TRANSCRANIAL NEAR INFRARED SPECTROSCOPY AND STIMULATION OF
PREFRONTAL COGNITIVE FUNCTIONS IN YOUNG ADULTS

by

SNEHAL NIWRUTTI HASE

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Abstract

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Snehal Niwrutti Hase, M.S.

The University of Texas at Arlington, 2015

Supervising Professor: Hanli Liu

Transcranial near infrared stimulation or Low Level Laser Therapy (LLLT) is an innovative technique shown to control neuronal function in cell cultures, animal models and clinical conditions. The aim of this study was to use near infrared spectroscopy (NIRS) to evaluate the physiological and functional effects of LLLT on prefrontal cognitive functions in young healthy adults. This study was divided into two parts: the first part investigated the physiological effects of LLLT and the second investigated the functional effects.

The aim of the first part of this study was to explore how a single session of LLLT altered the hemodynamic status of the prefrontal cortex. Two experiments were designed to irradiate the therapeutic laser at 1) the centre and 2) the right side of the frontal lobe, while monitoring the changes in hemoglobin concentrations in both hemispheres. The results obtained showed significant hemodynamic changes in responses to the laser stimulation in both the experiments (p<0.01).
The objective of the second part of this study was to evaluate whether a single session of LLLT could induce any beneficial effects on the functioning of prefrontal cortex in attention and short-term memory domains. This was achieved by instructing participants to perform two neurocognitive tasks, namely, Psychomotor Vigilance Task (PVT) and Delayed Match-to-Sample (DMS) memory task, before and after LLLT. PVT was used to evaluate individual’s sustained attention, while DMS memory task was used to examine short-term memory. Functional near infrared spectroscopy (FNIRS) was used to record the hemodynamic responses of the brain while the subjects were performing these tasks. Pre- and post-treatment results were compared to evaluate the effects of LLLT. Results in this part of study showed insignificant improvement in the performance of PVT after LLLT (p > 0.05), but significant improvement in the performance of the DMS task (p < 0.05).

The results in this study indicate that LLLT could lead to the development of non-invasive, performance-enhancing interventions in healthy humans, which in future might be applicable on those in need of neurorehabilitation.
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Chapter 1

Introduction

1.1 Functional Near Infrared Spectroscopy

Functional near infrared spectroscopy (fNIRS) is a noninvasive technique for mapping the human cortex. It uses the principles of near infrared spectroscopy (NIRS). Thus to understand fNIRS the basics of NIRS need to be understood. Human tissues are relatively transparent to light in the near infrared spectral window (650nm-1000nm). Some basic and important characteristics of NIR light are: 1) NIR light is either absorbed by pigmented compounds (chromophores) or scattered in tissues, 2) NIR light is able to penetrate human tissues, since the dominant factor in its tissue transport is scattering, which is typically about 100 times more probable than absorption, and 3) the relatively high attenuation of NIR light in tissue is due to the main chromophore, hemoglobin (the oxygen transport red blood cell protein) [1].

1.1.1 Optical Window

Optical absorption in biological tissue originates primarily from haemoglobin, melanin and water [2]. Lower wavelengths such as violet and ultraviolet appear to have poor tissue penetration, as light absorption by blood and tissue components is very high [3]. Similarly, water significantly absorbs energy at wavelengths higher than 1150 nm. In between these two wavelength regions, the NIR electromagnetic spectrum is termed as the optical window or therapeutic window for noninvasive optical imaging since absorption from blood is reduced significantly in this wavelength range (650nm-1000nm) [3]. In this window, scattering is the most dominant interaction that takes place which indicates that the propagating light becomes highly diffused. Also, light is able to propagate through tissue thickness of up to 15 cm for brain,
breast, and limbs [4]. The absorption spectra of oxygenated hemoglobin (HbO₂) and deoxygenated hemoglobin (Hb) in the optical window can be shown as follows:

![Absorption spectra of HbO₂ and Hb in the optical window](image)

**Figure 1: Absorption spectra of HbO₂ and Hb in the optical window [5].**

From figure 1 it can be seen that the absorption spectra for HbO₂ and Hb are different and can be separated by a few wavelengths. Thus changes in HbO₂ and Hb can be measured in this optical window which is the fundamental principle of optical imaging.

### 1.1.2 Behaviour of Light in Biological Tissue

Photons that enter tissue may undergo the following types of interaction with tissue: (1) absorption which may lead to radiation-less loss of energy to the medium, or induce either fluorescence or phosphorescence, and (2) scattering at unchanged frequency when occurring in stationary tissue or accompanied by a Doppler shift due to scattering by moving particles in the tissue (for example, blood cells) [6]. Most biological tissues are characterized by strong optical scattering and hence called as scattering media or turbid media [2]. When a tissue is illuminated with light, photons enter the tissue and propagate through it. The trajectory of these photons is affected due to scattering and absorption. Absorption of a photon can elevate an electron of a molecule from the ground state to an excited state. Thus, the electromagnetic energy of light is
transformed into internal energy of the absorber, such as thermal energy. Tissue’s ability to absorb photons is characterized by its absorption coefficient. Absorption coefficient $\mu_a$ is defined as the probability of photon absorption in a medium per unit path length. It has a representative value of $0.1\text{cm}^{-1}$ in biological tissue [2]. The reciprocal of $\mu_a$ is referred to as mean absorption length. Light attenuation in an absorbing-only medium is given by the following formula:

$$\frac{dI}{I} = \mu_a \, dx \quad (1.1)$$

where $I$ denotes the light intensity and $x$ denotes the distance along the light propagation direction. This equation represents the percentage of light being absorbed in interval $(x, x+dx)$ is proportional to the product of $\mu_a$ and $dx$ [2]. Integration equation 1.1 leads to the well-known Beer Lambert Law (BLL)

$$I(x) = I_o \exp(-\mu_a x) = I_o \, 10^{(-\varepsilon c x)} \quad (1.2)$$

where $I_o$ is the light intensity at $x=0$. The negative sign indicates that $I$ decreases as $x$ increases.

The optical properties of tissue, absorption and scattering, are wavelength dependent [2]. The absorption coefficient of biological tissue can be calculated by estimating the concentration of chromophores at particular wavelength based on the following equation:

$$\mu_a(\lambda) = 2.3 \times [\varepsilon_1(\lambda) \cdot C_1 + \varepsilon_2(\lambda) \cdot C_2 \ldots \ldots \cdot C_n] = 2.3 \times [\sum_{i=1}^{n} \varepsilon_i(\lambda) \cdot C_i] \quad (1.3)$$

Scattering in a tissue is characterised by its scattering coefficient $\mu_s$, which is defined as the probability of photon scattering in a medium per unit path length. It has a representative value of $100\text{cm}^{-1}$ in biological tissue. The reciprocal of $\mu_s$ is referred to as scattering mean free path. Optical scattering originates from light interaction with biological structures, which range
from cell membranes to whole cells. When a photon undergoes multiple scattering events, it is characterized by the reduced scattering coefficient $\mu_s'$, which is defined as follows:

$$\mu_s' = \mu_s (1 - g)$$  \hspace{1cm} (1.4)

$\mu_s'$ takes into account the degree of forward scattering i.e. the anisotropy factor ‘$g$’ [2]. The anisotropy factor ‘$g$’ is the mean of cosine of scatter angles and is in the range of 0.8 – 1.0 in biological tissues, as expressed by

$$g = <\cos\theta>$$  \hspace{1cm} (1.5)

As light propagates through a series of different layers of tissues, it undergoes a complex light scattering path. Series of photon migration simulation studies provided theoretical explanation of light propagation through tissue. Injected light diffuses in all directions inside the tissues of the head (scalp, skull, and subarachnoid space filled with cerebrospinal fluid) both before and after passing through the brain tissue, the sensitivity of each source-detector pair exhibits a banana-shaped profile (Refer figure 2).

![Figure 2: Illustrations of how light propagates through tissue [7].](image)

1.1.3 Modified Beer Lambert’s Law

In BLL, the light is undergoing only absorbance and no scattering takes place. Light travelling through biological tissue is highly scattered. Scattering adds to “path length” travelled
by the light. Thus the beer lambert law needs modification as the path traveled by light is not straight but exhibits a banana-shaped profile. The banana profile propagation of NIR light through the scalp can be quantified using Modified Beer Lambert’s Law (MBLL). The MBLL is an empirical formula to quantify this measured light intensity at the surface for a highly scattering medium like biological tissues. MBLL represented in equation 1.6 enables calculation of change in optical density as a logarithm of change in detected light intensity.

MBLL is expressed as follows

\[ OD = \log \frac{I_0}{I} = \epsilon c L = \frac{\mu_a L}{2.3} \]  \hspace{1cm} (1.6)

where OD is the attenuation measured in optical densities, \(I_0\) is the light intensity before the change of concentration, \(I\) is the light intensity after the change of concentration, \(c\) is the concentration of the absorbing species, \(\epsilon\) is the extinction coefficient of the absorbing species, and \(L\) is the path length through the tissue. The path length through the tissue can be disintegrated into the source-detector separation (d) and the differential path length factor (DPF). The DPF is a constant based on the optical properties of the tissue. It can be calculated by the following formula:

\[ L = d \times \text{DPF} \]  \hspace{1cm} (1.7)

Where \(d\) = source-detector separation distance and \(\text{DPF} = \text{Differential Path length Factor}\)

The change in optical density (\(\Delta OD\)) is directly in proportion to the change in absorption coefficient (\(\mu_a\)), which is the result of change in chromophore concentrations in tissue (Eq. 1.2). [10]. Change in optical density is given as:

\[ \Delta OD = \frac{\Delta \mu_a * L}{2.3} \]  \hspace{1cm} (1.8)
Thus this formula enables calculation of light attenuation along a scattering medium and has been applied in measuring neuronal activity associated with motor, visual and auditory tasks [3].

1.1.4 FNIRS Instrumentation

FNIRS principle can be implemented by three different techniques; each is based on the type of illumination used for the measurement. First type is the continuous wave (CW) mode, based on constant tissue illumination, which simply measures light attenuation through the head. The second method is the frequency-domain (FD) method in which, illuminating the head with intensity-modulated light is used to measure both attenuation and phase delay of emerging light. The third method is the time-domain (TD) technique in which, the head is illuminated with short pulses of light and is detected by the shape of the pulse after propagation through tissues. The quantitation of NIRS parameters depends on which NIRS technology is adopted. CW-based NIRS instrumentation measures only oxygenation changes of HbO₂ and Hb (with respect to an initial value arbitrarily set equal to zero) calculated using MBLL. For this study we have used a CW-based modality for measurements.

Our fNIRS device uses optic fibers and light emitting diode (LED) light sources at two NIR wavelengths (experiment 1: 690nm and 830nm & experiment 2: 750nm & 850nm) and avalanche photo diode (APD) detectors. One advantage of using fiber optics is that it is very suitable for any head shape and posture. FNIRS measurements can be performed in natural environments without the need for restraint or sedation [1]. Adequate depth of NIR light penetration (almost one half of the source-detector distance) can be achieved using a source-detector distance around 3 cm [1, 10]. The selection of the optimal source-detector distance depends on NIR light intensity and wavelength, as well as the age of the subject and the head region being measured [1].
1.2 Brain Physiology

1.2.1 Neurovascular Coupling

The brain is critically dependent on a continuous supply of blood to function. Therefore, the cerebral vasculature is endowed with neurovascular control mechanisms that assure that the blood supply of the brain is corresponding to the energy needs of its cellular constituents [11]. The regulation of cerebral blood flow (CBF) during brain activity involves the coordinated interaction of neurons, glia, and vascular cells [11]. Neurons and glia generate the signals initiating the vasodilation. Endothelial cells, pericytes, and smooth muscle cells act in concert to transduce these signals into carefully orchestrated vascular changes. This leads to CBF increase focused to the activated area and is temporally linked to the period of activation [11]. At times when the requirement for glucose and oxygen increases due to increased activity, the levels of glucose and oxygen drop causing local arteriolar dilation and subsequently increased CBF and cerebral blood volume [1]. This phenomenon that causes change in concentration levels of HbO$_2$ and Hb as a result of neuronal activity is called ‘Neurovascular Coupling’ [3]. The mechanisms underlying neurovascular coupling include ions, metabolic by-products, vasoactive neurotransmitters, and vasoactive factors released in response to neurotransmitters. A physiological increase of neuronal activity caused by voluntary movement increases cerebral metabolic rates for oxygen and glucose, and increases CBF [12]–[14]. The brain has little energy reserves and depends on a continuous supply of glucose and oxygen through cerebral blood flow [11], [15]. Thus, when CBF increases there is also an increase of oxygen availability. With the increased uptake of glucose and availability of oxygen, neurons are able to produce more ATP giving them more energy during stimulation. Surrounding capillaries deliver HbO$_2$ which releases its oxygen and takes away the CO$_2$, thus converting it to Hb. The following figure 3
summarizes the phenomenon of neurovascular coupling which is the principle or basis of using optical imaging techniques to study cortical function.

Figure 3: Phenomenon of neurovascular coupling [16].

1.2.2 Prefrontal Cortex

Prefrontal cortex (PFC) is the region of brain that covers the front portion of the frontal lobe. The PFC consists of Brodmann areas 9, 10, 11, 12, 46, and 47. It occupies a far greater proportion of the cerebral cortex in humans than in other animals, suggesting that it might contribute to those cognitive capacities that distinguish humans from animals [17]. Figure 4 shows the human cerebral cortex with PFC and the Brodmann areas.
Figure 4: Cerebral Cortex with PFC and the Brodmann areas [18].

Research indicates that there is a vital association between a person's personality and the functions of the PFC [19]. PFC has been associated in planning complex cognitive behaviour, personality expression, decision making, and moderating social behaviour [20]. It can synthesize information from a wide range of brain systems and exert control over behavior [19, 20]. The basic function of this region is organisation of thoughts and actions in agreement with internal goals. Damage of PFC results in deficits in concentration, orientation, abstracting ability, judgment, and problem solving ability; destruction of the orbital (frontal) lobe results in inappropriate social behaviour [19]-[21]. Researchers suggest that PFC helps to intelligently guide thought, action, and emotion, including the inhibition of inappropriate thoughts, distractions, actions, and feelings [17].

This study was designed to evaluate effects of transcranial LLLT on PFC functions in processing of attention, vigilance, and memory by instructing participants to perform two neurocognitive tasks before and after transcranial LLLT.
1.3 Low Level Light/Laser Therapy

Light is a form of electromagnetic (EM) radiation visible to human eye. EM waves have properties of frequency and wavelength of oscillations. These properties help characterise EM waves and form a spectrum which includes radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, x-rays and gamma rays. Infrared radiation is invisible radiant energy extending from the nominal red edge of the visible spectrum at 700nm to 1 mm (300 GHz). Non-thermal near infrared (700-2000nm) has penetrability of biological tissue and has been shown to have a wide range of therapeutic benefits as well.

Low Level Light/Laser Therapy (LLLT) is defined as use of directional low-power and high-fluence monochromatic or quasimonochromatic light from lasers or light-emitting diodes (LEDs) in the red to near infrared wavelengths (λ = 600–1100 nm) to modulate a biological function or induce a therapeutic effect in a non-destructive and non-thermal manner [22]. LLLT is commonly known as photobiomodulation. When nerve cells are targets the therapy is called photoneuromodulation. LLLT has also been referred to as near infrared light therapy.

LLLT is the use of light energy to alter biological functions. The fundamental principle of LLLT is energy conversion, which states that certain molecules in a living system are able to absorb photons that trigger signalling pathways in response to light [23]. This indicates that the molecules stimulated by light reach an excited state that temporarily changes its configuration and function. This causes activation of signalling pathways that affect cellular metabolism.

1.3.1 Properties of LLLT Source

LLLT varies from sunlight by the fact that LLLT uses monochromatic light which permits high specificity and targeted molecular biomodulation [22]. LLLT sources used can be either LED
arrays or lasers. Both these sources have been used for photobiomodulation of the brain. Laser sources produce highly coherent light energy in a single wavelength [22]. Laser sources allow high tissue penetration and produce a constant beam width. Laser features such as monochromatic, unidirectional and coherence allow delivery of significant levels of concentrated energy [22]. Thus the advantages of laser sources include high monochromaticity, their efficient fiber optic coupling and high tissue penetration [22]. LED sources produce non coherent light between a narrow range of wavelengths (4-10nm) [22]. This property of no coherence of LED’s is the reason for significant difference in the amount of energy delivered to a single unit area (cm²) of target compared to lasers [22]. Some advantages of LEDs include that LEDs can be fixed on arrays of ergonomic features that permits efficient energy delivery that is needed when the target organ has large surface area like brain. Secondly, LEDs generate insignificant amounts of heat in comparison to lasers, thus decreasing thermal injury risk [24].

1.3.2 Parameters of LLLT

Some important parameters to consider while performing LLLT are listed in the table 1 below
1.3.3 Cellular Targets of LLLT

Biological systems contain two types of molecules that can absorb light: specialized and non-specialized. The specialized molecules are called photoreceptors. These highly efficient molecules help in energy conversion. Examples are rods and cones opsins (retinal photoreceptors) and melanopsin (found in some retinal ganglion cells). The non-specialized molecules are called photoacceptor molecules. These can absorb light but are not integral to light receptor organs. Photoacceptors are not directly linked with processing of light [22]. Photoacceptors are more ubiquitous than photoreceptors. The components of photoreceptors/photoacceptors that are responsible for absorption of light are called chromophores. Chromophores are usually organic cofactors or metal ions within a protein structure and contain electrons that can be excited from ground state to excited state [22].
Numerous examples of chromophores exist in nature, such as chlorophyll in plants, bacteriochlorophyll in blue-green algae, flavoprotein and haemoglobin in red blood cells. The colours of chromophores are depend on the part of the range of light they absorb: chlorophyll is green, flavoproteins is yellow and haemoglobin is red [25, 26]. Thus when light is absorbed by the chromophore, the electrons get excited and jump from lower energy orbits to higher energy orbit. This energy can be stored by the system to perform various cellular tasks. This excitation causes molecular shape change which is related to the functional and intracellular metabolism changes. [21]. In cells, chromophores can comprise of resonating systems or metal complexes. In resonating systems, electron excitation by light happens within a structure that alternates single or double bonds, while in metal complexes electron excitation occurs within open or closed pyrrole rings that allow binding of transition metals [22]. This link of chromophores and respiratory enzymes has major implication for functional neural regulation [22].

In LLLT the photoacceptor targeted by the NIR light is the mitochondrial enzyme cytochrome C oxidase (CCO). Mitochondrion is called the power house of a eukaryotic cell. Its primary function is cellular respiration which is conversion of ingested nutrients to usable energy (ATP) using oxygen through the process of oxidative phosphorylation and electron transport chain. Basic principle behind cellular respiration is a three-step process. First step involves glycolysis which is breaking down of glucose into pyruvate. The second step is the TCA cycle where the pyruvate is converted to acetyl COA, and last step is oxidative phosphorylation that contains the electron transport system (ETS) and chemiosmosis. The ETS consists of a series of electron carriers like nicotinamide adenine dinucleotide (NADH), reduced Flavin adenine dinucleotide (FADH2) and a sequence of proteins complexes/enzymes called cytochromes (b,c,a,a3). High energy electrons pass through the ETS to the final electron acceptor generating a
proton gradient. This gradient is used by ATP synthase to produce ATP. CCO is one of the four membrane bound complexes that are embedded in the inner membrane of mitochondria. Figure 5 shows the illustrations of mitochondrion, as well as of the electron transport chain and oxidative metabolism.

Figure 5: Illustrations of mitochondrion, ETS & oxidative metabolism [25].

CCO is an effective energy-converting device as it not only generates an electrochemical gradient through redox reactions but also is a rate limiting step required for formation of ATP [27]. CCO is a multicomponent transmembrane protein complex that is a part of the ETS in the mitochondria. CCO contains four redox metal centers: Cuₐ, Cuₐ, Hem a and Hem a₃. Cytochrome C is oxidized to CCO by catalytic transfer of electrons among the four redox metal centers [22].

These metal centers decide the different light absorption peaks for CCO (see figure 6): Cuₐ is reduced at 620 nm (range 613.5–623.5 nm), and Cuₐ oxidized and 825 nm (range 812.5–846 nm), Cuₐ reduction and oxidation is at 760 nm (range 750.7–772.3 nm), and 680 nm (range 667.5–683.7 nm) respectively [28]. In neural tissue, CCO is the most abundant metalloprotein
and wavelength peaks in its absorption spectrum (670 nm and 830 nm) are highly correlated with its peaks in catalytic activity and with ATP content in vitro [24].

![Figure 6: Structure of Complex IV (cytochrome c oxidase) [21].](image)

1.3.4 Mechanism of Action of LLLT

The mechanistic effects of LLLT are divided into two: 1) Primary effects and 2) Secondary effects.

1.3.4.1 Primary Effects

Primary effects are changes occurring in photoacceptor molecules on excitation due to light. Primary effects occur when the target tissue is being exposed to light. Current research suggests that, there are three types of primary effects [22]. The first and important effect is the redox change taking place in the components of the respiratory chain. LLLT induces reduction or oxidation of CCO. Changes in the redox state of CCO indicate that there is a change in electron flow. LLLT increases in oxygen consumption and mitochondrial membrane potential. This activates the mitochondrial permeability transition pore [29, 30]. These changes have been associated with accelerated electron flow in the ETS in the mitochondria.
The second likely effect is the generation of free radicals; this includes singlet oxygen via direct photodynamic action and superoxide ion via one electron auto-oxidation. The importance of this effect is that, reactive oxygen species are not only damaging by-products of respiration but they have an important role in cellular signaling [22].

The third primary effect of LLLT is limited transient “heating” of the absorbing chromophore taking into account electric or light oscillations [30]. This effect has been less characterized and it is believed to complement the other two proposed primary effects. This seems, by all accounts, to be more summed up and influences all particles in the target tissue, including water. It can be bolstered that LLLT has the capacity to reinforce hydrogen bonds and induce large size hydrogen bonds networks that permit quick energy transfers due to resonant intermolecular energy transference. Subsequently, LLLT can bring about non equilibrium electrical fluctuations that bias Brownian movement and induce mechanisms that support electron pumping without heat transfer [31].

1.3.4.2 Secondary Effects

Secondary effects are a consequence of the primary effects i.e. they occur after the light exposure. These effects include a series of biochemical reactions that change cellular homeostasis [22, 32, 33]. Secondary effects include activation of second messengers with subsequent modulation of enzyme function and gene expression [22]. Secondary are exceptional on the grounds that they can happen hours and even days after light exposure and they implicate the activation of signaling pathways that result in amplified macro effects. Since they happen as a major aspect of cascade reactions, secondary effects have a tendency to be pleiotropic [22]. LLLT triggers the retrograde signaling pathway from the mitochondria to the nucleus. This signaling pathway generates adaptive responses to stress by communicating with the nucleus via
mitochondria, which contain the photoacceptors. The nucleus then respond by changing levels of gene expression [22]. The beginning period of this pathway has been suggested to be an increment in the NAD/NADH proportion and mitochondrial between layer potential, separation of nitric oxide (NO) from its coupling site in CCO, and change of the ATP pool. In a stressed cell, NO created by mitochondrial NO synthase displaces oxygen from CCO, which brings about a downregulation of cellular respiration and a consequent reduction in the generation of ATP [25]. By separating NO from CCO, LLLT stops the dislodgment of oxygen from CCO and thereby helps unimpeded cellular respiration (Refer to figure 7) [34].

Figure 7: Nitric oxide can bind to copper centers in CCO & inhibit respiration [25].

Even small changes in ATP alter cellular metabolism [22]. ATP activates P2 receptors (P2X and P2Y) to induce inward calcium currents and release of calcium from intracellular stores [33]. Increased cellular ATP created by LLLT may add to the constructive outcomes, both by raising cellular energy levels and by upregulating the cyclic AMP molecule (biochemically formed from ATP) that is included in numerous signaling pathways [25].
Oxygen acts as a final electron acceptor in the ETS and in the process is converted to water. Part of the oxygen that is metabolized produces reactive oxygen species (ROS) as a natural by-product. ROS (for example: superoxide and hydrogen peroxide) are chemically active molecules that play an important role in cell signaling, regulation of cell cycle progression, enzyme activation, nucleic acid and protein synthesis [35]. As seen earlier since LLLT promotes oxygen metabolism, it also subsequently increases ROS production. In turn, ROS activates certain redox-sensitive transcription factors, such as nuclear factor-κB [NF-κB] and activator protein 1, which leads to the upregulation of various stimulatory and protective genes [25].

LLLT is also shown to stimulate the production of deoxyribonucleic acid (DNA) and RNA [36] and increase the production of proteins [37]. It additionally modulates enzymatic movement [38], influences intracellular and extracellular pH and quickens cell metabolism [37]-[39]. LLLT has shown to stimulate the expression of multiple genes related to cellular proliferation, migration, and the production of cytokines and growth factors [40]. Therefore the ultimate effect of LLLT is expected to be activation of transcription factor which is responsible for the modulation of host’s downstream cellular and tissue responses. Thus the secondary effects of LLLT involve changes in gene expression, which impact mitogenic signaling, surface molecule expression, and expression of proteins regulating inflammatory, redox states, and apoptosis [22]. Figure 8 illustrates the mechanism of LLLT on the cellular and molecular level.
1.3.5 Effects of LLLT on nervous tissue

Many recent researches support that LLLT has an enhancing and protective effect in the nervous tissue. Neurons are highly specialised cells with chief requirement of aerobic energy production. Mitochondrial aerobic metabolism in neurons is the foundation for electrophysiological, neuroplastic, and neuroprotective functions including repolarization of cell membranes in neurotransmission, synapse formation and cell survival [22]. Energy is required by the neurons for important functions like neuronal networks, data integration in space and time and sensory processing that includes vision, activation of motor function and expression of higher cognitive functions like memory. Research has proved that neuronal dysfunction, neurological impairment, and neurodegeneration is caused due to impaired mitochondrial oxidative metabolism [41]. Thus mechanisms targeted at enhancing mitochondrial metabolism are postulated to help the function of brain.
1.4 Focus of this Study

Transcranial LLLT consists of applying monochromatic light directly to the head with wavelengths between the “optical window” in the red to near infrared optical region. Since the spectral band of the therapy is between ~620-1150nm, the process is also called near infrared stimulation.

The aim of this study is to use fNIRS to evaluate the physiological and functional effects of transcranial LLLT on prefrontal cognitive functions in young healthy adults. This study first investigated the physiological effect of transcranial LLLT and then the functional effect of transcranial LLLT. So far, very few studies have been conducted to assess the physiological effects of transcranial LLLT on prefrontal cognitive functions. Most of these studies have evaluated the effects of transcranial LLLT by observing the changes in behavioral responses and postulating the underlying cellular mechanism that causes them. Only one study has so far looked at the changes in hemodynamic responses taking place during and after transcranial LLLT. Schiffer et al used a very basic oximeter for the measurement of total concentration of hemoglobin. This study aimed at not only evaluating the behavioral results but also the changes in hemodynamic responses taking place before and after transcranial LLLT using a high-performance fNIRS system. In this study changes in HbO₂ and Hb (ΔHbO₂ and ΔHb) concentrations were measured and ΔHbT (= ΔHbO₂ + ΔHb) and HbD (= ΔHbO₂ - ΔHb) concentrations were calculated. Behavioral and hemodynamic results were associated to understand the neural activation patterns and observe if the prefrontal cognitive functions are improved physiologically after transcranial LLLT.
1.5 Outline of this Thesis

Chapter 1 gives a brief introduction about fNIRS, brain physiology and LLLT. Chapter 2 investigates the physiological effect of transcranial LLLT by evaluating the changes in the hemodynamic responses before and after transcranial LLLT. This chapter contains a brief description of the subjects; protocol and instrumentation used for the experiments, and the results obtained. Chapter 3 examines the functional effect of transcranial LLLT by assessing changes in the behavioral and hemodynamic responses while cognitive tasks were performed. It has a description of the subjects, protocol and instrumentation used for the experiment. Chapter 3 also gives the data analysis methodology and results obtained after comparing the pre- and post-treatment task performances. Chapter 4 discusses the obtained results for both parts of the study. It also talks about the limitations and future work of the study.
Chapter 2
Physiological Effects of Transcranial LLLT

The aim in this part of study is to explore how a single treatment of transcranial LLLT alters the hemodynamic status of the PFC. Two experiments were designed to evaluate changes in hemoglobin concentration in both hemispheres when the therapeutic laser was shined at 1) the center or 2) the right side of the frontal lobe.

2.1 Ethical Approval and Subjects

The protocol for these experiments was approved by the University of Texas at Arlington’s Institutional Review Board and compliant with the applicable NIH guidelines. Participants were recruited from the local community of the University of Texas at Arlington through direct person-to-person solicitation. Interested individuals were screened by one of the investigators in this study to determine whether they met the eligibility criteria. Healthy young adults of either sex, of age group ranging from 18-40 years from any race and ethnic background were considered. The exclusion criteria for the experiments were as follows: diagnosed with a psychiatric disorder, history of a neurological condition, history of several brain injury, history of violent behaviour, have ever been institutionalized/imprisoned, currently intake of any medicine or drug, currently pregnant. Eligible participants were scheduled immediately for both experiments that would be at least two weeks apart.

On the day of each experiment, one of the investigators guided the participants through the consent form with an oral explanation and then the participant was given the opportunity to read through the consent form and ask questions. Signed consent form was obtained from each participant before the experiment started. After this, a confidential one-page medical history was
completed by the participant, primarily to evaluate whether there is any evidence that a participant is predisposed to depression or any other psychological disorder. Another brief record of participant's demographics (gender, age, and handedness) was also obtained.

2.2 Instrumentation

2.2.2 Treatment Laser

The LLLT treatment was administered with a 1064-nm laser provided by Cell Gen Therapeutics, LLC (Model CG-5000 laser, HD Laser Center, Dallas, TX, USA). This laser is FDA-cleared and is safe for various uses on humans, such as for improving circulation, temporary relief of muscle and joint pain, muscle spasm, stiffness associated with arthritis, and relaxation of muscle tissue [42] (see Fig. 9). The laser received approval from the University of Texas at Arlington Environmental Health and Safety Department and a standard operating procedure for the laser was approved by the University Laser Safety Officer. All of the investigators who operated the CG-5000 laser in this study had been trained and certified by Dr. Rudy Rivera, the Laser Safety Officer and Director of Research, Education and Operations from Cell Gen Therapeutics.

The laser was operated at power of 3.5 Watts. The diameter of the laser beam from CG-5000 is 4 centimetres. Thus irradiance (or power density) used was $\sim 3500 \text{ mW} / (4\pi \text{ cm}^2) = 280 \text{ mW/cm}^2$ which is the same as those used in previous studies [42, 43]. At the power level described, the energy emitted by the CG-5000 is low, exposure to it is not harmful to tissue, and it causes negligible heat and no physical damage [42]. Similar settings are used clinically by Cell Gen Therapeutics for the treatment of lower back pain, sciatica, and migraine headaches [42].
Figure 9: The CG-5000 high-density laser for brain stimulation (left) and laser head that delivers light to skin (right)

2.2.2 FNIRS System

The fNIRS system used in this part of the study to measure the hemodynamic responses of the brain to transcranial LLLT is a continuous-wave (CW) system (CW-2 TechEn, Inc.). This system is built with a series of frequency-encoded lasers and an array of avalanche photo-diodes (APDs) [44]. The laser light has two wavelengths, 690 nm and 830 nm, which is delivered through optical fibers to the subject’s head, and the reflected light through the human brain is collected back by detector fibers. Both source and detector fibers have a diameter of 3 mm. The laser power from CW-2 is less than a few milliwatts, much lower than the power to cause any detectable heat effects on the skin. Laser sources are located on the left of the system while the APD detectors are on the right of the unit (see Fig. 10). Both have connectors that fit standard SMA connectors for fibers. There are screw dials to the left of each laser that can manually adjust the laser output power. Green LEDs next to each detector connector indicates light saturation. The data acquisition card is placed in the center of the unit, connecting the unit to the controlling computer. The LEDs to the right of this port indicates system status. Green light indicates 1) when the system is turned on and 2) when the computer is properly connected [44].
Data acquisition is done using a software interface that uses the MATLAB data acquisition toolbox and thus requires MATLAB to be installed on the controlling computer. The NIRS-2 acquisition interface performs four basic functions: 1) Laser controls 2) Detector gain controls 3) Acquisition controls and 4) Data display. The sampling rate for the measurement was 200 Hz.

![CW2 brain imaging system](image1.png)

Figure 10: CW-2 brain imaging system showing light sources and detectors

2.3 Protocol

Two experiments were conducted to study the hemodynamic responses during and immediately after transcranial LLLT treatments, by following a same treatment protocol. The therapeutic laser was shot at different location during each experiment, 1) the center or 2) the right side of the frontal lobe. The laser was shot at two different locations to evaluate if there was any regional difference in the hemodynamic responses, if different site of the brain was treatment.

In each experiment, an fNIRS probe was placed on subject’s forehead as shown in Figure 11. The probe used for measurement had two sources and two detectors and provided four
channels of data, as shown in Figure 11. The probe was placed bilaterally and symmetrically on the subject’s forehead. The separation between the source and detector was 3.5 cm. The probe assembly was made with low-weight optical fibers (TechEn Inc., Boston, MA) and thin polyethylene film for the participants’ comfort during the experiment, which was held in place with Velcro strips. Once the probe was in place, the subject was instructed not to move so as to avoid any big motion artifacts resulting from movement. Then the baseline readings were taken for the first 2 minutes of the experiment. Then transcranial LLLT treatment were administered. The laser parameters were set as described in section 2.1.2. The laser’s power output was automatically calibrated by an internal mechanism. The CG-5000 has a handheld 4-cm diameter aperture through which NIR Light was directed on the subject’s head. The experimenter controlled the “on” and “off” switch of the therapeutic laser with a button. The treatment was given for a total of 10 minutes. It was divided into 1 minute cycles. Each one-minute treatment cycle was marked by a timer counting down and by a beep from the apparatus. After LLLT had been administered the participants were asked to sit for another 7 minutes during which fNIRS data was recorded. The aim of this recovery segment was to evaluate if the hemodynamic changes that took place during the treatment were maintained or reversed immediately after LLLT. Thus fNIRS data acquisition was initiated 2 minutes prior to the LLLT treatment and ceased 7 minutes after the treatment. Thus, the total data acquisition time was ~20 minutes. (See Fig.12).
In the first experiment, the site of laser treatment was at the center of the forehead. The cortex area treated was approximately the ventromedial prefrontal cortex (Bordmann Areas 10, 32) [45]. In the second experiment, the treatment site of transcranial LLLT was on the right side of the forehead, similar to that reported in Schiffer et al. 2009. Schiffer et al study showed a beneficial effect of near-infrared light stimulation on mood; however, instead of treating both hemispheres, this study targeted the right side of the forehead since the right frontal pole region is associated with sustained attention and memory [42], [46]-[48]. Figure 12 shows the diagrammatic representation of the LLLT stimulation protocol.

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**Figure 11: NIRS Probe geometry for LLLT Protocol showing the sites of treatment**

**Figure 12: LLLT Stimulation Protocol**
The transcranial LLLT was conducted in a locked room with black walls and no reflective surfaces. The experimenters locked both themselves and the participants inside the room, which has a sign on the outer door indicating that the apparatus was in use, and made sure that 1064nm protective eyewear was worn by all individuals present in the room, though the precaution was also taken that while transcranial stimulation was done, light was not shined in the eyes. In addition to the protective eyewear provided, subjects were instructed to keep their eyes closed during the treatment.

2.4 Data Processing and Analysis Methodology

The data obtained from fNIRS was processed in MATLAB. Data was processed for individual subjects and then group averaging across subjects was done. The raw data for each subject was plotted, and visually inspected to exclude data points associated with significant data discontinuities or high noise. The data during transcranial LLLT treatment had a lot of interference and discontinuities; therefore, the data points between two consecutive treatments were selected for processing. Thus after screening, a total of 17 good data points were picked up from the baseline, during the 10-minute treatments and from the 7 minutes of recovery. After the qualified data was collected, changes of hemoglobin concentrations relative to the baseline, $\Delta [\text{HbO}_2]$ and $\Delta [\text{Hb}]$, were computed following the modified Beer–Lambert Law [49]. These concentrations were calculated for both 690nm and 830 nm wavelength. Changes in total hemoglobin ($\Delta \text{HbT}$) concentration and hemoglobin concentration difference ($\Delta \text{HbD}$) were also calculated. $\Delta \text{HbT}$ is derived from $\Delta \text{HbO}_2$ plus $\Delta \text{Hb}$, and $\Delta \text{HbD}$ derived from $\Delta \text{HbO}_2$ minus $\Delta \text{Hb}$. This process was done for both the right and left hemispheres at the individual and group level for both experiment. Figure 13 shows raw data with interference and processed data without interference.
2.5 Statistical Analysis

One-sample t-test was performed to examine whether the treatment-induced hemodynamic responses were statistically significant. The normality of the data was checked by calculating the skewness of the data. A two-tailed, unpaired t-test was performed to evaluate if any of the 17 data points were statistically significant at a confidence interval of 99%. The null hypothesis of the t-test stated that there was no significant difference between the data obtained and baseline reading and the alternative hypothesis was that there is a significant difference between the data obtained and baseline reading. The test was done for all measured and calculated signals for both of the experiments.

Figure 13: Raw data with interference and processed data without interference
Two-sample t-tests were performed to evaluate if the left versus right hemisphere data were statistically significant. The normality of the data was checked by calculating the skewness of the data. A two-tailed, unpaired t-test was performed to evaluate if any of the 17 data points for all subjects between the left and right hemisphere were statistically significant at a 99% confidence interval. The null hypothesis of this t-test stated that there was no significant difference between right and left hemisphere data and the alternative hypothesis was that there was a significant difference between the right and left hemisphere data. The test was done for all measured and calculated signals for both of the conditions.

Another two-tailed, unpaired t-test was performed to evaluate if any of the 16 data points for all subjects for central head versus right head stimulation were statistically significant at a 99% confidence interval. The null hypothesis of this t-test stated that there was no significant difference between central head and right head stimulation data and the alternative hypothesis was that there was a significant difference between the central head and right head stimulation data. The test was done for all measured and calculated signals for both hemispheres.

2.6 Results

Both experiments had 9 subjects each. Seven subjects that participated in experiment one also volunteered to participate in experiment two. The subject demographics are as shown below:
2.6.1 Results of transcranial LLLT on the central head

The results of hemodynamic responses obtained from experiment 1 are shown in Figures 14 and 15. The results showed that LLLT evoked approximately equal hemodynamic changes in both hemispheres. A clear $\Delta$HbO$_2$ increase as well as $\Delta$Hb decrease was seen. The group averaged results for both hemispheres showed that an increase of HbO$_2$ and a decrease of Hb had similar amplitudes in both hemispheres. The results also showed that the $\Delta$HbT concentration values remained approximately close to zero. This signified that $\Delta$HbT did not change much during the experiment. For both hemispheres, the results for $\Delta$HbD showed a maximum increase, as high as 3 µM in change of hemoglobin concentration.

The statistical analysis confirmed significant changes for all concentrations except $\Delta$HbT, as marked in Figs. 14 and 15. The $\Delta$HbO$_2$ concentration in the left hemisphere showed 11 out of 17 data points to be statistically significant from the baseline; while the right hemisphere showed 6 ($p < 0.01$) (see Fig. 14). $\Delta$Hb concentration results from the left hemisphere showed 14 significant data points, while $\Delta$Hb results from the right hemisphere showed all the 16 points ($p$...
As seen in Fig. 15, ΔHbT did not show any statistically significant change from all the data points in either of the hemispheres, indicating that there was no change in HbT during and after transcranial LLLT. ΔHbD revealed 15 significant data points in the left hemisphere, on the other hand, while the right hemisphere showed 12. The above results suggested that ΔHbO₂, ΔHb, and ΔHbD significantly changed during and after transcranial LLLT. Errors bars shown in the figures are based on standard error of means (SEM).

Statistical analysis was also performed to evaluate if the left versus right hemisphere data had any significant difference. None of the measured concentrations showed any significant difference at any time points for all the subjects. Thus, for central head stimulation, there is no difference in the hemodynamic responses between the two hemispheres.

Figure 14: Group-Averaged ΔHbO₂ and ΔHb concentration results for central head stimulation (N=9).
Figure 15: Group-Averaged ΔHbD and ΔHbT concentration results under central head stimulation (N=9)

2.6.2 Results of transcranial LLLT on the right forehead

The results of experiment 2 are shown in Figures 16 and 17. The results showed that LLLT evoked a clear ΔHbO₂ increase on the left hemisphere as well as ΔHb decreased in both hemispheres. It was clear that the increase of ΔHbO₂ was higher in the left hemisphere and the decrease of ΔHb was similar in both hemispheres. As shown in Fig. 17, ΔHbT values for the right hemisphere remain approximately close to zero. However, the left hemisphere showed an increase in HbT. For both hemispheres, the results for ΔHbD showed maximum increases, as high as 4 μM in change of concentration.

In statistical analysis, the left hemisphere showed significant difference in all ΔHbO₂, ΔHb, ΔHbT, and ΔHbD concentration to LLLT while the right hemisphere showed a difference only in ΔHb and ΔHbD concentration. The details of the analysis were as follows: The ΔHbO₂
concentration in the left hemisphere showed significant difference at all 17 time points (p<0.01), while the right hemisphere did not show any significant difference at all time points. ΔHb concentration results for the left hemisphere showed significant difference at 15 time points (p<0.01), while right hemisphere showed the same at all the time points. ΔHbT from the left hemisphere showed significant difference at 8 data points during the LLLT treatment, but ΔHbT did not show any statistically significant difference at all data points in the right hemisphere. For ΔHbD concentrations, the left hemisphere showed significant difference at all data points, while the right hemisphere showed the same at only 10 time points. The above analysis suggests that ΔHb and ΔHbD concentrations in both hemispheres significantly increase with respect to the baseline during and after transcranial LLLT, while ΔHbO₂ and ΔHbT concentrations exhibit significant changes only in the left hemisphere, contralateral to the LLLT stimulation side.

Figure 16: Group averaged ΔHbO₂ and ΔHb concentration results for Right Forehead Stimulation (N=9)
Figure 17: Group-Averaged $\Delta$HbD and $\Delta$HbT concentration results under Right Forehead Stimulation (N=9)

Statistical analysis was also performed to evaluate if the left versus right hemisphere data had any statistically significant difference. These results are shown in Fig. 18, 19 and 20. The amplitudes of $\Delta$HbO$_2$, $\Delta$HbT concentrations showed 3 significant differences at 3 data points out of 17 over all the subjects and $\Delta$HbD concentration showed 5. The left hemisphere seemed to have significant increases in all three concentrations than the right side. But the true/significant increases occurred only during the 10-min LLLT stimulation period, not after the intervention. On the other hand, $\Delta$Hb did not show any significant difference between the two hemispheres at all of the time points. In summary, right head stimulation created more increases in $\Delta$HbO$_2$, $\Delta$HbT and $\Delta$HbD concentrations, but these differences disappeared right after the LLLT intervention. No difference in $\Delta$Hb concentration was observed between the hemispheres.
Figure 18: Significant changes in $\Delta HbO_2$ concentration between the two hemispheres at time points during and after LLLT.

Figure 19: Significant changes in $\Delta HbT$ concentration between the two hemispheres at time points during and after LLLT.
Figure 20: Significant changes in \( \Delta \text{HbD} \) concentration between the two hemispheres at time points during and after LLLT

2.6.3 Result comparison between central-head vs. right-head stimulation

Statistical analysis was done to evaluate statistically significant difference between the results induced by central-head and right-head stimulation. A two-tailed, unpaired t-test was performed on the results obtained from both conditions in both hemispheres. Right hemisphere results did not show any significant difference for all concentrations in response to the central-head versus right-head LLLT stimulation. The results from the left hemisphere did show significant differences at a few time points for \( \Delta \text{HbO}_2 \), \( \Delta \text{HbT} \) and \( \Delta \text{HbD} \) concentrations - as shown in Fig. 21, 22 and 23. No significant difference was seen in \( \Delta \text{Hb} \) concentration under these two stimulation conditions. It can be concluded that there were significant differences in \( \Delta \text{HbO}_2 \), \( \Delta \text{HbT} \) and \( \Delta \text{HbD} \) concentrations generated by central-head versus right-head stimulation only from the left hemisphere; \( \Delta \text{Hb} \) concentration data had no difference by the two simulation conditions.
methods and showed no difference between the two hemispheres. Also, right hemisphere data showed no difference for any concentrations between central-head versus right-head stimulation.

Figure 21: Significant changes in $\Delta$HbO$_2$, $\Delta$HbT concentration during and after LLLT obtained by central vs. right head stimulation
Figure 22: Significant changes in ΔHbO₂, ΔHbT, ΔHbD concentration during and after LLLT obtained by central vs. right head stimulation

Figure 23: Significant changes in ΔHbO₂, ΔHbT, ΔHbD concentration during and after LLLT obtained by central vs. right head stimulation
Chapter 3

Functional Effect of Transcranial LLLT

The aim of this part of the study was to evaluate whether single session of transcranial LLLT had any beneficial effects on brain functions in young healthy adults. The experiment was designed to evaluate effects of transcranial LLLT on prefrontal cortex functions in processing of attention, vigilance, and memory by instructing participants to perform two neurocognitive tasks before and after transcranial LLLT.

3.1 Ethical Approval and Subjects

The protocol for this experiment was approved by the University of Texas at Arlington’s Institutional Review board and compiled with all the applicable federal and NIH guidelines. Healthy young adults of either sex, of age ranging from 17-40 years from any ethnic background were considered. The exclusion criteria for subject participation were as follows: diagnosis of psychotic disorder, history of violent behavior, history of neurological condition, current pregnancy, or prior institutionalization or imprisonment [42]. Participants were recruited over the course of one semester from the local community of the University of Texas at Arlington through direct person-to-person solicitation. Eligible participants were then scheduled for the experiment.

On the day of each experiment, one of the investigators guided the participants through the consent form with an oral explanation and then the participant was given the opportunity to read through the consent form and ask questions. Signed consent form was obtained from each participant before the experiment started. After this, a confidential one-page medical history was completed by the participant, primarily to evaluate whether there is any evidence that a
participant is predisposed to depression or any other psychological disorder. Another brief record of participant's demographics (gender, age, and handedness) was also obtained.

3.2 Instrumentation

3.2.1 FNIRS System

A high-performance fNIRS system (Cephalogics LLC, Boston, MA) [50] was used to acquire each participant’s prefrontal hemodynamic activities during performance of the tasks. The system used light-emitting diodes (LEDs) at two wavelengths (750 and 850nm) as light sources and avalanche photodiodes (APDs) as detectors. The data sampling rate was 10.8 Hz. The geometry of fNIRS probe used for the study is shown in Figure 24. It consisted of 12 sources and 12 detectors (6 light sources and 6 detectors on each hemisphere). The probe was placed bilaterally and symmetrically on the participant’s forehead. It was positioned just above the eyebrows with the midpoint ~ 3.5 cm in distance from the nasion (see Fig. 24). The fNIRS probe provided a total of 32 measurements (channels) when only the nearest source-detector pairs were considered (the nearest source-detector distance was 3 cm) [51]. Other larger source detector distances were not included because their signals were too weak to be scientifically meaningful [51]. The probe assembly was constructed with low-weight optical fibers (TechEn Inc., Boston, MA) and thin polyethylene film to ensure participants’ comfort which was held in place with Velcro strips during the experiment [51].
Figure 24: NIRS Probe geometry for Task Protocol showing the site of treatments. Red circles represent light sources, blue squares represent detectors, and green lines represent the nearest source-detector pairs (channels) to measure the brain activities. Dotted green channels are noisy channels which were excluded while data processing.

3.2.2 Treatment Laser

The treatment laser used is the same as in section 2.2.1. All the specifications and parameters of the laser were same as in previous section.

3.3 Tasks

The tasks used in this study were reported in a paper published by Gonzalez-Lima et al [42]. “The PVT and DMS task were implemented using an open source programming language called the Psychology Experiment Building Language (PEBL). The PVT is a neurocognitive test in attention domain [48]. As shown in Fig. 25 (a), when the test started, participants looked at a small ‘+’ fixation point which appeared shortly at the center of a computer screen. After the
fixation pointed vanished, a black screen was presented for a random interval ranging from 1 second to 10 seconds. Then, a bright timer counting in millisecond appeared at the center of the screen. Participants were instructed to respond via button press as rapidly as possible to stop the time counting. The final counter value corresponded to the participant’s reaction time and was displayed onscreen for 1 s, thus providing feedback for that particular trial. Participants were given at most 30 s to make a response before the computer aborted a trial. The data gathered during the PVT included each trial’s inter trial interval in seconds, reaction time in milliseconds, and a code number indicating whether the trial was a success (response in less than 30 s), a lapse (no response in 30 s), or a false alarm (response with a button press prior to the onset of the cue). On completion of task these information about each trial’s success/failure and reaction time was stored as log files for later analysis.

The DMS task is a neurocognitive test in short-term memory domain. As shown in Fig. 25 (b), it entailed viewing a 4 X 4 grid of brightly colored squares with a unique, randomly-generated pattern for each of 30 trials. The grid of 16 squares consisted of 7, 8, or 9 red-colored squares, the rest of the grid had yellow squares. The participant memorized the stimulus grid and then, with a key press, the stimulus grid disappeared. After 3.5 s, two stimuli grids were then presented on screen (a “match” and a “non-match”). The “match” one was identical to the previous stimulus, while the “non-match” contained 1–2 randomly switched squares. The participant indicated which stimulus was the correct “match” with a key press. “Correct” or “Incorrect” was displayed for one second after each trial to provide feedback. Correct/incorrect status and memory retrieval latency (reaction time) for each trial were stored as log files for later analysis.”
3.4 Experimental Procedures

The protocol used was same as the one used and published by Barrett et al. in ‘Neuroscience’ journal [42]. The entire experiment was conducted in three separate phases: 1) pretreatment tests, during which the participants completed the PVT and DMS for the first time; 2) transcranial LLLT, during which the participants received laser treatment which was administered on the right frontal lobe; and 3) post-treatment tests, during which the participants repeated the PVT and DMS. The entire experiments took about 45 minutes.
3.4.1 Pretreatment Protocol

Before the experiment started, the subject was asked to sit comfortably and the fNIRS probe was placed on his/her forehead. The probe was positioned as shown in figure 24 and as described in section 3.2.1. Hair under the source and detectors was split out for better signal. Then the participants were given a short 1 minute practice for each of PVT and DMS tasks. After this the participants completed one session of PVT tasks which consisted of 40 trials (approximately five minutes long); inter trial intervals were set to be random between 2 and 10 seconds. Thus, the average inter trial interval was around 6 s [42]. Then the participants took part in the DMS task. The participants were informed that while there was no time limit for either studying the target or choosing the match, they should try to ‘be as fast as possible, while still being accurate.’[42]. One DMS block had 30 trails and approximately lasted for 5 minutes. Participants' behavioural measures (reaction time and success rate) were recorded in the same way as the previous study by Barrett et al. The fNIRS data acquisition was initiated 20 seconds prior to starting the tasks and ceased immediately after the tasks were finished.
3.4.2 Transcranial LLLT

After the PVT and DMS Tasks were completed, transcranial LLLT was administered. The laser parameters were set as described in section 2.2.1. The laser’s power output is automatically calibrated by an internal mechanism. The laser stimulation site was same as in Barrett et al. paper. It was focused at the right frontal pole of the cerebral cortex, which is the most anterior region of the right prefrontal cortex (Brodmann’s areas 9 and 10) [42]. According to the 10–20 system used for EEG electrode placement, the forehead stimulation site was centered on the FP2 (right frontal pole) point [42]. It extended medially for a 4-cm diameter area from this point, and laterally for another 4-cm diameter area from this point [42]. The location of the stimulation on the forehead was like that shown in Fig. 1 of Schiffer et al. 2009, the first paper which showed a beneficial effect of near-infrared light on mood; however, this study targeted the right side of the forehead only, since the right frontal pole region is associated with sustained attention and memory [42], [46]-[48]. The treatment was given for a total of 8 minutes that were divided into eight 1-min cycles. The experimenter controlled the on/off of each cycle. Each one-minute treatment cycle was marked by a timer counting down and by a beep from the apparatus. Each subject received four one-minute treatments to each of two sites on the right forehead alternating between sites medial and lateral to the FP2 point. The transcranial laser stimulation was conducted in a locked room with black walls and no reflective surfaces. The experimenters locked both themselves and the subjects inside the room. A warning sign on the outer door indicated the treatment laser was in use. Laser (1064 nm) protective eyewear was worn by of all individuals present in the room, though the precaution was also taken that while transcranial stimulation was done, light was not shined in the eyes. In addition to the protective eyewear provided, subjects were also instructed to keep their eyes closed during the treatment.
3.4.3 Post-treatment Protocol

Immediately after the transcranial LLLT, the subjects were instructed to repeat the PVT and DMS tasks, as described above. A comparison between the pre- and post-treatment will reveal whether and how the LLLT treatment alters the participants’ neurocognitive functions.

3.5 Data Analysis Methodology

3.5.1 Data Screening and Processing

The fNIRS data from each subject was screened and processed using a publically available toolbox called HomER. For basic analysis, the HomER program allows the user to calculate hemodynamic changes in HbO$_2$ and Hb concentrations from raw light intensity time series acquired at different wavelengths [53]. The program contains a collection of signal processing tools for more advanced analysis, including band pass filters to remove instrumental or physiological noise contributions, subject motion correction filters [54], and principal component-based filters to remove systemic physiology [53, 55, 56]. HomER also contains numerous tools for block averaging and linear regression of stimulus epochs for conventional analysis of functional data, as blocked, event-related, and multiple condition experimental designs [53].

The raw data was visually scrutinized to remove trails from a task block which had significant data discontinuities. If the signal swing was 15% or more than the baseline intensities the trail was excluded, this large signal swing may have been caused due to motion artifacts during the experiment. Then the selected data was filtered to remove any spontaneous hemodynamic fluctuations related to arterial pulsations and breathing. Low pass filtering was done at a cut-off frequency of 0.2 Hz this not only eliminates cardiac and blood pressure pulsations but also any electronic noise present. High pass filtering was done at a cut-off
frequency of 0.01 Hz to eliminate slow baseline drifts. Then changes in the hemoglobin concentrations in relation to initial baseline were calculated. Temporal profiles of each event were averaged across each test session to obtain an averaged, event-related hemodynamic response that was evoked by the PVT or DMS tasks.

3.5.2 General Linear Model Analysis

General linear model (GLM) analysis has been increasingly utilized to analyze fNIRS data over the last decade to identify cortical areas significantly stimulated by given tasks [57]-[60]. In this study, GLM analysis was used to analyze channel-wise hemodynamic responses in prefrontal cortex evoked by the tasks. The event-related analysis was done and the number of regressors depended on the tasks. In PVT, the first regressor accounted for the onset of the timer and second regressor for the reaction time. For DMS task, the two regressors accounted for the two phases of working memory, namely the encoding and recall phases. The regressors for each task were generated after convolving two corresponding boxcar functions with a hemodynamic response function (HRF) [59]. Here we used a standard HRF derived from BOLD fMRI [59]. By fitting the one/two regressors channel wise, the amplitudes of event-related hemodynamic responses to each memory phase were estimated [51]. Figure 27 and 28 depict the regressors’ positions in PVT and DMS task.

![Figure 27: Regressors of PVT](image-url)
3.5.3 Statistical Analysis

For behavioral analysis, one-tailed paired sample t-test was used to compare pre- and post- transcranial LLLT data. The normality of the data was checked by calculating the skewness of the data. The null hypothesis was defined as there is no significant difference in the reaction time/study time/recall time before and after the transcranial laser stimulation. The alternative hypothesis stated that there was a decrease in reaction time/study time/recall time after transcranial LLLT.

For hemodynamic measures, one-tailed paired sample t-test was performed to compare the beta values obtained during the GLM analysis for pre- and post-transcranial LLLT. The normality of the data was checked by calculating the skewness of the data. The null hypothesis was that there was no significant difference in the hemodynamic responses before and after the transcranial laser stimulation. The alternative hypothesis was that there is an increase in hemodynamic responses after transcranial LLLT.
3.6 Results

A total of 8 subjects were measured for this study (males = 7 and female =1). Table 3 shows the demographics of the subjects.

Table 3: Subject demographics for the task protocol

<table>
<thead>
<tr>
<th>Name</th>
<th>Gender</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7</td>
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<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>24</td>
</tr>
</tbody>
</table>

3.6.1 Behavioral Scores

Table 4 shows the results of average reaction time for the PVT before and after the transcranial LLLT. It also shows the average study/encoding time and recall/retrieval time taken for the DMS task before and after the transcranial LLLT.

For PVT test, the t-test indicates that the results did not show any significant difference in the reaction time before and after the transcranial LLLT.

For DMS test, significant difference in study/encoding and recall/retrieval time before and after the transcranial LLLT was seen.
Table 4: Behavioural results for PVT and DMS task

<table>
<thead>
<tr>
<th></th>
<th>PVT</th>
<th>DMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REACTION TIME (ms)</td>
<td>STUDY TIME (secs)</td>
</tr>
<tr>
<td>Pre LLLT</td>
<td>328.08</td>
<td>1.62</td>
</tr>
<tr>
<td>Post LLLT</td>
<td>330.65</td>
<td>1.28</td>
</tr>
<tr>
<td>p Values</td>
<td>0.07</td>
<td><strong>0.02</strong></td>
</tr>
</tbody>
</table>

3.6.2 Hemodynamic Responses

Hemodynamic response analysis was done using GLM. Beta values were obtained for HbO₂ and Hb concentrations. For this study only beta values for HbO₂ concentration were analyzed by plotting images that showed the activation areas of prefrontal cortex. These images were generated using MATLAB. A one-tailed paired sample t-test was performed for each channel to evaluate if the beta values were statistically significant.

3.6.2.1 Hemodynamic Response Evoked by the PVT Task

PVT tasks evoked robust HbO₂ increase in the prefrontal cortex. PVT-evoked Hb changes were much smaller. Figure 29 shows the HbO₂ changes before and after transcranial LLLT for PVT. From the results, it can be concluded that the highest HbO₂ changes were evoked at the site of the treatment.
Figure 29: Images of prefrontal activations for HbO2 concentration before & after transcranial LLLT for PVT Task

Statistical analysis was performed only for HbO2 concentrations. All 34 channels were found to be not significant. Therefore we failed to reject the null hypothesis which suggests that there was no significant difference in PVT reaction time before and after the transcranial LLLT. Table 1 in Appendix A shows the average of beta values for each channel and the p values obtained after t-test for each channel. Thus it can be concluded that though an overall increase in HbO2 concentration and improvement in performance of task after transcranial LLLT by subjects was seen, the change was not statistically significant.

3.6.2.1 Hemodynamic Response Evoked by the DMS Task

DMS task evoked an HbO2 increase during study/encoding phase, followed by undershoot during recall phase. For the study all of the study-related peaks, recall-related valleys, and the P-P amplitudes between two phases were analyzed. Figure 30 shows hemodynamic responses during the phases of DMS task.
DMS task results showed a robust increase in HbO$_2$ concentration after transcranial LLLT. Maximum activation was seen in the areas of the prefrontal cortex close to the site of treatment. Figure 31 shows the changes in the HbO$_2$ concentrations after the transcranial LLLT for the study/encoding phase of the DMS task and Figure 32 shows the same for recall/retrieval phase of the DMS task. Figure 33 shows the peak–peak amplitudes response between the two phases.

Figure 30: Illustrations of hemodynamic responses during the phases of DMS task

Figure 31: HbO$_2$ concentrations before & after transcranial LLLT for the study phase of DMS task
Figure 32: HbO$_2$ concentrations before & after transcranial LLLT for the recall phase of DMS task

Figure 33: HbO$_2$ concentrations before & after transcranial LLLT for peak-peak response

On performing the t-test on the beta values for DMS study/encoding event and the recall/retrieval event, 6 of the 34 channels were found to be significant. This suggested that the results showed significant difference in the DMS task performance the before and after the transcranial LLLT. Table 2 and 3 in Appendix show the average of beta values for each channels and the p values obtained after t-test for each channel. The tables also highlight the channels showing significant difference.
Chapter 4
Discussion and Future work

4.1 Discussion

In recent years, LLLT has been specially used in the areas of physical medicine and rehabilitation. Its applications started mainly for wound healing and pain relief, but over the recent years they have broadened to include diseases such as stroke, myocardial infarction, and degenerative or traumatic brain disorders [25, 45]. The aim of this study was to evaluate both the physiological and functional effects of single session of transcranial LLLT in young healthy adults.

Transcranial LLLT effect on cognitive functions in human subjects is a largely unexplored area and only a few studies have been done. In 2007, Lampl et al reported that infrared laser therapy to the head improved neurological outcome in controlled clinical trials of stroke. [61]- [63]. A study by Michalikova et al., 2008 showed that LLLT can improve working memory in middle-aged mice tested in a spatial navigation task [64]. In 2009, Schiffer et al reported that LED treatment to the forehead may alleviate depression in an uncontrolled pilot study of 10 patients. Another study reported of improved attention, executive function, and memory in two patients with chronic traumatic brain injury with the daily use of LLLT to the head [45]. In 2013, Barrett and Gonzalez-Lima reported the first controlled study of transcranial laser stimulation of psychological functions in humans [42]. All these studies assessed the behavioral scores to evaluate the effects of transcranial LLLT. This study evaluated behavioral as well as hemodynamic responses to understand the effects of transcranial LLLT.
4.1.1 Physiological Effects of Transcranial LLLT

The main findings in regard to the physiological effects of transcranial LLLT are summarized as follows:

1. HbT was calculated as sum of HbO₂ and Hb, and thus could provide a measure of cerebral blood volume [65]. Results in this study showed that the changes in HbT induced by LLLT were insignificant; indicating that the cerebral blood volume does not change.

2. HbD was calculated as the difference between HbO₂ and Hb, and could reflect changes in cerebral oxygen saturation (For example, an increase of oxygen saturation results in an increase of HbO₂ and a decrease of Hb). Therefore, a significant increase in HbD concentration after transcranial LLLT treatment could suggest an increase in regional cerebral oxygenation specific to frontal lobe [41].

3. Clear HbO₂ increase as well as Hb decrease was seen due to LLLT. This could indicate that there was a significant increase in cerebral oxygenation after transcranial LLLT.

4.1.2 Functional Effects of Transcranial LLLT

The second part of this study aimed at evaluating the functional effects of transcranial LLLT to identify if there was any improvement in the neural activation patterns. The main findings are summarized as follows:

1. For PVT test, the behavioral scores showed no significant difference before and after transcranial LLLT. The hemodynamic responses showed an increase in HbO₂ concentration after transcranial LLLT, which however was statistically insignificant.
2. The behavioral scores for DMS task showed significant difference in study/encoding and recall/retrieval times before and after the transcranial LLLT. DMS task evoked an HbO$_2$ increase during study/encoding phase, followed by undershoot during recall phase. Maximum activation was seen in the areas of the prefrontal cortex close to the site of treatment. Statistical analysis confirmed a significant difference in the activation amplitudes of the DMS task before and after transcranial LLLT.

A previous study by Soul et al. demonstrated that changes in HbD reflected changes in cerebral blood flow (CBF) over a wide range of intracranial pressure (ICP) in a model of acute hydrocephalus. [65] They measured CBF in cerebral cortex, white matter, and basal ganglia at each ICP with radioactive microspheres. Changes in HbO$_2$ and Hb were measured continuously by fNIRS. They observed that cerebral perfusion pressure declined with increasing ICP, and this decline was accompanied by significant decreases in HbD measured by fNIRS and CBF measured by radioactive microspheres. A strong correlation between changes in HbD and individual changes in CBF in cerebral cortex, white matter, and basal ganglia (all $p < 0.0001$) was found. [65]

Figure 34 shows the results obtained by Soul et al. In this figure the left panel shows the changes in ICP, MAP, Hb, HbO$_2$ and the resultant change in HbD during the experiment [65]. The right panel shows the relationship between change in HbD and the change in regional CBF in the cerebral cortex and cerebral white matter with accompanying $p$ values [65]. NIRS results in the left panel show a rising ICP and, in parallel, a declining HbO$_2$ and HbD [65]. These time courses are similar to the results obtained in this study except the trends are opposite (refer to Figure 14 and 15 in Chapter 2). By comparing with the results shown in this figure, we tend to
believe the significant increase in HbD concentration after transcranial LLLT may indicate not only an increase in cerebral oxygenation but also an increase in CBF.

Figure 34: (Left) Graphs displaying time course of changes in ICP, MAP, Hb, and HbO2 and the resultant change in HbD during one experiment. (Right) Graphs showing the highly significant relationship between change in HbD and the change in rCBF in cerebral cortex and cerebral white matter with p values [65].
The significantly increased cerebral oxygenation and blood flow during and after transcranial LLLT could be related to the following underlying mechanism:

The primary mechanism of improvement after LLLT suggested by many studies was an increase in ATP [25, 45, 61]. This mechanism suggests that NIR light during the treatment could have been absorbed by the mitochondria in the nervous tissue, leading to upregulation of cellular respiration. This increase in respiration occurs due to increased rate of oxidation and reduction of an electron transport system complex called cytochrome oxidase (CCO). This results in increase of mitochondrial products like ATP, NADH, protein, ribonucleic acid [22, 25]. Thus LLLT increases CCO, causing increase in oxygen consumption and mitochondrial membrane potential, and activating the mitochondrial permeability transition pore [22]. Therefore the increase in the concentration of HbO₂ could be due to the increase in oxygen consumption by the neurons. Figure 31 shows a flow chart summarizing the mechanism.

![Flow Chart](image)

**Figure 35:** Underlying mechanism at molecular and hemodynamic level during and after transcranial LLLT
Thus in this study, both PVT and DMS tasks resulted in a greater increase in HbO$_2$ concentration and a greater decrease in Hb concentration after transcranial LLLT. These changes suggest that overall greater amplitudes of activation induced by transcranial LLLT, could interpret the subjects’ improvement in the performance scores.

4.1.3 Regional Differences due to LLLT

In study of physiological effects of transcranial LLLT, results of right hemisphere versus left hemisphere for central head stimulation didn’t show any significant difference. These results suggest that transcranial LLLT induced an approximately systemic or global effect on the brain.

In study of neurological effects of transcranial LLLT, the results also showed an increase in HbO$_2$ concentrations in both the hemispheres. This suggests that increase in neural activity took place not only in the right hemisphere (the treatment site) but also in some regions of the left hemisphere. The human brain is a complex and dynamic system and is often represented as a structurally or functionally interconnected network that works to ensure both continuous processing and efficient information flow between interconnected units [66]. Thus the left and right hemispheres are functionally interconnected and this is the reason of activations seen in both the hemispheres during the tasks.

4.2 Limitations

At last, it is important to note a few limitations in the current study:

In evaluation of the physiological effects of LLLT, significant hemodynamic changes in response to the LLLT treatment was observed at a 99% confidence interval for a sample size of 9 subjects. However, in the evaluation of functional effects of LLLT, significant difference in task-evoked activations before and after the treatment was not observed at 95% confidence interval for sample size of 8 subjects. Compared to the directed physiological effects of the LLLT
treatment, the neurological effects of such a treatment seem more subtle in magnitude and therefore are more difficult to detect. Increasing the sample size in the second part of study will increase the chance of finding more conclusive results from the data.

Another limitation was absence of a placebo-controlled/ sham group. Performing the study with this group would rule out any possibility of placebo effects and would give a more complete and conclusive result.

The third limitation of this study was that the fNIRS signals were collected only from the prefrontal cortex. It has been largely unknown whether the LLLT treatment affects the functions of other brain regions. Future measurements across different regions of the cerebral cortex would help give a larger picture of the hemodynamic changes taking place following the transcranial LLLT.

Another limitation is that some of the obtained results could not be explained and need further study to understand and describe the underlying phenomenon. The central forehead stimulation results showed constant cerebral blood volume. This could not be explained as the increase in blood flow should increase the blood volume but this was not observed during central forehead stimulation. Second result that could not explained is the decrease in HbO₂ observed during the recall phase of the DMS Task. The HbO₂ concentration was lower than baseline during the recall phase of DMS Task, this is unexpected as there are studies that show tasks evoked robust hemodynamic activations during the encoding and retrieval phases [51]. Further study is needed to explain and interpret the underlying mechanism on these results.
4.3 Future Work

Currently fNIRS allows a non-invasive and relatively low-cost optical approach for measuring cerebral hemodynamics and oxygenation. However, this technique solely is still not sufficient to describe the complex physiological processes of the brain in response to the LLLT treatment. Especially, as our main findings above indicate, there is a suspicious change in CBF associated with the treatment. In future a technique that can independently measure cerebral blood flow or cerebral perfusion can be integrated with the current NIRS setup. This would comprehend the understanding of the physiological changes taking place in the cerebral cortex after transcranial LLLT. Such a technique is currently available, e.g., a diffused correlation spectroscopy (DCS) system. To address this improvement a DCS system with a software autocorrelator to measure CBF was developed. More details of this project are included in Appendix B.

Since LLLT is noninvasive treatment, future studies could test this treatment on a number of patient populations without additional concern on safety. Potential subjects could include those who need neuro-rehabilitation like Post Traumatic Stress Disorder (PTSD) patients. Studies on Post-traumatic stress disorder (PTSD)-related memory impairments have constantly implicated abnormal activities in the frontal and parietal lobes. Tian et al studied the involvement of the prefrontal cortex in working memory phases, which was assessed among veterans with PTSD and age- / gender-matched healthy controls. They reported that veterans with PTSD appeared to suppress prefrontal activity during memory retrieval [51]. They observed that deactivation was more pronounced in the right dorsolateral prefrontal cortex during the retrieval phase [51]. The study concluded that deactivations in PTSD patients might implicate an active inhibition of dorsolateral prefrontal neural activity during retrieval of working memory [51].
This study will be able to help such subjects, by enhancing their neural metabolism in the hypoactive regions, which in turn leads to functional improvement, as the site of the transcranial LLLT used in this study is close to the hypoactive regions mentioned earlier.

In summary, this study demonstrated that transcranial LLLT could be a non-invasive, non-pharmacologic, therapeutic, cytoprotective and performance-enhancing approach to improve brain functions like cognitive functions.
APPENDIX A

PVT and DMS GLM analysis Tables
Table A-1: Table showing average beta values and p values for HbO2 concentration of PVT task

<table>
<thead>
<tr>
<th>Channel</th>
<th>Pre-LLLT PVT Mean ±SD</th>
<th>Post-LLLT PVT Mean ± SD</th>
<th>p-Value</th>
</tr>
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<td>1</td>
<td>-1.15 ± 3.56</td>
<td>-0.71 ± 1.23</td>
<td>0.38</td>
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<tr>
<td>2</td>
<td>0.39 ± 1.44</td>
<td>1.51 ± 2.66</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>-0.18 ± 1.73</td>
<td>-0.08 ± 1.89</td>
<td>0.46</td>
</tr>
<tr>
<td>4</td>
<td>0.16 ± 2.92</td>
<td>1.66 ± 2.21</td>
<td>0.16</td>
</tr>
<tr>
<td>5</td>
<td>0.03 ± 2.61</td>
<td>0.36 ± 1.43</td>
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</tr>
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<td>-0.25 ± 1.17</td>
<td>0.50</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
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<td>0.24 ± 1.37</td>
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<td>32</td>
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Table A-2: Average beta values & p values for HbO2 concentration of DMS study task

(Significant channels are highlighted in red)

<table>
<thead>
<tr>
<th>Channel</th>
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<th>Post-LLLT DMS STUDY TIME</th>
<th>p-Value</th>
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</thead>
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<td><strong>Mean ± SD</strong></td>
<td><strong>Mean ± SD</strong></td>
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<td>1.08 ± 1.33</td>
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<td>24</td>
<td>0.93 ± 1.17</td>
<td>0.11 ± 0.45</td>
<td>0.06</td>
</tr>
<tr>
<td>25</td>
<td>1.14 ± 2.05</td>
<td>0.18 ± 0.90</td>
<td>0.13</td>
</tr>
<tr>
<td>26</td>
<td>0.50 ± 1.53</td>
<td>0.10 ± 0.40</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table A-3: Average beta values & p values for HbO2 concentration of DMS recall task

<table>
<thead>
<tr>
<th>Channel</th>
<th>Pre-LLLT DMS RECALL TIME</th>
<th>Post-LLLT DMS RECALL TIME</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.54 ± 2.57</td>
<td>-0.06 ± 0.46</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>-0.65 ± 2.05</td>
<td>-0.10 ± 0.49</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>-0.83 ± 1.69</td>
<td>-0.22 ± 0.33</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>-0.63 ± 1.91</td>
<td>-0.32 ± 0.33</td>
<td>0.31</td>
</tr>
<tr>
<td>5</td>
<td>-1.03 ± 1.77</td>
<td>-0.18 ± 0.23</td>
<td>0.10</td>
</tr>
<tr>
<td>6</td>
<td>-0.67 ± 1.95</td>
<td>0.01 ± 0.34</td>
<td>0.19</td>
</tr>
<tr>
<td>7</td>
<td>-0.56 ± 1.45</td>
<td>-0.16 ± 0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>9</td>
<td>-0.82 ± 1.39</td>
<td>-0.30 ± 0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>10</td>
<td>-0.54 ± 1.30</td>
<td>-0.25 ± 0.41</td>
<td>0.23</td>
</tr>
<tr>
<td>11</td>
<td>-0.95 ± 1.45</td>
<td>-0.23 ± 0.31</td>
<td>0.06</td>
</tr>
<tr>
<td>12</td>
<td>-0.62 ± 1.40</td>
<td>-0.27 ± 0.34</td>
<td>0.22</td>
</tr>
<tr>
<td>13</td>
<td>-0.34 ± 0.99</td>
<td>-0.07 ± 0.29</td>
<td>0.27</td>
</tr>
<tr>
<td>15</td>
<td>-0.22 ± 1.51</td>
<td>-0.16 ± 0.31</td>
<td>0.45</td>
</tr>
<tr>
<td>18</td>
<td>-0.24 ± 1.48</td>
<td>-0.19 ± 0.89</td>
<td>0.47</td>
</tr>
<tr>
<td>19</td>
<td>-0.15 ± 2.23</td>
<td>-0.03 ± 0.50</td>
<td>0.44</td>
</tr>
<tr>
<td>20</td>
<td>-0.41 ± 1.86</td>
<td>-0.21 ± 0.58</td>
<td>0.36</td>
</tr>
<tr>
<td>21</td>
<td>-0.88 ± 2.26</td>
<td>-0.08 ± 0.25</td>
<td>0.16</td>
</tr>
<tr>
<td>22</td>
<td>-0.53 ± 1.82</td>
<td>-0.26 ± 0.54</td>
<td>0.33</td>
</tr>
<tr>
<td>23</td>
<td>0.03 ± 1.57</td>
<td>-0.06 ± 0.52</td>
<td>0.43</td>
</tr>
<tr>
<td>24</td>
<td>-0.67 ± 1.75</td>
<td>-0.31 ± 0.53</td>
<td>0.25</td>
</tr>
<tr>
<td>26</td>
<td>-0.80 ± 1.96</td>
<td>-0.17 ± 0.45</td>
<td>0.16</td>
</tr>
<tr>
<td>27</td>
<td>-0.54 ± 1.26</td>
<td>-0.17 ± 0.52</td>
<td>0.20</td>
</tr>
<tr>
<td>28</td>
<td>-0.59 ± 1.42</td>
<td>-0.29 ± 0.70</td>
<td>0.26</td>
</tr>
<tr>
<td>29</td>
<td>-0.62 ± 1.16</td>
<td>-0.22 ± 0.40</td>
<td>0.20</td>
</tr>
<tr>
<td>30</td>
<td>-0.12 ± 1.12</td>
<td>0.15 ± 1.06</td>
<td>0.33</td>
</tr>
<tr>
<td>32</td>
<td>-0.06 ± 1.48</td>
<td>0.16 ± 0.76</td>
<td>0.33</td>
</tr>
</tbody>
</table>
Table A-4: Average beta values & p values for HbO2 concentration of DMS P-P response

(Significant channels are highlighted in red)

<table>
<thead>
<tr>
<th>Channel</th>
<th>Pre-LLLT DMS P-P Response</th>
<th>Post-LLLT DMS P-P Response</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.20 ± 5.41</td>
<td>0.17 ± 0.71</td>
<td>0.16</td>
</tr>
<tr>
<td>2</td>
<td>1.73 ± 2.72</td>
<td>0.17 ± 1.12</td>
<td>0.04</td>
</tr>
<tr>
<td>3</td>
<td>1.91 ± 2.70</td>
<td>0.40 ± 0.60</td>
<td>0.06</td>
</tr>
<tr>
<td>4</td>
<td>1.66 ± 3.02</td>
<td>0.44 ± 0.81</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>2.94 ± 3.74</td>
<td>0.26 ± 0.72</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>1.67 ± 3.34</td>
<td>0.26 ± 0.58</td>
<td>0.13</td>
</tr>
<tr>
<td>7</td>
<td>1.69 ± 2.32</td>
<td>0.42 ± 0.39</td>
<td>0.09</td>
</tr>
<tr>
<td>9</td>
<td>2.25 ± 3.11</td>
<td>0.47 ± 0.93</td>
<td>0.05</td>
</tr>
<tr>
<td>10</td>
<td>1.07 ± 2.09</td>
<td>0.40 ± 0.74</td>
<td>0.14</td>
</tr>
<tr>
<td>11</td>
<td>2.58 ±3.11</td>
<td>0.39 ± 0.71</td>
<td>0.02</td>
</tr>
<tr>
<td>12</td>
<td>1.83 ± 2.84</td>
<td>0.47 ± 0.54</td>
<td>0.09</td>
</tr>
<tr>
<td>13</td>
<td>0.59 ± 1.70</td>
<td>0.17 ± 0.35</td>
<td>0.25</td>
</tr>
<tr>
<td>15</td>
<td>0.42 ± 2.32</td>
<td>0.21 ± 0.60</td>
<td>0.41</td>
</tr>
<tr>
<td>16</td>
<td>0.00 ± 0.00</td>
<td>0.00 ±0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>17</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>18</td>
<td>1.32 ± 2.25</td>
<td>0.19 ± 1.37</td>
<td>0.13</td>
</tr>
<tr>
<td>19</td>
<td>0.97 ± 1.96</td>
<td>-0.07 ± 1.10</td>
<td>0.07</td>
</tr>
<tr>
<td>20</td>
<td>1.24 ± 2.18</td>
<td>0.15 ± 0.94</td>
<td>0.08</td>
</tr>
<tr>
<td>21</td>
<td>2.28 ± 2.92</td>
<td>0.07 ± 0.74</td>
<td>0.03</td>
</tr>
<tr>
<td>22</td>
<td>1.48 ± 4.01</td>
<td>0.31 ± 0.94</td>
<td>0.20</td>
</tr>
<tr>
<td>23</td>
<td>-0.12 ± 3.90</td>
<td>-0.03 ± 0.82</td>
<td>0.47</td>
</tr>
<tr>
<td>24</td>
<td>1.36 ± 3.09</td>
<td>0.42 ± 1.10</td>
<td>0.16</td>
</tr>
<tr>
<td>26</td>
<td>2.20 ± 3.31</td>
<td>0.24 ± 0.97</td>
<td>0.05</td>
</tr>
<tr>
<td>27</td>
<td>1.34 ± 1.95</td>
<td>0.22 ± 1.06</td>
<td>0.06</td>
</tr>
<tr>
<td>28</td>
<td>1.94 ± 3.62</td>
<td>0.39 ± 1.22</td>
<td>0.10</td>
</tr>
<tr>
<td>29</td>
<td>1.56 ± 1.72</td>
<td>0.33 ± 0.72</td>
<td>0.06</td>
</tr>
<tr>
<td>30</td>
<td>1.26 ± 2.63</td>
<td>0.03 ± 0.82</td>
<td>0.14</td>
</tr>
<tr>
<td>32</td>
<td>0.55 ± 2.76</td>
<td>-0.07 ± 0.72</td>
<td>0.27</td>
</tr>
</tbody>
</table>
APPENDIX B

Diffused Correlation Spectroscopy with a Software Autocorrelator
Diffused Correlation Spectroscopy with a Software Autocorrelator

The future work of my thesis indicated that a technique that can independently measure cerebral blood flow or cerebral perfusion can be integrated with the current NIRS setup. This would help comprehend the understanding of the physiological changes taking place in the cerebral cortex after transcranial LLLT. I have been working on the development of a diffused correlation spectroscopy (DCS) system which will help measure cerebral blood flow. This section of the appendix discusses the details of the mechanism, development and validation of DCS system

B.1 Introduction

Diffused correlation spectroscopy (DCS) is an optical technique that utilizes temporal fluctuations of light in the NIR spectral window (650 to 1100 nm) to non-invasively measure cerebral blood flow (CBF) [67]. DCS is an emerging technique which has been developed, extensively validated and employed for continuous noninvasive measurement of blood flow in deep tissue like brain, muscle and breast [68]. Studies on DCS suggest that DCS quantitatively determines relative changes of tissue blood flow quite well with respect to a baseline [67]. Studies have also concluded that, hybrid instrumentation combining DCS with NIRS gives the prospect for continuous non-invasive approximation of cerebral metabolic rate of oxygen extraction (CMRO$_2$) [67]. This would not only help us understand the CBF but also metabolic changes taking place after transcranial LLLT in the PFC.

B.2 Theoretical Background of DCS:

As mentioned in section 1.1.2 biological tissue can be characterized by its optical properties like absorption coefficient and scattering coefficient. When NIR light scatters through
moving scatterers, the attenuated light is phase shifted and causes speckle fluctuation at the
detector. The motion information of the scatterers is carried by the electric field of the attenuated
light and can be obtained from the electric field autocorrelation function [68] The function is
defined as follows:

\[ G(\vec{r}, \tau) = \langle E(\vec{r}, t)E^*(\vec{r}, t + \tau) \rangle \]  

(1)

Experimentally, it is easier to measure intensity than electric field of the attenuated light. Thus
the diffuse light electric field autocorrelation function is derived from the measured normalized
intensity autocorrelation [67, 68]. The normalized intensity autocorrelation is calculated as
follows:

\[ g(\vec{r}, \tau) = \frac{\langle I(\vec{r}, t)I(\vec{r}, t+\tau) \rangle}{\langle I \rangle^2} \]  

(2)

Equation 2 is calculated by using the Siegert relation: [68,69]

\[ g(\vec{r}, \tau) = 1 + \beta \frac{|G(\vec{r}, \tau)|^2}{\langle I(\vec{r}, t) \rangle^2} \]  

(3)

Where \( I(\vec{r}, t) \) is the detected diffusing light intensity at position \( r \) and time \( t \), the angle bracket
\( \langle .. \rangle \) represents an ensemble average, and \( \beta \) is a numerical factor related to the detector geometry,
number of detected speckles and other experimental parameters [68]. The Siegert relation is used
to obtain the electric field autocorrelation function with the assumption that the system is ergodic
i.e. the time average is equal to the ensemble average. [68] Previous studies have shown that
\( G(\vec{r}, \tau) \) satisfies the correlation diffusion equation. [69]- [71]. The mean square displacement of
the moving scatterers in the diffusion equation is represented by \( < \Delta r^2(\tau) > \). Two models that
describe this term are the Brownian motion and random flow model in biological tissues. Studies
have indicated that Brownian motion model presented better fitting in the most of cases, this
includes brain, muscle and tumor models [69, 72-76]. The measurements of $G(\vec{r}, \tau)$ can be fitted with this model to calculate a blood flow index to in turn quantify the relative blood flow [76].

B.3 Set Up

The DCS system was designed similar to Dong et al 2012 paper [68]. It consisted of a long coherence length (>10 m) continuous-wave laser at 785 nm (DL785-100-S, ~100 mW, CrystaLaser, Reno, Nevada, USA) as the source. The detector used was a photon-counting APD (SPCM AQRH-14-FC, Excelitas Technologies, Vaudreuil (Quebec), Canada), its output was transistor-transistor logic (TTL) pulses. The light from the laser was coupled through a multi-mode optical fiber (125 μm diameter) to the sample surface. The attenuated light was collected using a single-mode fiber operated at 785 nm (5-μm diameter) and fed to the APD. The TTL output generated by the APD was connected to the 32-bit, eight input channel counter/timer board with the maximum input rate of 80 MHz (PCI-6602, National Instruments)[80]. The counter/timer board was connected to a PC (CPU: Intel Core 2 Duo, RAM: 4-Gbyte) through a shielded BNC connector block for DAQ devices (BNC-2121, National Instruments). Figure B-1 shows a block diagram of the set up:

Figure B -1: Block diagram of the DCS system
B.4 Data Acquisition Principle

Data acquisition was done using LabVIEW (National Instruments), which is one of the most popular and powerful tool for interactive data acquisition. Two virtual instruments (VI) were designed for the DCS system in LabVIEW. The first VI was designed to generate the gate signal and the second VI was to read and write the photon counts from the APD into a file. The detector output pulses were counted over sampling time $\tau$ by the counter/timer board.[72] The PCI-6602 has maximum source frequency of 80 MHz, this means the fastest input pulses that can be counted are 12.5 ns ($1/80; 000; 000$ s) apart [68, 77, 78]. The PCI-6602 is the 32-bit counter and can count up to 4,294,967,295 ($2^{32} - 1$) before it rolls over [79]. The counter counted the events continuously and stored the data into the buffer. The buffer size was limited and overflowed in less than 1 second for time resolution of anything less than 2µs [68]. The sampling rate of the system can reach up to $\sim$400 kHz and thus enables the minimum lag time of $\sim 2.5$ µs, which is significantly shorter than the decay constant (between tens to hundreds of microseconds) in DCS applications [68]. For stable and smooth data acquisition the experiments were conducted at 4.0µs.

A gate signal was used to count the APD output pulses. The gate signal had a total time resolution of 4µs of which 3.4µs was “on” and 0.6µs was “off”. Buffered period measurement technique was used for counting the photon pulses. In buffered period measurement, source edges cause the count to increment while gate edges will buffer the instantaneous count and also reset the count register back to 0 for the next interval (Refer figure B-2). Thus for our application every APD pulse will increment the internal count register and every active gate edge will (1) stored the instantaneous count value in the task buffer and (2) reset the count register to 0. The period of gate signal is characterized in terms of the number of APD pulses that occur per
interval. To accomplish this, the DAQmx Timing VI was set up to use "Implicit" timing (Refer figure B-6). This means that the signal whose period is being measured implicitly sets the timing of sampling based on whenever its edges arrive [80]. Thus the net effect is to perform a "binning" measurement where the "periods" is read to find out how many APD pulses happened within each interval of the gate signal. When using buffered counter operations, the first acquired points represented bad data in period measurements and thus were ignored. The first data point is the measured interval between the instant when the counter is armed and when the first edge transition takes place on the counter GATE (refer figure B-2) [79]. Since there is no deterministic way of specifying when the counter is actually armed, the first value may be incorrect. Subsequent data points acquired will not have this problem and thus the first reading is ignored [79].

For efficient data acquisition, “queue” structure was implemented in the read and write VI (Refer figure B-6). The queue structure had two loops, the first loop was the “producer” loop, which was responsible for reading photons counts and the second ”consumer” loop was in charge
of writing the results in a file. The two loops worked in parallel for a faster speed [68]. The data was read and stored for every 1 second with 5 second delay between two consecutive reads.

For the first stage of the system design, the autocorrelation was not done in real time. In this stage correlation was calculated after the data had been acquired. In future the data acquisition and correlation will be done in real time. The snippets of the VIs are shown below:

![Image of Front panel of the gate signal VI](image-url)

**Figure B-3: Front panel of the gate signal VI**

![Image of Block diagram of the gate signal VI](image-url)

**Figure B-4: Block diagram of the gate signal VI**
Figure B-5: Front panel of the read and write VI

Figure B-6: Block diagram of the read and write VI
B.5 Efficiency Tests of VIs:

Two tests were performed to check if the VIs were recording the photon counts accurately and efficiently. The first test was done to determine the frequency and duty cycle of the gate signal at which the read and write VI was most efficient. The VI efficiency was checked at frequencies: 250 kHz, 333 kHz and 400 kHz for the duty cycle of 10%, 20%, 50%, 80% and 85%. After performing the tests it was concluded that the VI was most efficient at frequency of 250 kHz and 80% duty cycle. Table B-1 shows the results for frequency = 250 kHz.

Table B-1: VI efficiency test results for gate signal frequency = 250 kHz

<table>
<thead>
<tr>
<th>Duty cycle</th>
<th>10%</th>
<th>20%</th>
<th>50%</th>
<th>80%</th>
<th>85%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed counts</td>
<td>11</td>
<td>20</td>
<td>60</td>
<td>91</td>
<td>27</td>
</tr>
<tr>
<td>Expected Counts</td>
<td>16</td>
<td>32</td>
<td>80</td>
<td>128</td>
<td>136</td>
</tr>
<tr>
<td>Efficiency %</td>
<td>68.75</td>
<td>62.5</td>
<td>75</td>
<td>71.09375</td>
<td>19.85294</td>
</tr>
</tbody>
</table>

The second test was done to determine the photon counting efficiency of the read and write VI by varying frequency of the APD signal. The APD signal was simulated from a function generator. The VI was tested for sine and square waves at frequencies of 2 MHz, 10 MHz, 15 MHz, 20 MHz, 30 MHz and 40 MHz. For the APD signal as sine wave the VI showed a good efficiency of ~93% till 20 MHz frequency after which it gave zero counts. For the APD signal as square wave the VI showed a good efficiency of ~93% till 30 MHz frequency and at 40 MHz the VI efficiency dropped to 47%. Table B-2 and B-3 summarize the results of the second test.
Table B-2: Results of efficiency test for sine wave APD signal

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Observed Counts</th>
<th>Expected counts</th>
<th>Photon counting efficiency of VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 MHz</td>
<td>6 or 7</td>
<td>6.5</td>
<td>92.8%</td>
</tr>
<tr>
<td>10 MHz</td>
<td>32</td>
<td>34</td>
<td>94.1%</td>
</tr>
<tr>
<td>15 MHz</td>
<td>48</td>
<td>51</td>
<td>94.1%</td>
</tr>
<tr>
<td>20 MHz</td>
<td>0</td>
<td>68.5</td>
<td>0</td>
</tr>
<tr>
<td>20 MHz</td>
<td>0</td>
<td>68.5</td>
<td>0</td>
</tr>
<tr>
<td>20 MHz</td>
<td>0</td>
<td>68.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Table B-3: Results of efficiency test for square wave APD signal

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Observed Counts</th>
<th>Expected counts</th>
<th>Photon counting efficiency of VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 MHz</td>
<td>6 or 7</td>
<td>6.5</td>
<td>92.8%</td>
</tr>
<tr>
<td>10 MHz</td>
<td>32</td>
<td>34</td>
<td>94.1%</td>
</tr>
<tr>
<td>15 MHz</td>
<td>48</td>
<td>51</td>
<td>94.1%</td>
</tr>
<tr>
<td>20 MHz</td>
<td>64</td>
<td>68.5</td>
<td>93.4%</td>
</tr>
<tr>
<td>30 MHz</td>
<td>96</td>
<td>102.75</td>
<td>93.4%</td>
</tr>
<tr>
<td>40 MHz</td>
<td>64</td>
<td>136</td>
<td>47.1%</td>
</tr>
</tbody>
</table>

After a few modifications in the VI like disabling duplicate counting and increasing the number of counters that counted the photon counts, the efficiency of the VI increased. It increased to ~ 94% for all the frequencies. Table B-4 shows the results for this test.
Table B-4: Results of efficiency test for square wave APD signal after VI modification

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Observed Counts</th>
<th>Expected counts</th>
<th>Photon counting efficiency of VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 MHz</td>
<td>6 or 7</td>
<td>6.5</td>
<td>92.8%</td>
</tr>
<tr>
<td>10 MHz</td>
<td>32</td>
<td>34</td>
<td>94.1%</td>
</tr>
<tr>
<td>15 MHz</td>
<td>48</td>
<td>51</td>
<td>94.1%</td>
</tr>
<tr>
<td>20 MHz</td>
<td>64</td>
<td>68.5</td>
<td>93.4%</td>
</tr>
<tr>
<td>30 MHz</td>
<td>96</td>
<td>102.75</td>
<td>93.4%</td>
</tr>
<tr>
<td>40 MHz</td>
<td>128</td>
<td>136</td>
<td>94.1%</td>
</tr>
<tr>
<td>50 MHz</td>
<td>192</td>
<td>204</td>
<td>94.1%</td>
</tr>
<tr>
<td>60 MHz</td>
<td>160</td>
<td>170</td>
<td>94.1%</td>
</tr>
</tbody>
</table>

B.6 Data Processing Principle

The data obtained was stored in TDMS format, which is more efficient for storage of large data files. The output file had the photons counts for each active gate signal. MATLAB was used for processing of the data. The data was loaded into MATLAB and used to calculate the autocorrelation function. For this stage of the development, MATLAB’s inbuilt function “autocorr” was used for calculation of autocorrelation function. The “autocorr” function computes the sample autocorrelation function (ACF) of a univariate, stochastic time series [81]. The sampling frequency and the time lag were determined depending on the experiment performed. In future, an FFT-based correlation algorithm will be used to perform the autocorrelation in real time.
B.7 Validation of the DCS System

For validation of the system, intralipid phantom experiments were conducted. Intralipid phantom is a liquid that mimics properties of human or animal tissues. Intralipid phantoms play an important role in evolvement of diagnostic systems and most physical therapeutic interventions [76]. It can be used for the purposes of initial testing of system design, optimizing signal to noise ratio (SNR) in existing system [82]. In order to validate and assess the ability of the system, an intralipid phantom was made as shown in figure B-7 (b).

Intralipid phantom of different concentrations were used to mimic the characteristics of the tissue. The intralipid concentrations used were 0.3%, 0.5%, 0.7% and 1%. The validation test was also done with a solid phantom. The optical properties of the solid phantom were $\mu_a=0\text{cm}^{-1}$ and $\mu_s'=5\text{cm}^{-1}$. The schematic of the DCS experimental set up is shown in figure b-7(a). Reflection geometry was adopted in which the incident light was injected into the phantom by the source fiber and detected 1.0 cm away.

![Schematics of DCS experimental set up.](image1)

![Intralipid phantom with source and detector fibers](image2)

Figure B-7: (a) Schematics of DCS experimental set up. (b) Intralipid phantom with source and detector fibers
The data acquired was processed in MATLAB for all the concentrations of intralipid phantom and also for the solid phantom. The normalized autocorrelation function was obtained for all the intralipid concentrations and the solid phantom. The results obtained showed a similar trend to results of previous studies (refer figure b-8). The obtained results need to be fitted with the Brownian motion model to interpret if the phantom mimics human tissue characteristics. The Brownian motion model will in later stages help us calculate the blood flow index. Thus these results still need to be interpreted and evaluated properly to validate the system.

![Autocorrelation curves of the phantom with varied concentrations](image)

Figure B-8: Autocorrelation curves of the phantom with varied concentrations (0.3%, 0.5%, 0.7%, and 1%) as a function of delay time $\tau$.

Other validation tests that can be performed are flow phantom test and the human arm cuff occlusion experiment. These tests mimic the blood flow characteristics better and thus will be more apt for validation and assessment of the DCS system. On incorporating all the above
mentioned concepts this DCS system design will be an easy to operate, cost-effective, noninvasive system for the measurement of cerebral blood flow.

In conclusion, this implementation of DCS system with a software autocorrelator has a potential of being a real-time flow indicator and can be used in future in research and clinical settings.
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Biographical Information

Snehal Hase was born in Nasik, India on June 22\textsuperscript{nd} 1990. She completed her Bachelors in biomedical engineering from University of Mumbai, India in May 2012. She started as a biomedical engineer at Fortis Healthcare Hospitals. She worked on diagnosing and solving service and maintenance complaints for medical devices in the hospital. In fall 2013, she began her graduate studies at University of Texas at Arlington. To pursue her research interest in medical imaging she joined Dr. Hanli Liu’s research lab. Her research work during the graduate course involved transcranial laser stimulation and diffused correlation spectroscopy. She plans to pursue her interests in medical imaging by taking up related challenging projects in healthcare and allied fields.