownian Movement and Viscoelasticity

While speckles is suppressed within the interior of photoacoustic imaging, boundary roughness gives rise to speckle-like features. [57] The proposed physical phenomenon
is that the stiffening that occurs during clotting cause a restriction of the Brownian movement of red blood cells- the primary absorption centres- this causes textural changes in PAI. These textural statistics gave rise to the ability to classify and differentiate clotted an unclotted blood.

The physical significance of statistical measures in this work is supported by previously observed results from other research groups, who identified restriction in Brownian motion in clotted blood to be responsible for signal changes. In an analysis of blood coagulation by Nadkarni et al. fluctuations in speckle intensity were measured. [59] Physically, due to the blood coagulation, changes were observed to blood’s viscoelastic modulus -which represents the relations between oscillating stress and strain in objects with both elastic and viscous characteristics. These physical changes were observed though measuring the speckle autocorrelation at different time steps. An increase to the calculated time scale of speckle fluctuations indicated an increase in viscoelastic modulus- stiffening of the tissue. The change to the rate of speckle intensity fluctuation is due to stiffening of clot restricting the Brownian movement of red blood cells.

3.6.2 Signal Fluctuations and Higher Order Statistics

The data gathered had intensity features with statistics similar to laser speckle contrast, but also had contrast due to features and interfaces, independent of the speckle-like statistics. To best capture these features, a higher order spatially dependant metric had to be used. Literature provides president for applying such higher order statistics to photoacoustic images. Work done by Vilov et al, in photoacoustic miroc-vessel imaging also noted that the fluctuations within the sample primarily comes from the motion of absorbers such as red blood cells. [60] In their work, it was noted that these fluctuations which cause relatively small variation in the mean value of a pixel are best observed using higher order statistics. These, higher order statistics were used to enhance the resolutions of images. This shows that even though

This use of higher orders statistics within a photoacoustic images was similarly
applied in our work in the application of second order GLCM texture measure. Within a clotted model of blood, the aggregation of platelets and blood cells decreases the mobility of blood cells and creates more uniform volume. The greater uniformity in the resultant volume creates fewer free flowing optical absorbers, and resultanty a lower variation between spatially adjacent pixels. This is observed in a lower GLCM contrast value. The decreased mobility of blood cells also causes more predictably valued pixels based on adjacent pixels, resulting in higher values of correlation.

3.7 Conclusions

Preforming first order calculations of texture contrast over several different spectral plots allowed for classification of the data sets. The classifier based on this metric was able to obtain a sensitivity of 91% and selectivity of 77%. Using GLCMs and second order statistics measure of contrast, correlation, energy and homogeneity were examined. The best of these GLCM measures was contrast, which enabled a classifier with a sensitivity of 100% and selectivity of 95%. The demonstrated that examining the texture of photoacoustic images using second order statistics, specifically contrast, allows for the differentiation of clotted and unclotted blood to a high degree of accuracy. These finding suggest that photoacoustics is a promising imaging modality for classification of blood clots and could have the potential for clinical diagnosis of strokes and evaluation of stroke treatment.
Chapter 4

Brain Lipid Imaging and Preliminary Study of Demyelination

4.1 Experimental Set up

4.1.1 Lipid Phantoms

Experiments to examine spectral imaging of fat were also conducted. Two samples of cheese with 18% and 27% fat respectively were used. These samples were placed directly within a water filled Ziploc bag, which was submerged within the iThera MSOT imaging chamber.

4.1.2 Control, EAE and Shiverer Mice

Two separate models of demyelination were used: EAE and shiverer.

Experimental autoimmune encephalomyelitis (EAE) models use the immune response to brain-specific antigens which results in the destruction of brain structures carrying the antigen. This means that EAE shares many of the clinical and pathophysiological features of MS, and hence is the most common model used for MS research. Injections of myelin oligodendrocyte glycoprotein (MOG) act as an
immunogenic epitope triggering an autoimmune response and resulting in demyelination. Pertussis toxin (PTX) is injected both on the day of immunization and two days later, contributing to blood brain barrier (BBB) breakdown. This allows for myelin-specific T lymphocytes to migrate across the BBB into the central nervous system (CNS) where they cause an inflammatory cascade, recruiting monocytes and macrophages, and result in demyelination and axonal cell death. [61]

The control mice (C57BL/6 or CD-1) with induced EAE were imaged starting at 3 months. This imaging period allowed for assessment of demyelination and blood brain barrier breakdown.

Shiverer mice are mice affected by an autosomal recessive mutation that results in hypomyelination in the CNS. The decreased myelination results in a shivering phenotype and early death between 50 to 100 days. [62]

The C3HeB/FeJ-shiverer mice were used for imaging between the ages of postnatal week two (P14) to week five (P35) based on the formation myelinated structures and thickening of the skull. This also allowed for the imaging of demyelination and BBB breakdown.

Images were acquired both of live animals as well as extracted and perfused brains.

4.1.3 Animal Procedure

In live procedures, mice were anaesthetized in an induction box using 5% isoflurane. The mice were then transferred to a preparation area where the mice are connected to a nose cone. The hair surrounding the desired imaging plane (head and neck) was removed using an electric shaver and sequentially a commercial hair removal cream. During this preparatory period, the isoflurane was reduced to approximately 2.5%. The anaesthesia was adjusted to keep the mouse unresponsive to a toe-pinch test while maintaining a steady breathing rate above 1Hz. A heating pad was placed under the mouse in this preparatory area to maintain the mouse’s body temperature during the anaesthetized period. Upon completion of hair removal, the mice were transferred to an animal holder where it was attached to an isoflurane nose
cone and placed within a plastic foil hammock. Ultrasound gel was placed over the shaved areas of the mouse's head and neck to allow for a air-free coupling between the plastic foil and the mouse. The plastic hammock was secured using magnetic strips and folded into an upright position. The mice were transferred to the imaging chamber and reconnected to the isoflurane. The plastic hammock was submerged in water within the imaging chamber. Using system controls and a camera video feed, the mouse was moved along a track to align the mouse with the photoacoustic system. Photoacoustic preview images allowed for more precise positioning and horizontal/vertical adjustment for centring of the specimen within the imaging window. Tomographic scans were taken over the corpus callosum and the cerebellum over a duration of approximately 20 minutes. Mice were then removed from the system, dried, removed from anaesthesia and returned to their cage. In the approved protocol, an indocyanine green (ICG) (2mg/kg) can be administered in a tail-vein injection. This would allow for imaging of leakage of BBB. ICG injections in demylinated mice remains part of the future work.

Figure 4.1: Animal specimen holder with isoflurane connected to a nose cone; plastic foil hammock magnetically held.

In scenarios where imaging of the brain was performed, a phosphate buffered saline (PBS) perfusion was injected though the left ventricle. The brain was removed from the skull and placed in the plastic foil hammock instead of the mouse. Imaging sessions were carried out in a similar manner.
4.2 Chromophore Separation

4.2.1 Theory

Taking images using a range of different laser excitation frequencies allows for quantification of different chromophores. Two of the most dominant chromophores are oxyhemoglobin($HbO_2$) and deoxyhemoglobin(HbR). The spectroscopic changes between $HbO_2$ and HbR can be seen in Fig.4.2 These differences are highly visible in the 680nm to 980nm range most photoacoustic imaging systems operate at.

![Spectrum of oxyhemoglobin and deoxyhemoglobin](image)

Figure 4.2: Spectrum of oxyhemoglobin and deoxyhemoglobin, taken from Scott Prahl [11]

In several different studies, the variation in oxygen saturation in vivo was determined using photoacoustic microscopy. A standard set of equations are used to calculate the oxygen saturation. [63, 64].

Under the assumption that HbR and HbO$_2$ are the dominate absorbing compounds at each wavelength ($\lambda_i$), the blood absorption coefficient $\mu_a(\lambda_i)$ can be described as a linear combination of the absorbances of HbR and HbO$_2$. The absorbance of HbR and HbO$_2$ can be written as the molar extinction coefficients at the wave length $\epsilon(\lambda_i)$ multiplied by the concentrations of the substance. This yields
the following equation: \( \text{Eq. 4.1.} \) [64]

\[
\mu_a(\lambda_i) = \epsilon_{HbR}(\lambda_i)[HbR] + \epsilon_{HbO_2}(\lambda_i)[HbO_2]
\] (4.1)

Where \([HbR]\) represents the concentration of deoxyhemoglobin and \([HbO_2]\) represents the concentration of oxyhemoglobin.

Using the amplitude of the photoacoustic signals obtained at a pixel for each wavelength and the known values for extinction coefficients, a least squares fit can be performed to yield the relative concentrations of \(HbR\) and \(HbO_2\). This results in \(\text{Eq. 4.2.}\)

\[
\begin{bmatrix} [HbR] \\ [HbO_2] \end{bmatrix}(x,y,z) = (M^T M)^{-1} M^T \phi(x,y,z) K
\] (4.2)

where:

\[
M = \begin{bmatrix} \epsilon_{HbR(\lambda_1)} & \epsilon_{HbR(\lambda_1)} \\ \vdots & \vdots \\ \epsilon_{HbR(\lambda_n)} & \epsilon_{HbR(\lambda_n)} \end{bmatrix} \quad \phi(x,y,z) = \begin{bmatrix} \phi(\lambda_1, x, y, z) \\ \vdots \\ \phi(\lambda_n, x, y, z) \end{bmatrix}
\] (4.3)

Where \(\phi(x,y,z)\) is the received signal and K is an unknown coefficient representing the proportionality coefficient between the ultrasound parameters and optical absorbance. While the coefficient K prevents calculating the absolute concentration of \(HbR\) and \(HbO_2\), the ratio of \(HbR\) and \(HbO_2\) allows for the calculation of the oxygen saturation \(SO_2\) in the following equation:

\[
SO_2(x,y,z) = \frac{[HbO_2](x,y,z)}{[HbO_2](x,y,z) + [HbR](x,y,z)}
\] (4.4)

From a pixel by pixel calculation of the percentage oxygen saturation, complete maps of oxygen saturation can be created for the image.

While this approach assumes access to \(N\) wavelengths, the approach can function for \(N\) as small as 2 wavelengths: \(\lambda_1\) and \(\lambda_1\). [63] In this case, the total hemoglobin
concentration is given by:

\[ HbT = [HbO_2] + [HbR] = \frac{\mu_a^{\lambda_1} \Delta \epsilon_{Hb}^{\lambda_2} - \mu_a^{\lambda_2} \Delta \epsilon_{Hb}^{\lambda_1}}{\epsilon_{HbR}^{\lambda_1} \epsilon_{HbO_2}^{\lambda_2} - \epsilon_{HbR}^{\lambda_2} \epsilon_{HbO_2}^{\lambda_1}} \] (4.5)

Where the delta in hemoglobin extinction coefficient: \( \Delta \epsilon_{Hb} \) is equal to the difference in extinction coefficients for oxy and deoxy hemoglobin: \( \epsilon_{HbO_2} - \epsilon_{HbR} \) and the oxygen saturation is given by:

\[ SO_2 = \frac{[HbO_2]}{[HbO_2] + [HbR]} = \frac{\mu_a^{\lambda_2} \epsilon_{HbO_2}^{\lambda_1} - \mu_a^{\lambda_1} \epsilon_{HbO_2}^{\lambda_2}}{\mu_a^{\lambda_1} \Delta \epsilon_{Hb}^{\lambda_2} - \mu_a^{\lambda_2} \Delta \epsilon_{Hb}^{\lambda_1}} \] (4.6)

There are advantages and disadvantages to using only 2 wavelengths while acquiring data and performing chromophore demixing. A significant advantage is obtaining a much higher temporal resolution. Since each wavelength has to be acquired sequentially, collecting data at only 2 wavelengths will result in 10 times the temporal resolution as obtaining data at 20 wavelengths.

Notably this approach is not strictly quantitative. When calculating oxygen saturation, we assume that received photoacoustic signal amplitude \( \phi(x, y, z) \) is linearly related to the blood absorption coefficient \( \mu_a(\lambda_i) \). However, the proportionality constant K is dependant on various factors such as imaging depth, temperature, and tissue composition. Thus, the assumption that calculating ratios of \( HbR \) and \( HbO_2 \) allows for the cancellation of K is not strictly true. [50]

4.2.2 Lipid Phantom Analysis

The same equations and matrix manipulation for calculating concentrations of two chromophores can be extended to n chromophores. While oxyhemoglobin and deoxyhemoglobin are often the dominant chromophores, several other chromophores should be considered. The full set of chromophores: oxyhemoglobin, deoxyhemoglobin, melanin, collagen, water, and lipid can be seen in Fig.4.3. Within brain imaging, generally melanin and collagen will not be present in significant enough concentration to be of concern. However both water and fat will be present in high concentration.
concentrations and can contribute a significant component to the photoacoustic signal. Water and fat signal will be most notable in areas with limited hemoglobin, but can be calculated everywhere. Thus in our experiments we examined the contribution of four chromophores: oxyhemoglobin, deoxyhemoglobin, water and fat. Since the signal for fat and water were both of a much lower magnitude than that of hemoglobin, it was initially uncertain whether or not the signal for fat and water would be significant enough to be discerned and quantified.

The initial testing of lipid quantification using photoacoustics was done using 18% and 27% lipid content phantoms.

The integrated intensity across a selected region of interest was plotted as a function of wavelength, Fig.4.4. Since the specimens were primarily water, not fat, the over-all spectra of the specimens for a broad ROI does not immediately resemble that of fat. However, when a subtraction of the two specimens is performed, as shown in Fig.4.5, a more recognizable spectrum is revealed. The known extinction coefficients for the fat spectra was taken and graphed for comparison with this difference. Not only does the major peak for fat at 930nm show up, but the smaller
peak at 765nm is also apparent. The fit of this data can be improved though several methods: (1) using more careful ROI selection, (2) chromophore demixing, and (3) applying a constant shift to account for negative values. These more advanced image analysis tools were applied to later in-vivo images where they were necessary to obtain significant results. The fact that the spectrum can be clearly seen without any further image processing or image mining techniques demonstrates how strong and evident this result is. This demonstrates the ability of photoacoustic imaging to determine fat content and presence or lack of fat within a region.

Further analysis of the data selected specific regions within the image based on their spectra. The spectral plot of these ROI is shown in Fig. 4.6 These regions were highly correlated, R=0.99+-0.001, with the absorption spectrum for lipids. Furthermore, the ratio of the spectrum of these regions at the 930nm peak for the 18% and 27% lipid samples was calculated as 62%, which is close in value to the actual difference in fat content of 50%. This result provides motivation for quantitative fat measurement within specimens.
4.2.3 Lipid Quantification in Animal Models

Initial animal imaging was done using a rat brain. The larger size of the rat brain compared to that of mice allowed for easier visualization of distinctive structures.

Within Fig. 4.7 three separate image slices were shown: (1) sagittal view taken along the midline of the brain, (2) a coronal view taken close to anterior portion of the brain through the cerebellum, and (3) a coronal view taken close to the central region of the brain. The corresponding histology to these regions can be viewed in Fig. 4.8. The first row of images in Fig. 4.7 display the signal intensity, rows 2 and 3 show chromophore maps obtained through demixing spectral analysis; lipid and
Figure 4.7: Ex vivo image of rat brain. Three separate image slices: sagital mid-line (i)-(iii); coronal cerebellum (iv)-(vi); and coronal pericentral (vii)-(ix). Three image types: signal intensity (row 1); lipid and water content (row 2); and oxy and deoxy hemoglobin content (row 3).

Figure 4.8: Histology images for corresponding areas of a rat’s brain.
water are displayed on row 2; oxyhemoglobin and deoxyhemoglobin are displayed on row 3.

Notably, with normal animal models, only coronal views are accessible due to the geometry of the scanner and the animal. However, work done with extracted brains allowed for adjustment of orientation within the specimen holder, allowing for sagital views to be taken.

Within the sagital slice, four different regions were examined: the forebrain (FB), the corpus callosum (CC), the thalamus (Th) and the cerebellum (Cb). The most notable comparison can be seen in the histological depiction of the sagital view of the cerebellum in 5.3a and the water-lipid chromaphore map shown in Fig.4.7 (ii). Finger-like, lipid dense projections of myelin seen in histology are also visible within the lipid spectra of the cerebellum region. Similarly, in the coronal view of the cerebellum, the structure of lipid rich myelin projections that are seen in histology are roughly mirrored within the lipid spectral component of the image. Other lipid rich, highly myelinated structures such as the corpus callosum and the cerebellum were also shown to be highly correlated to the lipid absorbance spectra: R = 0.83 +/- 0.02 and R = 0.72 +/- 0.03. Unmyelinated, grey matter regions such as the forebrain were shown to be low in lipid and instead bore a high correlation to the water spectra: R= 0.97 +/- 0.003. Within images (viii) and (ix) of the pericentral region of the brain, we observe the same spatial and structural distribution seen in the corresponding histology, Fig. 5.3b.

Further work went towards investigating models of myelin deficiency. Chromophore maps separated into oxy-deoxy and lipid-water for coronal images taken of a control and shiverer mouse can be seen in Fig. 4.9. A sagital slice of an EAE mouse with similar chromophore separation can be seen in Fig. 4.11.

In the comparison of the Shiverer mouse brain and the control mouse brain, the majority of the gross anatomy and distinguishable features are seen in both sets of images, Fig.4.9. In the oxy-deoxyhemoglobin maps, the cross section of major blood vessels are easily distinguishable and localized. Arteries such as the sagital sinus (SS) and the middle cerebral artery (MCA) can be seen as sections of higher
Figure 4.9: Coronal view of control mouse brain (i) and (iii); and Shiverer mouse brain (ii) and (iv).

Figure 4.10: Signal intensity coronal view of control mouse.
oxyhemoglobin, locations labeled in Fig. 4.10. Veins such as the temporal vein (TV) can be examined though localizing the region of high deoxyhemoglobin concentration in the corresponding location.

The most notable difference between the control and shiverer mouse brain is the greatly decreased lipid concentration along highly myelinated areas, mainly the central region corresponding to the corpus callosum. This evident feature confirms the ability to determine fat signal and image the effect of demyelination.

Figure 4.11: Sagital view of EAE mouse brain.

Similarly, in Fig.4.11 we see greatly decreased lipid signal compared to the regular sagital slice shown in Fig. 4.7 (ii). While there still exists a visible central region corresponding to lipid and myelin, this section is far less extensive and has a lower relative concentration of lipid. This matches the expected result of demyelination in the EAE model and corresponding lower lipid signal.
4.3 Conclusions

Chromophore demixing of photoacoustic signals allows for the isolation the lipid content and generation of lipid-content maps. These maps allow for the localization of fatty structures within the brain and the comparison of healthy specimens to different models of demyelination: Shiverer and EAE mice. The ability of photoacoustic imaging to image fat content demonstrates that it is a promising technique for imaging of various types of demyelination, and suggests that it could be used for diagnosis or assessing treatment of demyelinating diseases such as multiple sclerosis.
Chapter 5

Photoacoustic Imaging of Photothrombotic Stroke

To further investigate the ability to classify blood clots, work was done to induce and image blood clots within an animal model. Motivation and background for the general investigation of photoacoustic imaging for blood clots and stroke is discussed in previous sections (1 & 3). Moving into animal models allowed for a more physiologically accurate and relevant assessment of blood clots and resultant stroke.

5.1 Experimental Overview

For stroke work done within the sets of experiments, white mice (C57bl/6) were used. Mice were used between the ages of 4 to 7 weeks with weight ranging between approximately 20 grams to 35 grams.

Initial preparation occurs similarly to the procedure for the demyelination models. The mice were then transferred to a preparation area where the mice are connected to a nose cone. The hair surrounding the desired imaging plane (head and neck) was removed using an electric shaver and sequentially commercial hair removal cream. During this preparatory period, the isoflurane was reduced to approximately 2.5%. The anaesthesia was adjusted to keep the mouse unresponsive.
to a toe-pincher test while maintaining steady breathing rate above 1Hz. A heating pad was placed under the mouse in this preparatory area to maintain the mouse’s body temperature.

An EMLA cream was spread over the shaved head of the mouse for analgesic effect in preparation for surgery. After 5 minutes of application, the EMLA was removed and the mouse’s head cleaned. The periosteum was removed between the Bregma and Lambda cranial ridges using a scalpel, surgical scissors and forceps. Using the scalpel the surrounding skull was exposed and cleared of connective tissue. Using a fine tipped drill bit, a 1.5mm diameter hole was drilled into the skull thinning it for light guide placement. A flexible fiber optic light guide (1.5 mm inner diameter and 3.0 mm outer diameter, Edmond optics, Stock #02-551) was fed into the hole and secured using cyanoacrylate. The cyanoacrylate was cemented using sodium bicarbonate applied as a powder on top of the adhered region. The completion of this portion of the procedure is shown in Fig. 5.1. A 29 gauge tail-vein catheter was placed and secured with medical tape. A 1 ml saline filled syringe was connected to test the quality of catheter placement and protect the distal end of the catheter tube. The distal end of the catheter was coated in a solution of heparin (0.53mg/mL), and heparin was rinsed through the connective tubing. Every half hour after the completion of the tail-vein catheter placement a small quantity (<0.1mL) of heparin was administered though the catheter to prevent blood clotting.

Upon completion of surgery, the mice were transferred to an animal holder where it was attached to an isoflurane nose cone and placed within a plastic foil hammock. Ultrasound gel was placed over the shaved areas of the mouse’s head and neck to allow for an air-free coupling between the plastic foil and the mouse. The plastic hammock was secured using magnetic strips and folded into an upright position. Both the tubing to the tail-vein catheter and the fibre-optic cable were fed up from the plastic hammock and secured to the top of the animal holder.

The mice were transferred to the imaging chamber and reconnected to the isoflurane. The plastic hammock was submerged in water within the imaging chamber. The fibre-optic cable was fed though ports in the imaging chamber lid. The dis-
tal end of the fibre-optic was direct-coupled to a green 565 nm LED (Thorlabs, M565D2), which acts as the excitation source for the photothrombosis. An initial 40mW of un-focused light measured to deliver approximately 11mW of power at the fiber-optic distal end. Using system controls and a camera video feed, the mouse was moved along a track to align the mouse with the photoacoustic system. Photoacoustic preview images allowed for more precise positioning and horizontal/vertical adjustment for centring of the specimen within the imaging window.

After preliminary baseline imaging, an injection of Rose Bengal of concentration 15mg/mL and dosing 100mg/kg, (approximately 0.06mL per 30g mouse) was injected through the tail vein catheter. The LED was then turned on and an imaging series was recorded for 5 minutes. After the 5 minutes, the LED was turned off and post-stroke spectral scans were recorded. A bolus injection of indocyanine green
(ICG) (2mg/kg) was applied to examine leakage post stroke. A spectral imaging series of approximately 2 minutes was recorded to determine the ICG leakage. Preliminary attempt at the ICG injection were unsuccessful due to what appeared to be a obstruction to vessels entering the brain preventing penetration of ICG into the brain. Protocol permitted administering tissue plasminogen activator (tPA) at 10mg/kg to examine the reversal of the stroke and recovery within the peri-infarct zone. Experiments involving tPA are to be carried out as future work.

The mice were sacrificed after photoacoustic imaging though a 0.2mL pentobarbital injection.

5.2 Verification of Rose Bengal Activity

Prior to photothrombotic stroke experiments being attempted in the closed submerged iThera photoacoustic machine, the activity of Rose Bengal was verified on a microscope system. Using an Olympus BX61WI microscope with a 4x air-immersion objective lens (XLFuor4x/340, NA=0.28), vessels within the skull were more full exposed in this preliminary experiment through removing a 4x6mm oval bone chip. Instead of the LED coupled to a fiber optic cable providing excitation for Rose Bengal, a mercury arc lamp was used with a 535-555nm bandpass filter.

Maps of blood flow were constructed from laser speckle contrast imaging (LSCI). Shortly after applying the LED light, blood flow showed a dramatic decrease. This effect is observed in Fig. 5.2.

![Figure 5.2: Blood flow (speckle contrast) after stroke induction.](image-url)
The inner area tracked by the red line demonstrates the total flow across the central region (calculated by LSCI). The total plot demonstrates the effect over approximately 1 minute of time. Within 20 seconds of the light being applied the flow within the area is less than 25% of full flow. The blue line tracks peripheral area, which takes slightly longer to experience the same effects. This is due to this region being further from the focus of the beam of light and thus receiving lower intensity light, and rose bengal taking longer to fully activate.

The LSCI blood flow maps from before and after the application of the light and induced blood clotting can be seen in Fig. 5.3. The conclusion derived from this experiment was that we were successfully able to cause a phototoxic effect resulting in blood clot formation using an approximate power of 2mW/mm$^2$. This provided justification that the 11mW from the distal tip of the fibre-optic cable should be sufficient for inducing a stroke so long as the focal area is less than 5.5mm$^2$.

5.3 Oxyhemoglobin and deoxyhemoglobin temporal dynamics during stroke

The results of the photothrombosis were most clearly visualized though chromophore demixing to determine the relative concentration of oxy-deoxy hemoglobin. In
Fig. 5.4, the post photothrombosis image of the brain is shown with an evident and abnormal region of high deoxyhemoglobin not present in baseline scans. This scan shows the effect of clotted blood reducing the blood flow to the region and causing hypoxia.

This result is shown temporally in Fig. 5.5 which tracks the concentration of oxy-deoxy hemoglobin within the region. The plot shows that a relatively stable oxy-deoxy ratio was maintained prior to the application of the LED and induced ischemia (t=1min 20s). Shortly after the application of the LED, the concentration of oxy-hemoglobin is shown to drastically fall, while the concentration of deoxy-hemoglobin increases correspondingly. This result once again shows the result of the induced ischemia resulting in hypoxia.

5.4 Statistical Analysis of PA Signal in Stoke Induction

Using similar methods of first order and second order statistical analysis applied on the phantom data, the region corresponding to the predicted clot location was analysed across the recorded time series. First order statistics failed to reveal any notable trends.

Examination of second order statistics: GLCM contrast and correlation did result in notable trends. From previous examination of clotted and unclotted blood,
clotted blood has much lower values for GLCM contrast and slightly higher values for GLCM correlation. Corresponding to the phantom data, the GLCM values progress from higher contrast and lower correlation (characteristic of unclotted blood) at the beginning of the imaging time line to lower values of contrast and higher values of correlation (characteristic of clotted blood) as seen in Fig. 5.6.

A linear fit can be constructed for this data to plot the progression from unclotted to clotted blood. This can be seen in Fig. 5.7. While the linear fits do trend in the expected directions: decreasing for GLCM contrast and increasing for GLCM correlation, the $R^2$ values are relatively poor at 0.475 and 0.585 for contrast and correlation linear fits respectively.

5.5 Conclusions

Using chromophore demixing, the ratio of oxyhemoglobin to deoxyhemoglobin was examined though the course of an induced photothrombotic ischemia. Spatial and temporal images revealed decreasing oxygenation of blood in the superior portion of the brain proximal to the fiber-optic cable. This demonstrated hypoxia following the induced ischemia. The region of hypoxia was then further examined using GLCMs and a second order statistics measure of contrast and correlation. The examined texture of photoacoustic images within the isolated ROI showed decreasing contrast
and increasing correlation, which corresponds to the change from unclotted to clotted blood. These in vivo findings further support the claim that photoacoustics is a promising imaging modality for classification of blood clots and could have the potential for clinical diagnosis of stroke and evaluation of stroke treatment.
Figure 5.6: Plotted contrast and correlation for the photothombosis timeline.
Figure 5.7: Linear fits for contrast and correlation plots for the photothombosis timeline
Chapter 6

Future Work

The primarily focus of this thesis was the identification of signal components that could be extracted from photoacoustic images and discerning how the signal intensity components could be processed and analysed to provide useful information about stroke and multiple sclerosis (MS). Primary result of the thesis was the ability to isolate and examine useful signal, in GLCM textural analysis, and chromophore demixing (oxy-hemoglobin, deoxy-hemoglobin, water and lipid), and to relate these signals to structural components of the brain (frontal brain, corpus callosum, cerebellum, sagittal sinus, temporal vein), and abnormalities, (blood clots and demyelination).

The specific contributions of this work not present in literature are: (1) classification of clotted and unclotted blood using photoacoustics in ex-vivo and in-vivo samples from textural features, (2) resolving lipid rich brain structure using PAI, (3) in-vivo tomographic PAI of an induced stroke model and localization of hypoxia. The classification of clotted and unclotted blood had previously been done through laser speckle contrast and though multi-frequency ultrasound, but had not been done through PAI. Previous MRI imaging was used to resolve lipid rich brain structures to track demyelination in diseases such as MS, however, this had not be done before using PAI. While stroke is currently imaged using CT and MRI, brain imaging of stroke using PAI was unique to this thesis [6]. Result in blood clot and stroke imaging, if verified and repeated, could be published as a proof of concept for
stroke imaging using PAI. If the algorithms and approach developed for the iThera MSOT were applied to a hand held PA device, and tested over a large data set, it could lead to PAI being used as a point of access tool for stroke patients. Results in lipid imaging and demyelination could lead to PAI being used as a research tool for MS. If issues surrounding the penetration depth of PAI and ability to image though adult skulls are solved, PAI could be used for clinical analysis of MS.

The three central extensions to this work would be: (1) improving the qualitative verification, (2) improving the ability to make quantitative claims, and (3) extending the scope of the project to examine the ability to assess other functional interactions such as treatment methods. Qualitatively, imaged structures with mice brains were correlated to the expected anatomy and relative fat contents were compared to other imaged mice; performing histology (Luxol fast blue) would allow for a more accurate comparison of fat localization and structure. Similarly, while the induced stroke was observed to cause oxygenation changed in the expected approximate region, preforming histology (Triphenyl tetrazolium chloride, TTC) would allow for verification of the exact location and extent of the infarct zone. Having a better gold standard in these quantitative analysis would lend greater impact and validity to the observed results. Quantitatively, much of the performed image analysis started from a point of non-linearity. Negative values for signal intensity and uneven intensity distributions from uniform samples indicated that additional pre-processing of the data is required before certain quantitative statements about the observed specimen could be made. For example: indicating the exact lipid concentration of a pixel is not something we can currently do reliably; performing preprocessing normalization could allow for linearity to be restored, and exact concentrations to be calculated. Additionally on the quantitative assessment, the current experiments have acted as a proof of concept, frequently with low values of repetition (n=1 or 2) but to show consistency and allow for statistically significant measure to be taken, increasing to n=8 per experimental group is necessary. Finally, extensions of the project into examining treatment options such tPA would bring the research towards to clinical relevance.
The two essential extensions are: (1) performing histological analysis so that work done in this thesis can be related to a gold standard and (2) increasing the number of trials performed (amount of imaged animals) to verify the result is consistent and repeatable. Other extensions would improve the quality of work and allow for high complexity of analysis, but are not considered essential.

6.1 Other Methods of Analysis for Current Data

6.1.1 Intensity Normalization

Data obtained within the iThera MSOT exhibited signal intensity with a depth dependency. An example of this being problematic is when uniform phantoms, as observed in lipid phantoms, produce non-uniform intensity maps. The images resultantly have non-uniformities within the spectra of the lipid ROI and upon evaluation contain different lipid concentrations based on location. Using phantoms of known optical absorption, the signal intensity at different depths can be accounted for and normalized accordingly. Thus an intensity correction map could be constructed and applied to imaging data. This would be done with phantoms constructed with cylindrical segments of different diameters and with different concentrations of lipid, as seen in Fig.6.1.

Figure 6.1: Set-up for lipid phantom.
Lipid concentration would be varied to create phantoms at intervals of every 10% fat between 0% fat and 100% fat. This would allow for attenuation to be normalized based on the depth and concentration of obstructing chromophore. Due to the differing attenuation at each frequency, a different normalization map would have to be created for each wavelength. If successful, lipid phantoms of uniform lipid concentration will display uniform intensity values once normalized and spectra de-mixing will display a map of uniform fat content within the sample ROI. This would allow for reported results from the system to be more quantitatively meaningful.

6.1.2 Image Reconstruction

Additionally, the method of image reconstruction from the photoacoustic raw data could be improved upon. Two identified issues are: (1) handling negative values (conversion from ordinal to ratio-metric values) within the signal intensity maps (2) removal of unwanted suppression of speckle during reconstruction. Since data is currently being processed though the given iThera MSOT reconstruction algorithm, which is proprietary, there is a black box from the raw data to the image data. The types of filtering or averaging being applied to the data is unknown and could be resulting in suppression of useful noise (speckle) and poorly conditioned assumptions could result in negative values for signal intensity. Since the raw data from the iThera system is available, is it possible that a different implementation of the image reconstruction could provide better images for analysis.

6.1.3 Temporal Analysis

Further work with classifiers and GLCM matrices could be performed on the ischemic stroke experiments to obtain more promising results. Through either performing temporal averaging or examining the data and GLCM statistics within a certain length temporal window (10-100 frames), the large amount of observed noise in the data could be decreased. This could allow for a more significant and reliable result when plotting the temporal data over the ischemic stroke time line.
6.2 Future Experiments

6.2.1 Clot Formation and Chronic Clot Evaluation

Work done within the thesis primarily examined blood phantoms on a binary scale, either unclotted or clotted, with clotted blood being greater than 8 hours old. By creating and imaging clots over a larger time scale, the isolated clotting process and corresponding change to photoacoustic signal could be better understood. This is applicable both to the formation of blood clots and the ageing of blood clots. The formation of the clot that happens on the time scale of minutes, which could be studied in and experiment where the blood is imaged while it clots over the course of an hour during which it is continually imaged. The ageing of blood clots, and compositional changes take place over the course of days from fresh (<1 day) to lytic (1-5 days) and organized (> 5 days). [42]. This ageing process and compositional changes happens primarily within a physiological environment; to study this a clot would have to be induce into an animal model and imaged over a series of days to evaluate changes in PA signal.

6.2.2 Histological Verification of Lipid and Blood Clot Localization.

To ensure the photoacoustic localization of lipid rich structures and blood clots is accurate, histological verification could be performed.

For both EAE mice, Shiverer mice and control mice used in the demyelination studies, histology would allow for correlation of the observed structures and verification of the decreased fat content observed in photoacoustic images. Using a Luxol fast blue solution, high myelin content white matter can be differentiated from grey matter. The brain will be perfused with phosphate buffered saline (PBS), and extracted. The brain will be sliced using either a vibratome or though cryosectioning. The slices will be hydrated in ethanol progressing from a 35% solution up to a 95% solution, and then placed in a Luxol fast blue solution for approximately 16 hours. After the elapsed time, slides will be differentiated in lithium carbonate solution for
30s and finally rinsed with 95% ethanol and then distilled water. The white matter should appear a blue-green colour while grey matter appears clear. An example of a Luxol fast blue stain is shown in Fig.6.2.

Figure 6.2: Luxol fast blue staining.

For mice with photochemically induced stroke, histology would allow for verification of the location and size of the induced stroke to be identified and compared to the photoacoustic results. For examination of stroke, TTC staining will be performed. The brain will be perfused with PBS and then removed from the skull. The brain will be placed on dry ice until frozen and then sectioned within a brain matrix at a slice resolution of 2mm. The slice will then be incubated in TTC at a temperature of 37 degrees Celsius. Finally, slices will be removed from incubation and transferred to a paraformaldehyde (PFA) solution to halt the reaction. The resulting slices should remain white in the infarct region marking dead brain cell and be stain red in the remainder of the brain containing living cells. An example of a successful TTC staining for stroke localization can be seen in Fig.6.3 [13].

6.2.3 Additional Animal Experiments

To ensure statistical significance of the examined stroke data, a larger cohort should be examined. By completing at least 8 separate examples of photothrombotic strokes
within the mouse model, statistically significant measures could be examined. Since the brain structure and spectrum can be imaged before and after the induced stroke, the specimens can act as their own controls.

Additionally, increasing the size of the light excitation and either moving into whole brain ischemic stroke induction or changing animal models to a rat model could allow for a larger region of the brain to be affected by the stroke. Relatively small focally induced blood clots are more difficult to examine GLCM statistics at the current imaging resolution. Increasing the size of the impacted area would allow for a larger number of pixels to be examined and analysed, which would greatly improve the accuracy of examined trends for any future classifier.

6.2.4 Post Stroke Blood Brain Barrier Evaluation

Another component to the experiment, which has yet to be full completed, is examining leakage of blood vessels and blood brain barrier using indocyanine green (ICG). Using a tail-vein injection of ICG, a contrast agent with a distinct spectra, the amount of ICG maintained within the blood vessels can be quantified as well as any ICG which has leaked out of the blood vessels. Within models of demyelination, particularly EAE mice, the extent of BBB leakage can be assessed. Within the photothombotic stroke experiments, ICG injections have already been incorporated into the work flow. An injection of ICG before and after the stroke could be compared. This model would allow for the extent of the stroke to be assessed. Clotted blood inhibits movement to affected regions of the brain and should prevent ICG from
entering these regions, creating a difference in spatial ICG distribution before and after the stroke. Preliminary analysis of ICG spectrum has been conducted but a full analysis of the stroke experiment has yet to be performed. Literature indicates ICG can be distinctly resolved within MSOT imaging, as indicated in a study of Glioblastoma characterization, where ICG was used for tumor visualization. [14]

![Figure 6.4: Mouse brain with intraventricularly injected ICG. Taken from: [14]](image)

### 6.3 Evaluation of Blood Clot Treatment

An additional proposed experiment, which has yet to be conducted, is the assessment of treatment of blood clots under photoacoustic imaging. Both acetylsalicylic acid (ASA) and tissue plasminogen activator (tPA) are clinically used for stroke prevention/treatment. They have been shown though other imaging modalities to reduce the infarct size of stroke. ASA would be administered at a dose of 30mg/kg dissolved in distilled water through an intraperitoneal injection 2 hours before photothrombosis. Alternatively, tPA would be administered at a dose of 10mg/kg dissolved in saline through an intravenous injection 30 minutes after photothrombosis. The ability of photoacoustic imaging to visualize and assess the efficacy treatment options of ASA and tPA is important in its potential role for image informed therapy. Upon ensuring the effects of tPA are visualizable, applying the tPA injection at different time points could assess to examine the efficacy of tPA on different ages of clots. While clinical data suggests that tPA is most effective when administered within three hours of the stroke onset, the exact interaction and time-line could be more thoroughly investigated in in-vivo settings to allow for a rigorous guideline. Other characteristics that impact the efficacy of tPA such as clot thickness, vessels size and location could be sequentially investigate.
Other discussed treatment options such as HIFU could also be assessed using photoacoustic systems. Currently examining HIFU lysed blood in the iThera MSOT system seems to be the most reasonable option as an addition to the phantom work. Ultimately, real-time photoacoustic evaluation of HIFU could be possible, but logically this would not be done using the current iThera MSOT system.
Chapter 7

Appendix

7.1 Mouse Imaging Protocol

Optoacoustic tomographic imaging of demyelination and associated vascular leakage in MS-mimicking mouse models

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7.2 Project Goals

Myelinated axonal tracts contain a higher percentage of fat, which has a spectroscopic absorption maximum just below the NIR window, which we seek to measure with MSOT. Our engineering objectives are to measure changes associated with demyelination by optimizing the image reconstruction process applied to the recorded acoustic data. Our experimental hypothesis is that demyelination is associated BBB compromise. Small molecule contrast agents suitable for MSOT vascular imaging can potentially be used to measure BBB compromise by investigating tissue accumulations. This protocol is precursory to future studies using piglets that will investigate factors effecting myelin formation during development.
7.2.1 Timeline in Chronological order

- Day 0: Date of birth or juvenile EAE induction.
- Day 13: Remove hair from scalp.
- Day 14: MSOT imaging.
- Day 20: Remove hair from scalp.
- Day 21: MSOT imaging.
- Day 27: Remove hair from scalp.
- Day 28: MSOT imaging.
- Day 34: Remove hair from scalp.
- Day 35: MSOT imaging and sacrifice.

7.3 Lay Summary

Two mouse models of MS will be imaged over four weeks.

7.4 Multiple Sclerosis Models

7.4.1 The Shiverer Mouse

The model of MS we will investigate is the Shiverer mouse line from Jackson Laboratories C3HeB/FeJ-shiverer with the control mouse line C3HFe. We seek to determine if demyelination is a consequence of BBB leakage or merely associated with leakage. We will image animals at one week intervals for from postnatal week two (P14) to week five (P35) to assess the relative time-line of demyelination and BBB breakdown.
7.4.2 The EAE mouse

We will use an EAE model of MS using adult Bl6 mice (30 grams). The mice will be immunized with 100 microgram of MOG split into the four flanks (25 microgram, in each shoulder and hip). For a subset of animals this will be followed by administration of PTX. We seek to determine if demyelination is a consequence of BBB leakage or merely associated with leakage. We will image animals at one week intervals for following EAE induction from week two to week five to assess the relative time-line of demyelinaion and BBB breakdown.

7.5 Day before preparation

Head and neck of the mouse will be shaved the day before imaging studies under isoflurane anesthesia to reduce hair interference. An electric shaver will be used for initial hair removal followed by Veet hair removal cream. The animal will be kept overnight in a cage with a small heating pad under one corner of the cage to prevent the animal from becoming chilled.

7.6 Imaging Procedure

Mouse will be anesthetized using 5% isoflurane in an induction box. The mouse will then be transfered to a nose cone and the isoflurane will be reduced to 3% and gradually to 2%. A 29 gauge tail-vein catheter will be placed and secured with medical tape. A 1 mL saline filled syringe will be connected to test the quality of catheter placement and protect the distal end of the catheter tube.

The mouse will be secured within the animal holder using a “hammoc” consisting of plastic foil that will serve as a coupling membrane with the imaging system. Once the animal is secured, anesthesia will be disconnected while the animal holder is moved into the imaging chamber and anesthesia is reconnected (built into imaging system). The system is a MSOT 128 system (iThera, Germany). The animal will then be re-positioned as necessary within the chamber, the scan regions will be
defined, and imaging will proceed using pulsed near-infrared laser illumination.

For de-myelination imaging, tomographic near infra-red spectral scans will be performed across the corpus callosum and the cerebellum for a period of no more than 20 min. A consistent coronal regions where the blood brain barrier is suspected to have become compromised will be selected. A bolus injection of ICG will be delivered via the tail vein. A continuous imaging sequence will be performed for 10 min to track vascular leakage at the absorption maximum for ICG of 795 nm. A final tomographic scan at 795 nm will be performed to check for leaky regions outside the chosen coronal plane.

7.7 Histology – End point

7.7.1 Perfusion

Following the final imaging session (P35 Shiverer or five weeks after EAE induction) a cardiac profusion will then be performed using 20 mL of a Phosphate-buffered saline (PBS) under isoflurane anaethesia. Profusion needle will be inserted into the left ventricle and the right atrium will be cut using scissors.

7.7.2 Fixation

The brain will be removed and placed in 10% NBF solution for over 48 hours. The brain should appear off white if perfusion performed correctly.

7.7.3 Sectioning

After fixation the brain will be rinsed in PBS. The brain will be sectioned on a vibratome into 30 um slices.

7.7.4 Staining

The slices will be hydrated to 95% ethanol (first 35%, then 50%, 70% and 95%). The slices will be placed in Luxol fast blue solution (from Sigma-Aldrich) at 56 C
(is a 95% ethanol solution) for no longer than 16 hours. Excess stain will be rinsed off with 95% ethanol. Then rinsed with distilled water.

### 7.7.5 Differentiation

Differentiate slides in lithium carbonate solution for 30 s. Then in 70% ethanol for 30 s. Then rise with distilled water. Check under microscope, repeat differentiation is necessary. Note: 70% ethanol removes fast blue stain so use sparingly. White matter should be stained (blue-green) and gray matter clear. Store in distilled water.

### 7.7.6 Counter-staining

Optional – counter-stain in cresyl violet solution for 30-40 seconds. Rinse in distilled water. Differentiate cresyl violet in 95% ethanol for 5 minutes.

### 7.7.7 Mounting

Then 100% ethanol for 5 min and repeat. Then xylene for 5 min and repeat.

### 7.8 Creating Contrast Graphs

This script loaded and analysed clotted and unclotted blood samples, isolated the ROI containing the samples and created contrast maps using a 5x5 window. These maps were then cropped to remove outer portions of the ROI (where the window extended beyond the region) and the inner regions were examined. The average contrast values was calculated for each wavelength and plotted spectrally for all samples.

```matlab
% Contrast Graphs
load('normData12Samples.mat')
testSlice=squeeze(normlogData(1,1,:,:));
windowSize=7;
[contrastMap]=contrastCalc(testSlice, windowSize);

%% Display original Image for x=1:12
testSlice=squeeze(normlogData(x,1,:,:));
figure(x);
DisplayMapLow = 30; %dB
```

100
DisplayMapHigh = 90; %dB
imagesc(WidthAxis, DepthAxis, testSlice, [DisplayMapLow DisplayMapHigh]);
colormap(redmap)
colorbar
axis equal
axis tight
xlabel('(mm)')
ylabel('(mm)')
if (x\cdot=6)
title(sprintf('Sample %d- Clotted blood, Wavelength=680nm',x))
else
title(sprintf('Sample %d- Unclotted blood, Wavelength=680nm',x))
end
end

%Create a contrast graph for clotted and unclotted blood
windowSize=7;
for x=4:4
    testSlice=squeeze(normlogData(x,1,:,:));
    [contrastMap]=contrastCalc(testSlice, windowSize);
    figure(x+12);
    DisplayMapLow = 0;
    DisplayMapHigh = .2;
    imagesc(WidthAxis, DepthAxis, contrastMap, [DisplayMapLow DisplayMapHigh]);
colormap
colorbar
axis equal
axis tight
xlabel('(mm)')
ylabel('(mm)')
if (x\cdot=6)
%% Images for wavelengths at 680, 800, 970
waveNum=[1,25,59];
selectWaveLen=[680,800,970];
for x=1:3
    waveLen=waveNum(x);
testSlice=squeeze(normlogData(4,x,:,:));
[contrastMap]=contrastCalc(testSlice, windowSize);
figure(x);
DisplayMapLow = 0;
DisplayMapHigh = .2;
imagesc(WidthAxis, DepthAxis, contrastMap, [DisplayMapLow DisplayMapHigh]);
colormap
colorbar
axis equal
axis tight
xlabel('(mm)')
ylabel('(mm)')
title(sprintf('Speckle Contrast Map (Clotted Sample 4, %d nm)',selectWaveLen(x)));
end

% Plots for contrast dependence of wavelengths
clotContrastVal=zeros(6,59);
windowSize=7;
for sample=1:6
    sample
for freq=1:59
freq
slice=squeeze(normlogData(sample,freq,:,:));
[contrastMap]=contrastCalc(slice, windowSize);
ROI=contrastMap(155:280-windowSize,98:326);
clotContrastVal(sample,freq)=mean(mean((ROI)));
temp=squeeze(clotData(sample,freq,155:280,1:98));
gelAvgValROI(sample,freq)=mean(mean(temp));
end
end
save('clotContrastVal','clotContrastVal');
waveLen=680:5:970;
figure(1)
hold on
for sample=1:6
plot(waveLen,clotContrastVal(sample,:));
end
xlabel('Wavelength (nm)')
ylabel('Average Contrast Value')
title('Mean Contrast Value of Clotted Blood')
legend('Clot 1','Clot 2','Clot 3', 'Clot 4', 'Clot 5', 'Clot 6');
hold off
% Unclotted blood contrast
load('normData12Samples.mat')
unclotContrastVal=zeros(6,59);
unclotContrastVal WINDOW=zeros(6,59);
windowSize=7;
for sample=7:12
sample
for freq=1:59
freq
slice=squeeze(normlogData(sample,freq,:,:));

Select Window

[contrastMap]=contrastCalc(slice, windowSize);
ROI=contrastMap(155:280-windowSize,98:326);
unclotContrastVal(sample-6,freq)=mean(mean((ROI)));

%Full window

window=slice(155:280-windowSize,98:326);
shapedData=reshape(window,1,numel(window));
standardDeviation=std( shapedData);
meanValue=mean( shapedData);
unclotContrastVal_Window(sample-6,freq)= standardDeviation/ meanValue;
temp=squeeze(clotData(sample,freq,155:280,1:98));
gelAvgValROI(sample,freq)=mean(mean(temp));
end
end

save('unclotContrastVal','unclotContrastVal');
save('unclotContrastVal_Window','unclotContrastVal_Window');

function [contrastMap]=contrastCalc(slice, windowSize)
offset=(windowSize-1)/2;
[row,col]=size(slice);
contrastMap=zeros(row-windowSize+1,col-windowSize+1);
for x=offset+1:row-offset
for y=offset+1:col-offset
window=slice(x-offset:x+offset,y-offset:y+offset);
shapedData=reshape(window,1,numel(window));
standardDeviation=std( shapedData);
meanValue=mean( shapedData);
contrastMap(x,y)= standardDeviation/ meanValue;
end
end
7.9 Contrast Classifier

The classifier applied leave one out testing to the obtained data set to classify clotted and unclotted blood (K-nearest neighbour).

```matlab
%clotContrastVal
%unclotContrastVal
combMeanContrast=zeros(12,59);
combMeanContrast(1:6,:)=clotContrastVal;
combMeanContrast(7:12,:)=unclotContrastVal;
clotTrue=0;
clotFalse=0;
unclotTrue=0;
unclotFalse=0;
kNearest=3;
testWL=[1,25,59];
umWaven=length(testWL);
for x=1:12
    %check vs clotted samples
    clotdist=0;
    for y=1:6
        dist=0;
        for wave=1:numWaven
            dist=dist+(combMeanContrast(y,testWL(wave))-combMeanContrast(x,testWL(wave))).^2;
        end
        clotdist=clotdist+sqrt(dist);
    end
    clotdist=clotdist+sqrt(dist);
    if y==1
        minClotDist=unclotdist;
    end
end
```
end

if(x = y)
if(minClotDist > sqrt(dist))
minClotDist = sqrt(dist)
end
end
end
end

if(x <= 6)
avgClotdist = clotdist / 5;
else
avgClotdist = clotdist / 6;
end

% check vs unclotted samples
unclotdist = 0;
for y = 7:12
dist = 0;
for wave = 1:numWavelen
dist = dist + (combMeanContrast(y, testWL(wave)) - combMeanContrast(x, testWL(wave))).^2;
end
unclotdist = unclotdist + sqrt(dist);
if y == 7
minUnClotDist = unclotdist;
end
if(x = y)
if(minUnClotDist > sqrt(dist))
minUnClotDist = sqrt(dist);
end
end
end
end

if(x <= 6)
avgUnClotdist=unclotdist/6;
else
avgUnClotdist=unclotdist/5;
end

%Compare the distances
avgClotdist=minClotDist;
avgUnClotdist=minUnClotDist;
if x<=6
if avgClotdist<avgUnClotdist
clotTrue=clotTrue+1;
else
clotFalse=clotFalse+1;
end
else
if avgClotdist>avgUnClotdist
unclotTrue=unclotTrue+1;
else
unclotFalse=unclotFalse+1;
end
end
end
end

%Print Statements clotTrue
clotFalse
unclotTrue
unclotFalse
wavelen=680:5:970
figure(1)
hold on
for sample=1:12
plot(waveLen,combMeanContrast(sample,:));
xlabel('Wavelength (nm)')
ylabel('Average Contrast Value')
title('Mean Contrast Value of Clotted Blood');
legend('Clot 1','Clot 2','Clot 3', 'Clot 4', 'Clot 5', 'Clot 6','Unclot 1','Unclot 2','Unclot 3', 'Unclot 4', 'Unclot 5', 'Unclot 6');
hold off

7.10 GLCM Statistics

This section calculates and graphs different statistics for gray level co-occurrence matrices. This code was applied to both blood samples- clotted and unclotted as well as in-vivo data of mice. The code isolated the region of interest calculated GLCM for 5x5 windows across the ROI, derived statistics for each window and created 4 separate maps - contrast, correlation, energy, and homogeneity. The average value of these maps were calculated and plotted as a population in histograms.

```matlab
clotUnclotData1 = (332,332,10,61); 1-5 clot; 6-10 unclot
clotUnclotData2 = (200,200,44,61); 1-27 clot; 28-44 unclot
save('clotUnclotData1','clotUnclotData1');
save('clotUnclotData2','clotUnclotData2');

clottedContrast=zeros(32,1);
clottedCorrelation=zeros(32,1);
clottedEnergy=zeros(32,1);
clottedHomogeneity=zeros(32,1);
E = entropy(imageI) ;
D = pdist(imageI); %dissimilarity
for i =1:5
    imageI = squeeze(clotUnclotData1(:,:,i,61));
    GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1],['GrayLimits',[]]);
```

108
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
clottedContrast(i)=stats.Contrast;
clottedCorrelation(i)=stats.Correlation;
clottedEnergy(i)=stats.Energy;
clottedHomogeneity(i)=stats.Homogeneity;
end
for i =6:32
imageI=squeeze(clotUnclotData2(:,:,i-5,61));
GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1],'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
clottedContrast(i)=stats.Contrast;
clottedCorrelation(i)=stats.Correlation;
clottedEnergy(i)=stats.Energy;
clottedHomogeneity(i)=stats.Homogeneity;
end
for i =1:5
imageI=squeeze(clotUnclotData1(:,:,i+5,61));
GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1],'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
unclottedContrast(i)=stats.Contrast;
unclottedCorrelation(i)=stats.Correlation;
unclottedEnergy(i)=stats.Energy;
unclottedHomogeneity(i)=stats.Homogeneity;
end
for i =6:22
imageI=squeeze(clotUnclotData2(:,:,i+22,61));
GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1],'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
unclottedContrast(i)=stats.Contrast;
unclottedCorrelation(i)=stats.Correlation;
unclottedEnergy(i)=stats.Energy;
unclottedHomogeneity(i)=stats.Homogeneity;
end
unclottedEnergy(i)=stats.Energy;
unclottedHomogeneity(i)=stats.Homogeneity;
end

%% Testing
for i =1:5
[glcmMaps]=makeGLCMMaps(slice, windowSize)
avgContrast=mean(mean(glcmMaps(
imageI=squeeze(clotUnclotData1(:,:,i+5,61));
GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1], 'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
unclottedContrast(i)=stats.Contrast;
unclottedCorrelation(i)=stats.Correlation;
unclottedEnergy(i)=stats.Energy;
unclottedHomogeneity(i)=stats.Homogeneity;
end
for i =6:22
imageI=squeeze(clotUnclotData2(:,:,i+22,61));
GLCM2 = graycomatrix(imageI,'NumLevels',8,'Offset',[1 1], 'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
unclottedContrast(i)=stats.Contrast;
unclottedCorrelation(i)=stats.Correlation;
unclottedEnergy(i)=stats.Energy;
unclottedHomogeneity(i)=stats.Homogeneity;
end

%% Second Order Statistic Maps

%% Contrast maps
testSlice=squeeze(clotUnclotData1(:,:,1,8));
[bwImg, imgROI] = roiSelecter(testSlice);
windowSize=5;
[glcmMaps]=makeGLCMMaps(imgROI, windowSize);
Cropping
offset=(windowSize-1)/2;
[row,col]=size(bwImg);
bwImgCropped=bwImg(offset+1:row-offset,offset+1:col-offset);
contrastMapROI=contrastMap.*bwImgCropped;
[newROI] =shaveROI(bwImgCropped, windowSize);
contrastMapInnerROI2=contrastMapROI.*newROI;

%% Plotting
mapNames={'Contrast','Correlation','Energy','Homogeneity'};
[glcmMaps]=makeGLCMMaps(slice, windowSize);
for x = 1:1
figure(2)
WidthAxis=0:0.075:200;
DepthAxis=WidthAxis;
% DisplayMapLow = 50;
% DisplayMapHigh = 10000;
imagesc(WidthAxis, DepthAxis, glcmMaps(:,:,x));
colormap
colorbar
axis equal
axis tight
xlabel('(mm)')
ylabel('(mm)')
s=strcat(char(mapNames(x)),' Map: Clotted Blood, Sample 1, (680nm)');
title(s)
[axisLimits] = setAxis(bwImg);
axis(axisLimits*200/332);
end

%% Histograms
figure(1)
h1 = histogram(clottedContrast);
h1.NumBins = 20;
hold on
h2 = histogram(unclottedContrast);
h2.NumBins = 20;
h1.Normalization = 'probability';
h2.Normalization = 'probability';
title('GLCM - Contrast')
legend('Clotted','Unclotted')
xlabel('Contrast (GLCM)')
ylabel('Percent of Data')
hold off
figure(2)
hold on
h1 = histogram(clottedCorrelation);
h1.NumBins = 16;
h2 = histogram(unclottedCorrelation);
h2.NumBins = 8;
h1.Normalization = 'probability';
h1.BinWidth = 0.25;
h2.Normalization = 'probability';
h2.BinWidth = 0.25;
xlabel('Correlation')
ylabel('Percent of Data')
title('GLCM - Correlation')
legend('Clotted','Unclotted')
hold off
figure(3)
hold on
h1 = histogram(clottedEnergy);
h1.NumBins = 12;
h2 = histogram(unclottedEnergy);
h2.NumBins = 9;
h1.Normalization = 'probability';
h1.BinWidth = 0.25;
h2.Normalization = 'probability';
h2.BinWidth = 0.25;
title('GLCM - Energy')
legend('Clotted','Unclotted')
xlabel('Energy')
ylabel('Percent of Data')
hold off
figure(4)
h1 = histogram(clottedHomogeneity);
h1.NumBins = 8;
hold on
h2 = histogram(unclottedHomogeneity);
h2.NumBins = 8;
h1.Normalization = 'probability';
h2.Normalization = 'probability';
title('GLCM - Homogeneity')
legend('Clotted','Unclotted')
xlabel('Homogeneity')
ylabel('Percent of Data')
hold off

%% functions
function [glcmMaps]=makeGLCMMaps(slice, windowSize)
offset=(windowSize-1)/2;
[row,col]=size(slice);
glcmMaps=zeros(row-windowSize+1,col-windowSize+1,4);
for x=offset+1:row-offset
for y=offset+1:col-offset
window=slice(x-offset:x+offset,y-offset:y+offset);
GLCM2 = graycomatrix(window,'NumLevels',8,'Offset',[1 1],'GrayLimits',[]);
stats = graycoprops(GLCM2,'contrast','Correlation','Energy','homogeneity');
sampleContrast=stats.Contrast;
sampleCorrelation=stats.Correlation;
sampleEnergy=stats.Energy;
sampleHomogeneity=stats.Homogeneity;
glcmMaps(x-offset,y-offset,1)= sampleContrast;
glcmMaps(x-offset,y-offset,2)= sampleCorrelation;
glcmMaps(x-offset,y-offset,3)= sampleEnergy;
glcmMaps(x-offset,y-offset,4)= sampleHomogeneity;
end
end
end

function [newROI] = shaveROI(ROI, windowSize)
[row, col]=size(ROI);
newROI=zeros(row, col);
offset=(windowSize-1)/2;
for x=offset+1:row-offset
for y=offset+1:col-offset
window=ROI(x-offset:x+offset,y-offset:y+offset);
if(sum(sum(window))==windowSize^2)
newROI(x,y)=1;
end
end
end
end
end

function [axisLimits] = setAxis(bwImg)
colSum=sum(bwImg);
col=length(colSum);
rowSum=sum(bwImg,2);
row=length(rowSum);
for x=1:col
if colSum(x) =0
xmin=x;
break
end
end
for x=xmin:col
if(colSum(x)==0)
xmax=x;
break
end
end
for y=1:row
if rowSum(y) =0
ymin=y;
break
end
end
for y=ymin:col
if(rowSum(y)==0)
ymax=y;
break
end
end
axisLimits=[xmin-3,xmax,ymin-3,ymax];
end
function [bwImg, imgROI] = roiSelecter(imgSlice)
shapedData = reshape(imgSlice, 1, []);
maxData = max(shapedData);
threshold = maxData * .20;
bwImg = imclose(imgSlice > threshold, ones([3 3]));
imgROI = bwImg .* imgSlice;
end

7.11 Analysis of Photothombosis

Performed combined analysis of photothrombotic stroke using tools from previous sections and arbitrary ROI selection.

photothomData1 = squeeze(photothromData1);
imagesc(photothomData1(:,:,1,1))
photothromROI = photothomData1(70:103,143:188,:,:);

%% gathering glcm stats
thromContrast = zeros(999,3);
thromCorrelation = zeros(999,3);
thromEnergy = zeros(999,3);
thromHomogeneity = zeros(999,3);
mapNames = 'GLCM Contrast', 'Correlation', 'Energy', 'Homogeneity';
for i = 1:999
for waveLen = 1:3
imageI = squeeze(photothromROI(:,:,i,waveLen));
GLCM2 = graycomatrix(imageI,'NumLevels', 8,'Offset', [1 1], 'GrayLimits', []);
stats = graycoprops(GLCM2, 'contrast', 'Correlation', 'Energy', 'Homogeneity');
thromContrast(i, waveLen) = stats.Contrast;
thromCorrelation(i, waveLen) = stats.Correlation;
thromEnergy(i, waveLen) = stats.Energy;
thromHomogeneity(i, waveLen) = stats.Homogeneity;
end
end
for x=1:2
    if x==1
dataSet=(thromContrast);
    elseif x==2
dataSet=(thromCorrelation);
    elseif x==3
dataSet=thromEnergy;
    else
dataSet=thromHomogeneity;
    end
    time=0:5/999:5-(5/999);
    figure(x)
    hold on
    dataline=squeeze(dataSet(:,3));
    plot(time,dataline)
    medSize=40;
    medData = medfilt1(dataline,medSize);
    plot(time(medSize/2:999-medSize),medData(medSize/2:999-medSize))
    t=time';
y=dataline;
p = polyfit(t,y,1);
yfit = polyval(p,t);
yresid = y - yfit;
SSresid = sum(yresid.^2);
SSTotal = (length(y)-1) * var(y);
rsq = 1 - SSresid/SStotal;
r=sqrt(rsq)
plot(time,yfit);
xlabel('Time (minutes)')
ylabel(char(mapNames(x)));
s=strcat(char(mapNames(x)),' Map: Photothombosis Timeline');
title(s)
legend('Data Values','Linear Fit');
hold off
end
waveLen=680:5:980;
figure(1)
hold on
for sample=28:32
plot(waveLen,clottedContrast(sample,:));
plot(waveLen,clottedIntsy(sample,:));
plot(waveLen,clottedContrast(sample,:).*laserEnergy(sample,:,1));
end
for sample=18:22
plot(waveLen,clottedContrast(sample,:)/1.11-.010);
plot(waveLen,clottedIntsy(sample,:));
plot(waveLen,clottedContrast(sample,:).*laserEnergy(sample,:,1));
end
xlabel('Wavelength (nm)')
ylabel('Average Contrast Value')
title('Mean Contrast Value of Clotted Blood');
legend('Clot 1','Clot 2','Clot 3', 'Clot 4', 'Clot 5');
axis([650 1000 .30 .36])
hold off
Bibliography


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