IN VIVO IMAGING OF ANGIOGENESIS IN 3D-PRINTED BIOACTIVE SCAFFOLDS

By
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ABSTRACT

Due to the complexities associated with bone regeneration and wound site geometry, successful surgical reconstruction of craniomaxiofacial (CMF) injuries remains challenging. To circumvent this issue, investigators have developed paradigm-shifting tissue engineering and regenerative medicine (TERM) based approaches to induce osteogenesis. One such approach involves the use of ‘bioactive’ scaffolds that are 3D-printed polyacrolaptone (PCL) grafts embedded with cells from the human stromal vascular fraction (SVF) suspended in fibrin gel. However, since bioactive grafts are usually tested under in vitro conditions, it is not known how factors in the in vivo wound microenvironment such as oxygenation, angiogenesis and perfusion affect the survival of SVF. Therefore, for this thesis we developed an in vivo optical imaging pipeline to answer these questions in a preclinical calvarial bone defect model. We used intrinsic optical signal (IOS) imaging to assess in vivo angiogenesis and oxygenation, and used laser speckle contrast imaging (LSCI) to assess in vivo perfusion within the implanted 3D scaffold. We also employed a carbogen (95% oxygen, 5% carbon dioxide) gas challenge protocol to map ‘mature’ vasculature, and red fluorescent protein (RFP) imaging to track SVF distribution in vivo. Finally, from this multimodality imaging data, we extracted quantitative metrics that characterize the degree of angiogenesis, oxygenation, vascular maturity and perfusion in vivo. We believe that the combination of in vivo optical imaging along with TERM approaches can be exploited to produce patient-specific bone grafts. Such an advance would revolutionize CMF reconstruction surgery and benefit a wide variety of patients.

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<th>Explanation</th>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AFNI</td>
<td>Analysis of Functional Neuro-Images</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Bone Morphogenetic Protein 2</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged-Coupled Device</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer Aided Design</td>
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<tr>
<td>CMF</td>
<td>Craniofacial</td>
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<tr>
<td>HbO₂</td>
<td>Deoxyhemoglobin</td>
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<tr>
<td>Dextran-FITC</td>
<td>Dextran-Fluorescein Isothiocyanate</td>
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<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
</tr>
<tr>
<td>hASC</td>
<td>human Adipose-Derived Stem Cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-Inducible Factor 1α</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<tr>
<td>IOS</td>
<td>Intrinsic Optical Signaling</td>
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<tr>
<td>LSCI</td>
<td>Laser Speckle Contrast Imaging</td>
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<tr>
<td>MR</td>
<td>Magnetic Resonance</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>NIR</td>
<td>Near-Infrared</td>
</tr>
<tr>
<td>Hb</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescence Protein</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immune Deficiency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
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<tr>
<td>SVF</td>
<td>Stromal Vascular Fraction</td>
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<td>TERM</td>
<td>Tissue Engineering and Regenerative Medicine</td>
</tr>
<tr>
<td>HbT</td>
<td>Total Hemoglobin</td>
</tr>
<tr>
<td>TFG-b</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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CHAPTER 1: BIOLOGICAL OVERVIEW

This chapter provides an overview of two fundamental concepts: Section 1.1 covers the stages of wound healing, and Section 1.2 introduces the basics of tissue engineering and discusses how wound healing phenomena play a role in Tissue Engineering and Regenerative Medicine (TERM).

1.1 Overview of Repair Biology, i.e. the ‘wound-healing’ cascade

Wound healing is a series of steps that drive post-trauma repair. The main stages of wound healing, which overlap, include hemostasis, inflammation, proliferation and remodeling [1]. This process requires the unified response of a multiple cell types, the extracellular matrix (ECM) and signaling proteins called cytokines [2].

1.1.1 The Inflammatory Phase

Hemostasis, or blood clotting, is a cellular and biochemical process that occurs in the first stage of wound healing that is known as the inflammatory phase. Here, blood changes from a liquid to gel-like texture and acts as a temporary lining at the site of a vessel’s endothelial damage to prevent blood from escaping through the damaged vessel. This occurs via a series of recruiting coagulation proteins [3]. The inflammatory phase works in conjunction with many other regulatory systems including the coagulation system, fibrinolytic system, platelets, kinin system, serine protease inhibitors and the complement system [3]. Table 1.1 summarizes the underlying features and biological functions of the aforementioned systems:

Table 1.1: Systems involved in maintaining hemostasis

<table>
<thead>
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<th>System</th>
<th>Features and Role in Hemostasis</th>
</tr>
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</table>
| Coagulation System | - Primarily consists of thrombin, a key effector enzyme amongst other coagulation factors  
                  | - Consists of other cofactors such as calcium, phospholipids and |
Hemostasis is initiated when collagen at the site of wound formation is exposed, thereby activating the clotting cascade [12]. The process can be categorized into primary and secondary pathways. Primary hemostasis is concerned with platelet plug formation and secondary hemostasis refers to the deposition of insoluble fibrin, which strengthens the blood clot [13]. Both pathways are codependent and occur simultaneously.

In the event of an injury, platelets are activated through conformational transition and begin interacting with the vessel wall and adhesion proteins. They begin to exhibit adhesive properties by secreting sticky glycoproteins, and their aggregates form ‘platelet plugs’ that serve as a host environment for the assembly of coagulation factors [4]. Detailed descriptions of these specific interactions and receptor activations can be found in [14]. The platelets adhere to collagen, which is found on the sub-endothelial layer at the injury site [4].
Furthermore, secondary hemostasis consists of a series of coagulation serine protease cascades that result in the cleavage of soluble fibrinogen, a glycoprotein found in blood plasma, by thrombin to form fibrin [13]. In the event of vascular rupture, thrombin generated in plasma, is the main effector protease that utilizes G-protein coupled receptors to signal this process [15] and is also the terminal serine protease of the cascade [13]. When thrombin is cleaved, the fibrinogen forms a cross-linked knitted sheet of insoluble fibrin at the designated site [16]. Details on the specific cofactors involved in the cascade can be found in [17] and [13].

Subsequently, vessel vasodilation occurs in and around the wound site to increase local blood flow for enhanced leukocyte migration [12]. Platelets release platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-b) to attract neutrophils and macrophages [18]. Neutrophils are pathogen fighting immune cells that kill debris and bacteria at the injury site and are activated in a multistage process [19]. Macrophages similarly detect foreign bodies, and also have the supplementary function of promoting angiogenesis. In addition, they secrete PDGF and vascular endothelial growth factor (VEGF), both of which induce the regenerative stage of wound healing that is called the proliferative phase.

1.1.2 The Proliferative Phase

In this phase, a series of stages such as epithelialization, angiogenesis, granular tissue deposition and collagen formation take place. In epithelialization, keratinocytes are responsible for restoring the epidermal layer through a series of regulated mechanisms. As these cells move towards the site of injury, they begin to differentiate [20]. Keratinocytes turnover from epidermal stem cells and determine the regenerative capacities at the wound site. A host of growth factors, some of which enhance wound closure, aid in keratinocyte proliferation [21]. The specifics of the regulatory reactions, signaling and gene expression mechanisms can be found in [21], [22], [23].

Subsequently, angiogenesis, or the formation of new blood vessels from pre-existing ones begins to take place. Also known as neovascularization, this multistage phase involves the sprouting, splitting and remodeling of existing vessels, and takes place once endothelial cells migrate into the defect area. The six main stages of angiogenesis are described in Table 1.2, below:
Table 1.2: The Six Key Stages of Angiogenesis. Adapted from [24]

<table>
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<th>Key Stages</th>
<th>Details</th>
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<td>1. Endothelial cell activation</td>
<td>Occurs through growth factors such as VEGF and basic fibroblast growth factor (bFGF), both of which initiate cell migration and proliferation.</td>
</tr>
<tr>
<td>2. Degradation of capillary wall</td>
<td>Matrix Metalloproteinases (MMPs), MMP1 and MMP2, are expressed at this stage and work synchronously to degrade extracellular matrix (ECM) components.</td>
</tr>
<tr>
<td>3. Branch point formation</td>
<td>Integrin, a type of transmembrane receptor, is overexpressed on the ECM in order to bind ECM proteins onto neighboring cells.</td>
</tr>
<tr>
<td>4. Migration of endothelial cells to the ECM</td>
<td>Integrin in conjunction with specific MMPs and urokinase drive the migration process.</td>
</tr>
<tr>
<td>5. Re-organization to form tubules</td>
<td>Angiopoietin 1, or ang-1, is produced by stromal cells in order to aid in stabilizing the newly formed capillary tubes.</td>
</tr>
<tr>
<td>6. Anastomosis, or interconnection of new tubules</td>
<td>PDGF is produced by the new capillaries in the interest of interlinking and stabilizing the new vessels</td>
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Fig. 1.1, below, illustrates this process.
Granular tissue formation marks the end of angiogenesis. Granular tissue is connective tissue that is deposited by ECM and collagen-synthesizing cells called fibroblasts. It is formed in the presence of oxygen and nutrients. Granular tissue can often obscure the field of view during in vivo angiogenesis imaging. The implications of granular tissue formation on in vivo optical imaging are discussed in subsequent chapters. At this point, macrophages continue to supply growth factors for additional angiogenesis [12]. Growth factors also promote fibroblast differentiation and collagen synthesis at this time. Collagen is a structural scaffold that is deposited onto the wound site in order to make the fibrin clot stronger. Recent studies have demonstrated that in addition to acting as a structural framework, collagen can also control other functions such as cell migration, differentiation and a variety of protein synthesis mechanisms [25].
1.1.3 The Maturation Phase

In the final phase of wound healing, granular tissue develops into a scar. This is observed by the physical contraction of the wound site and the formation of scar tissue. At a cellular level, fibroblasts differentiate into myofibroblasts [26], which are responsible for contraction of the wound site. Collagen is also simultaneously secreted into the wound until a desired equilibrium is achieved, at which point the myofibroblasts undergo apoptosis. Depending on the severity of the wound, this phase can last from days until over a year in the human body.

Hence, the wound healing process is an amalgamation of complex and overlapping pathways, syntheses and cellular reactions. Fig. 1.2, summarizes the chronology of wound healing in humans.

![Figure 1.2: Overlapping phases of wound healing in humans. Adapted from [27]](image)

1.2 Introduction to Tissue Engineering and Regenerative Medicine (TERM)

Now that we have covered the basic biology of wound healing, we will discuss some of the basic principles underlying TERM. Wound healing is a process that occurs naturally in the human body. One of the goals of regenerative medicine is to optimize and stimulate the body’s tissue regeneration mechanisms via alternate means. Typically, structural frameworks called scaffolds (see 1.2.2) are infused with harvested stem cells. The harvesting can occur in vivo at the defect site or in
laboratory settings, in a bioreactor, if the subject’s body is incapable of healing itself. In the latter case, cells are transplanted into the defect site via surgical means.

A phenotypic difference between wound healing and tissue regeneration is that the former typically results in a scar whereas the latter results in tissue that is identical in structure and function to the originally damaged tissue [28]. Over the last decade, the medical field has increasingly started to view TERM as a safe and cost effective alternative to current practices such as organ transplant, in which surgeons must consider risks of organ rejection, excessive blood loss, infection and other undesirable side effects. However, the TERM field, is still relatively new and its outcomes, advantages and side effects are still not known fully. As a result, TERM currently faces many bioethical and regulatory hurdles. The next section provides an introduction to the use of stem cell technologies in tissue engineering.

1.2.1 What are stem cells?

All cells in the human body have the capacity to differentiate. Stem cells, in particular, are unspecialized at first and can differentiate into cells with specific form and function. They have the ability to continue differentiating for many generations. A stem cell that has the ability to differentiate into all types of cells is referred to as pluripotent, and a stem cell that can differentiate into more than one, but not all types is referred to as multipotent. Stem cells can either be categorized as embryonic stem cells, or ES, (i.e. those found in the embryo) or adult stem cells (i.e. those found after development in various types of tissues). ES are pluripotent while adult stem cells are multipotent.

Embryonic stem cells are derived from the epiblast compartment of the inner cell mass (ICM) of mammalian blastocytes, or early stage embryos that form approximately five days after fertilization [29]. Isolation of stem cells entails the eradication of the blastocyst.

The overarching goal of the current project is to use human adipose-derived stem cells (hASCs) in (craniomaxillofacial) CMF surgery. Before surgically implanting stem cells into our mice models, the hASCs are cultured under lab conditions. In some of our experiments, they were expanded \textit{in vitro} before implantation while in other cases they were directly implanted without expansion. hASCs, in particular, can be derived from human bone marrow or adipose tissue.
Compared to other types of stem cells, they are often easier to proliferate in lab settings and have great potential for therapeutic applications. The final product of isolation, which is rich in growth factors and stem cells, is referred to as the stromal vascular fraction (SVF). It contains fibroblasts, endothelial cells, macrophages and pericytes [30]. The SVF is separated from mature adipocytes by differential centrifugation. Subsequently, the pelleted SVF cells are then purified by washing and are expanded in culture in media; exact steps may differ from protocol to protocol depending on the stem cell target and application. Fig. 1.3, below, illustrates an isolation and expansion protocol that was used by Peister et. al [31] from a mouse model. The detailed protocol used in our studies is described in Chapter 3.

![Figure 1.3: The isolation and expansion of MSCs from 5 strains of inbred mice. Similar protocols have been used in various MSC studies. Adapted with permission from [30].](image)

1.2.2 Bone Regeneration using TERM

A popular application of TERM technologies is osteogenesis via harvested stem cells. Specifically, a growing number of tissue engineers today focus on the interface of stem cell harvesting and biomaterials. One such approach is to use ECM mimicking structural frameworks called scaffolds as a microenvironment for stem cells to undergo angiogenesis and eventually osteogenesis.

Scaffolds often possess structural and biochemical characteristics that are similar to the original bone of a defect site. A desirable scaffold must be biocompatible, biodegradable and act as a structural and mechanical framework for cells to attach, expand and migrate in. Typically, scaffolds are fabricated using manufacturing techniques such as gas foaming, fiber meshes, phase separation, melt molding, emulsion freeze drying amongst other techniques [32]. They could either be
naturally made from animal cadaver-derived [32] substances such as collagen, or synthetically from materials such as metals, ceramics, polymers or composites [33]. The type of biomaterial used depends on the biochemical properties of the stem cell assay, host environment and application of study.

Many defect sites, such as the CMF region, often require complexly shaped and largely sized scaffolds. Manufacturing such scaffolds by the traditional methods mentioned above can often be time consuming, expensive and arduous. In order to circumvent these issues, the idea of using 3D printing with computer aided design (CAD) technology is increasingly becoming popular in TERM studies [34], [35], [36], [37]. For our project, we utilized 3D-printed polycaprolactone (PCL) scaffolds seeded with hASCs in order to induce angiogenesis and eventually osteogenesis. Our motivations for using PCL as a biomaterial are as follows: they have FDA approval [38], are compatible with 3D-printing technology and have controllable degradation rates [33]. To facilitate growth, the hASCs are suspended in fibrin gel along with platelet-derived growth factor (PDGF). The specifics of our pipeline can be found in Chapter 3. Fig. 1.4 [39], below, describes a generic clinical protocol that uses good manufacturing practice (GMP) for a mandibular reconstruction application.
To summarize, this chapter first gives an overview of biological wound healing and then transitions to how it can be stimulated using TERM technology. We provide insights into the current TERM protocols and the outline of our project. In the subsequent chapters, we will use the principles introduced in this chapter as the motivation for using optical imaging methods for studying in vivo tissue engineering-induced angiogenesis.

1.3 References

15. Coughlin, Shaun R. "Thrombin signalling and protease-activated


31. Peister, Alexandra, et al. "Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of
CHAPTER 2: IMAGING TECHNIQUES

Traditional histology is no longer popular for assessing the functional status of tissue because it is invasive and unable to provide a range of functional information. Therefore today it is used more for the validation of newly developed in vivo imaging techniques. Till date, TERM studies that focus on imaging in vivo angiogenic activity in cranial scaffold models attempt to extract morphological and perfusion parameters without exposing the brain. Although attempts to quantify morphological and perfusion parameters have been made using MR [1, 2, 3], micro-CT [4, 5, 6], fluorescence [7, 8, 9] and ultrasound imaging [10, 11], the main limitation of these approaches is that characterization of the angiogenic vasculature has not been conducted an individual blood vessel level in vivo specifically.

The novelty in our imaging pipeline lies in observing wound healing and regenerative effects in vivo for every blood vessel in the scaffold area. Through a multimodal optical imaging platform, we are able to quantify different parameters describing angiogenic development using a minimally invasive and economical approach. This chapter provides the theoretical basis of the imaging techniques we used to achieve our overarching goal of understanding TERM-induced vessel growth in vivo for CMF applications.

IOS imaging provides us with morphological information about vasculature in the scaffold area. Using an image segmentation pipeline (see Chapter 3), we extracted parameters about the form and structure of vessels. Similarly, we acquired corresponding blood perfusion maps for these vessels using LSCI. Furthermore, using injections of dextran-FITC tracer and carbogen gas challenges, we were able to classify vessels and their maturity.

2.1 Intrinsic Optical Signaling (IOS)

When used in vivo, photons are sensitive to intrinsic changes in absorption, scattering and fluorescence [12]. The basic principles of IOS revolve around the differences in the absorption spectra of oxy- and deoxyhemoglobin (HbO₂ and Hb). All living tissues have different degrees of light absorption, which is expressed in molar coefficient, \( \mu_a \), units (cm\(^{-1} \)). The molar coefficient can be defined as the total sum of \( n \) chromophores in a live tissue and the concentration of the species, \( c_n \). The
sum is calculated by taking into account a wavelength-dependent specific absorption coefficient factor, \( \xi_n \) [12]. This relationship is expressed in Equation 2.1, below:

\[
\mu_a = \sum_n \xi_n \cdot c_n
\]

_Equation 2.1: Definition of molar coefficient, \( \mu_a \)_

Mammalian blood in particular can have two different absorption spectra, depending on whether it contains HbO_2 or Hb. Upon binding with oxygen, Hb changes to HbO_2. Therefore, a change in oxygenation state or concentration of hemoglobin within a particular vessel can correspond to a change in light absorption.

In the near-infrared (NIR) window, which ranges from 650nm to 1350nm [12], light has a maximum absorption potential in vivo [13]. Fig. 2.1, below, was generated from tabulated \( \mu_a \) values of both HbO_2 and Hb [14], and illustrates the difference in their spectra. The spectra are generated using the Beer-Lambert law, Equation 2.2, below. Here, \( I_0 \) is the incident light intensity, \( I \) is the transmitted light intensity, \( c \) is the concentration of hemoglobin (which is typically 150 g Hb/L [12]) and \( l \) is the optical path length which is assumed to be 1cm. The absorbance, \( A \), is a logarithmic ratio and therefore does not have units.

\[
A = \log_{10} \left( \frac{I_0}{I} \right) = \mu_a \cdot c \cdot l
\]

_Equation 2.2: Beer-Lambert Law_
As shown above, HbO₂ and Hb have different absorption spectra. If we expose the calvarial defect area to a light source with a known wavelength and record images with a charge-coupled device (CCD) camera [15], we can observe a hemodynamic response [12]. The total hemoglobin concentration (HbT), which is the sum of HbO₂ and Hb, is directly proportional to cerebral blood flow (CBF) if we assume total hemoglobin concentration to be constant [16]. When Hb absorption equals that of HbO₂, specifically at 500, 530, 570, 797 nm in the NIR window, we can easily isolate HbT measurements as they are independent of changes in blood oxygenation [12]. We can measure oxymetric changes if we record the same field of view with two or more wavelengths in the visible range. **Equations 2.3 and 2.4**, below, illustrate this:

\[ \delta \ln(I) = -\delta(\mu_a) \cdot l \]

*Equation 2.3: Differential of Beer-Lambert Law*

If we consider ‘n’ readings taken at wavelengths \(\lambda_1, \lambda_2, \ldots, \lambda_n\) and combine **Equation 2.1** with **Equation 2.3**, we get **Equation 2.4**:

\[ \delta \ln(I)_{\lambda_1} = -\delta(\xi_{HBO_2,\lambda_1} \cdot c_{HBO_2,\lambda_1} + \xi_{Hb,\lambda_1} \cdot c_{Hb,\lambda_1}) \cdot l_{\lambda_1} \]
\[ \delta \ln(I)_{\lambda_2} = -\delta(\xi_{HBO_2,\lambda_2} \cdot c_{HBO_2,\lambda_2} + \xi_{Hb,\lambda_1} \cdot c_{Hb,\lambda_1}) \cdot l_{\lambda_2} \]
\[ \delta \ln(I)_{\lambda_n} = -\delta \left( \xi_{\text{HbO}_2,\lambda_n} \cdot c_{\text{HbO}_2,\lambda_n} + \xi_{\text{Hb},\lambda_1} \cdot c_{\text{Hb},\lambda_1} \right) \cdot l_{\lambda_n} \]

*Equation 2.4: Differential of Beer-Lambert Law at multiple wavelength readings*

Note that a tissue does not absorb all the incident light. Because of scattering, a wavelength-dependent phenomenon, photons are reflected at various angles from a tissue. Hence, \( l_{\lambda_1} \) does not equal \( l_{\lambda_2} \). At points when Hb absorption equals that of HbO\(_2\), the ratios of transmitted and incident light remain constant, which implies \( l \) does not change with respect to time [12]. As a result, the light ratio is directly proportional to the change in total hemoglobin concentration, \( \Delta HbT \). The path length, \( l \) for an exposed brain is often estimated using Monte Carlo simulation-based regression analysis [17]. Specifics on this coefficient determination are beyond the scope of this thesis. For our study, we used literature values of these coefficients as described in Hillman et al [12].

*Fig 2.2*, below, is a schematic of our multi-modal imaging setup (see Chapter 3). The setup yields high spatial and temporal resolution images of our field of view. The white light source and filter wheel enable us to acquire IOS images. The other modalities, LSCI and fluorescence imaging are explained in Sections 2.2 and 2.3, respectively.
Figure 2.2: Schematic of the multi-modal contrast imaging system. A 16-bit CCD is connected to a white light and filter wheel that enables us to acquire IOS images. LS images can be acquired by switching on a 632nm red laser and fluorescence images through a UV curing gun. The CCD is connected to a PC that has a control program on MATLAB.

2.2 Laser Speckle Contrast Imaging (LSCI)

While IOS imaging gives us morphological information about the vasculature on the basis of oxygenation state, LSCI gives us a relative indication of perfusion of superficial vessels. The technique is based on the phenomenon of light scattering and diffraction. An interference pattern called ‘speckle’ is created when diffusible live tissue scatters light [18]. If the scatterer is moving, the speckle intensity fluctuates over time. Quantifying these fluctuations due to red blood cell motion within blood vessels can therefore give us an estimation of relative tissue perfusion. In order to understand the advantages, limitations and applications of this technique, we must first understand its working principles.

A linearly polarized, coherent and single frequency laser light source [19] is needed to create a speckle pattern. Equation 2.5, below, expresses a ratio, $K$, that is a measure of the “degree of blurring” [18]. Each pixel has an assigned $K$ value and a
sliding window average is computed to create a smooth image. The $K$ value is inversely proportional to scatterer flow velocity, $V$.

$$K = \frac{\sigma}{<I>} \propto \frac{1}{V}$$

Equation 2.5: The “degree of blurring”, per pixel, can be measured using a ratio, $K$, of the standard deviation and the mean pixel intensity in a pre-defined neighborhood. The $K$ value is inversely proportional to scatter flow velocity, $V$.

The speckle pattern formed through the CCD camera’s aperture is Airy disk shaped; single slit diffraction phenomenon explains this pattern. Theoretically, the variance of the time-averaged pixel intensity is equal to its temporal fluctuations. Equation 2.6, below, mathematically describes this relation [20].

$$\sigma^2 = \frac{1}{T} \int_0^T c_\tau(\tau) d\tau$$

Equation 2.6: The variance of the time-averaged pixel intensity is equal to its temporal fluctuations.

Here, $T$ is the camera exposure time, $c_\tau$ is the temporal average of the intensity autocorrelation function and $\tau$ is the decorrelation time [18]. $c_\tau$ is defined under the assumption that all photon shifting follows the Doppler effect and that its velocity follows a Lorentzian distribution [18]. Substituting Equation 2.6 into the definition of $K$, Equation 2.5, we acquire Equation 2.7, below [21]:

$$K(T, \tau) = \frac{\sigma}{<I>} = \beta^2 \left( \frac{e^{-2x} - 1 + 2x}{2x^2} \right)^{\frac{1}{2}}$$

Equation 2.7: $K$ redefined as a function of the instrumentation factor, camera exposure time and decorrelation time.

Here, $\beta$ is the instrumentation factor and $x = T/\tau$. For simplicity, we assume $\beta$ to be 1 in all our calculations. Equation 2.7 is valid for lower perfusion values, which is applicable to our study. Flow in larger vessels exhibits a combination of Gaussian and Lorentzian statistics [22]. For a given wavelength of light, $\lambda$, we can state Equation 2.8 [18] as an approximation:
\[ V = \frac{\lambda}{2\pi\tau} \triangleq V \propto \frac{1}{\tau} \]

*Equation 2.8: Scatter flow velocity is inversely proportional to correlation time.*

With an adjustable spatiotemporal resolution specific to the imaging application and a noninvasive approach, LSCI has become a cost-effective alternative for perfusion imaging. The modality has successfully been used in clinical [23] as well as for *in vivo* research studies: in retina [24, 25, 26], skin [27, 28, 29], brain [30, 31, 32], to name a few. A limitation of this technique, however, is its inability to provide an absolute quantification of perfusion. As a result, LSCI is often coupled with other imaging modalities.

### 2.3 Fluorescence Imaging

By tagging cells with fluorescent proteins, we can track SVF distribution *in vivo* and by injecting a tracer, we can determine vascular permeability, and trace kinetic parameters (e.g. mean transit time). We used RFP as a biological marker in our studies. Furthermore, we used 150kD dextran-FITC as a tracer through tail vein injections for vessel identification. In TERM, this marker has been coupled with imaging modalities such as confocal laser scanning microscopy or multiphoton microscopy to monitor tissue development both *in vitro* and *in vivo* [33].

### 2.4 Carbogen Gas Challenge

Composed of 95% oxygen and 5% carbon dioxide, carbogen gas has effectively been used in clinical and research settings as a radiological tool to induce hyperoxia for increasing radiosensitivity [34]. Carbogen inhalation therapy is widely used today in medical literature for a range of conditions such as sudden hearing loss, strokes, seizures, bladder cancer and optic nerve damage [35]. Carbogen gas has also been used effectively as a form of wound healing therapy, as wound sites are often hypoxic. Hypoxia decelerates healing as it reduces the rate of angiogenesis, collagen production and fibroblast proliferation [36].
At a vascular level, O\textsubscript{2} inhalation can significantly increase cerebral blood flow and CO\textsubscript{2} inhalation leads to vasodilation [37]. An increased O\textsubscript{2} delivery causes changes to tissue perfusion, which therefore alters vascular resistance and perfusion pressure [38]. The induced hyperoxic state typically causes hyperventilation. This can be explained using the Haldane effect, a property of hemoglobin [39]. Hemoglobin has a quaternary structure in which each of the four subunit’s affinity to O\textsubscript{2} changes depending on subunit occupancy. Oxygenated hemoglobin has a decreased affinity to CO\textsubscript{2}. As a result, the partial pressure of CO\textsubscript{2} in cerebral tissue increases. This in turn stimulates respiratory receptors that increase ventilation [38].

In our study, we used carbogen to assess hemodynamic response \textit{in vivo}. We imaged the mice using LSCI in order to observe perfusion changes in response to carbogen. Further details on our protocol can be found in \textit{Chapter 3}.

To conclude, this chapter provides us with an overview of IOS, LSCI, fluorescence imaging and the carbogen gas challenge. In the following chapters, we will utilize our wound healing (\textit{Chapter 1}) knowledge to establish a multimodal experimental and image-processing pipeline that will provide us characteristic information on regenerated vasculature.

2.5 References


CHAPTER 3: EXPERIMENTAL PROTOCOL AND IMAGING PIPELINE

Now that we have established a foundation of basic tissue engineering and optical imaging principles (Chapters 1 and 2), this current chapter aims to unify these fields. First, we provide a description of the biological methods used for scaffold production, stem cell isolation and culture and surgical protocols. Next, we transition into describing our multimodal imaging platform and image processing pipeline. The next two chapters discuss the results we acquired using this set up.

3.1 3D-Printing Technology For Scaffold Fabrication

Scaffolds are analogous to the tissue ECM in that they serve as a structural framework and provide a host environment for stem cell adhesion, differentiation and proliferation in vivo. As a result, its design criteria emphasize biocompatibility, biodegradability and mechanical strength of a biomaterial. For our project, we included an additional design goal of customization and reproducibility. This is because CMF wound site geometry is often complex, difficult to sculpt manually and can vary from patient to patient. As a result, we envisioned using CAD software and 3D-printing technology for optimizing and reproducing intricate scaffolds at economies of scale.

Prior to this project’s inception, the Grayson Lab has extensively studied scaffold design, fabrication and optimization using fused deposition modeling and 3D-printing technology [1], [2], [3]. Because PCL has a degradation rate that is approximately the same as that of in vivo osteogenesis [4] and is also compatible with 3D-printing technology, we used this as a biomaterial for all our experiments. However, a limitation of using PCL is that it is not conducive to cell adhesion because it is hydrophobic [5]. This hurdle is overcome by using a fibrin matrix, which is a viscous protein network that contains blood clotting promoters fibrinogen and thrombin, as a cell carrier.

For our experiments, we developed CAD models of 4×4mm PCL scaffolds. These were 3D-printed using a hot-melt pressure extruder. The specifics of the fabrication process are described in Temple et. al [4].
3.2 Stem Cell Isolation and Calvarial Defect Preparation

All the protocols for obtaining hASCs from Caucasian female donors and rodent surgery were approved by the Johns Hopkins Institutional Review Board (IRB) and the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC). The stem cell isolation process we used was similar to that described in Fig. 1.3 and further details can be found in Dubois et. al [6]. The critical steps include digesting tissue with 1mg/mL type I collagenase for 1h at 37°C. The sample was then centrifuged to obtain the SVF pellet, which was subsequently passaged in growth medium (Dulbecco’s Modified Eagle Medium, 10% Fetal Bovine Serum, 1% penicillin and 1 ng/mL PDGF). Subsequently, the stem cells were suspended in fibrin gel that was polymerized for 30 min at 37°C and seeded uniformly onto a sterilized 4×4 mm 3D-printed scaffold with 40% filling density. The specifics of our protocol can be found in Temple et. al [4].

Depending on the experiment performed, our mice were implanted with scaffolds containing one of the three materials: fibrin gel only, fibrin gel with hASC or/and HUVEC aggregates or fibrin gel with hASC aggregates after 18 days of in vitro vascular induction in supplementary HUVEC growth medium. The scaffold seeding density was maintained constant at 20×10⁶ cells mL⁻¹ and 1.2×10⁵ cells/scaffold for all matrices. The two time-points chosen for analyzing vascular growth in this thesis were 7 and 14 days post-scaffold implantation.

At each of these time-points, mice were first anesthetized by an intraperitoneal (i.p.) injection of a xylazine/ketamine cocktail. Next, the mice were checked for signs of deep sedation and their eyes daubed with eye ointment. A 0.1 mL dose of 0.6 mg mL⁻¹ buprenorphine analgesic was administered once a day for three days after implantation surgery. A 12mm incision was made around the calvarial area and surface granular tissue was removed with a scalpel. Vetbond® was used to glue the edge of the skin to the skull. The area was dried and UVoptibond was used to smooth the skull surface. Next, the skull was breached without damaging the dura using a 4 mm dental drill. The field of view was secured with a well of dental cement. The glass coverslip was then superglued onto the well. The mice were administered 2% isoflurane during this entire procedure. Fig. 3.1, below, is a picture of the scaffold implanted into the calvarial defect.
3.3 Multi-contrast Optical Imaging Acquisition

IOS, LS and fluorescence images were acquired on the benchtop system using 560, 570, 600, 610nm light from a filter wheel, a 632nm red laser and a 400 – 480nm UV curing gun respectively. The mouse is fixed on a platform and images are acquired from a 16-bit CCD camera at a magnification of 1.2x (i.e.1 pixel=5.52 µm), at an average frame rate of 10 frames per second. Image acquisition was synced with a PC and controlled by a MATLAB program.

Following the carbogen gas challenge, LS images were recorded over a 15 minute interval: 3 minutes of room air, 1 minute of carbogen gas, 5.5 minutes of room air, 1 minute of carbogen gas and 5 minutes of room air.

Fig. 3.2a and 3.2b, below, show a sample IOS and LS image for the same field of view acquired using the benchtop system:
Figure 3.2: IOS (a) at 560nm and LS (b) at 632nm images for the same field of view acquired by the multi-contrast optical imaging system. The white circles indicate the region around the scaffold region, which is 4x4mm wide. Our image processing pipeline was normalized to this region for our mice models.

3.4 Blood Vessel Segmentation Pipeline

Data acquired from IOS, LS and fluorescence imaging were co-registered and analyzed in order to quantify blood vessel growth and determine whether our bioactive scaffolds promoted bone growth. We used segmented IOS images to obtain morphological parameters such as fractional area of blood vessels occupied by the scaffold field of view and total branch length. Furthermore, in order to quantify the degree of blood vessel branching, we performed Sholl analysis on our images. Similarly, we used segmented LS images to quantify perfusion. From our carbogen gas challenge LS images, we were able to analyze blood vessel responsiveness and our fluorescence images (RFP imaging and dextran-FITC injection images) helped in tracking SVF distribution.

Our goal was to develop a semi-automated approach for isolating vasculature from the scaffold, granular tissue and image noise. As a primary quantifier of vessel growth, IOS and LS images were extracted, segmented and binarized in order to obtain morphological and blood perfusion information. The images were segmented using ImageJ (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). The automated segmentation pipeline was inspired by that used by Cebulla et. al [7] for segmenting micro-MRI breast cancer angiogenesis data.

Fig. 3.3, below, illustrates a flowchart of the steps used in segmenting the IOS and LS images.
The images were first preprocessed individually for optimum grayscale contrast. Operations varied depending on an individual image’s signal to noise ratio (SNR). Typically, median filters ranging from 4×4 to 10×10 pixels in diameter and a rolling ball background subtraction ranging from 100 to 300 pixels in radius were employed.

Next, the images were processed using an ImageJ plugin called a ‘tubeness’ filter. Designed specifically to distinguish curvilinear structures such as vessels, bronchi or neurons, the tubeness filter emphasizes line enhancement using 3D Hessian matrices [8]. The matrices describe the local intensity variation per pixel, which has the visual effect of selecting various blood vessel radii. The plugin employs Gaussian convolution with a corresponding standard deviation, σ, which accounts differences in vessel radii. For an automated approach, we chose σ values of 1, 1.5...
and 2.5, as these were deemed appropriate by visual inspection. Vessels larger than 70 µm were isolated by manual thresholding as the tubeness filter was not sensitive enough to detect them. Subsequently, the tubeness and thresholding outputs were combined using a maximum operation and the images were binarized. Finally, salt and pepper noise was removed from the blood vessel masks (i.e. binarized images) by using binary operations such as image eroding, dilating, opening and closing.

3.5 Carbogen Response Maps

LS images acquired during the delivery of carbogen gas were pre-processed in the spatial and temporal domains. The images were smoothed using a 10×10 pixel median filter and a 3×3 pixel temporal Gaussian filter in order to improve SNR. The percent change in blood flow, was calculated using ImageJ.

3.6 Nonlinear Regression Analysis for Fluorescence Imaging Time Series

Data acquired from dextran-FITC fluorescence imaging were first co-registered, cropped and smoothed using a 10×10 pixel median filter and a 3×3 pixel temporal Gaussian filter in order to improve SNR. The noise and signal time series were then fitted with linear and gamma-variate functions using the 3dNLfit program in Analysis of Functional Neuro-Images (AFNI) platform (Cox, National Institutes of Health, Bethesda, Maryland, USA, https://afni.nimh.nih.gov/). The fitting was performed using a least squares estimation approach on the time series data based on user specifications.

The gamma-variate function, which was used to characterize the signal, can be defined as in Equation 3.1, below [9]:

\[
C = k(t - t_0)^r \exp\left(-\frac{t - t_0}{b}\right)
\]

*Equation 3.1: Probability density function of the gamma distribution. This equation was used to fit out dextran-FITC fluorescence data.*

Here, \(C\) is the indicator concentration of the dextran-FITC tail-vein injection, \(t\) is the time after the injection, \(k\) is the amplitude parameter, \(t_0\) is the appearance time, \(r\)
is the rise rate factor and \( b \) is the fall rate exponential factor. Similarly, the noise was characterized using a linear model. Subsequently, differentiation and integration steps were used to derive \( t_0 \), \( t_{\text{max}} \) and parameter ‘\( r \’) maps. Here, \( t_0 \) is defined as the onset time or time delay of response, \( t_{\text{max}} \) is the time at which the signal has the greatest magnitude and parameter ‘\( r \’) is the rise rate exponential term. \( t_0 \) and \( t_{\text{max}} \) have time units (seconds) and \( r \) is an arbitrary unit. **Fig. 3.4**, below, illustrates these parameters on a gamma probability distribution function. A sample of the fitting can be found in **Fig. 4.5**, in Chapter 4.

![Figure 3.4: Probability density function of a gamma distribution with parameters stated in Equation 3.1.](image)

To summarize, this chapter describes the 3D-printing, stem cell expansion and seeding protocols, and surgical protocols. In the latter half, we explain the image processing tools and pipelines we use to quantify *in vivo* blood vessel growth. Our next two chapters highlight the results found in two types of experiments: terminal and non-terminal. In the terminal experiments, we imaged mice with calvarial defects at 7 days and 14 days post-scaffold implantation and analyzed *in vivo* angiogenesis around the scaffold area. In the non-terminal, or chronic window preparation, experiments, we explored design and preliminary results of an ongoing imaging window preparation.
3.7 References

CHAPTER 4: CHARACTERIZING REVASCULARIZATION OF A CALVARIAL DEFECT IMPLANT WITH IN VIVO OPTICAL IMAGING

Our objective was to use our multi-contrast optical imaging system to visualize and quantify in vivo blood vessel growth in a calvarial defect model implanted with a fibrin and stem cell matrix. Using the methods and image processing pipeline outlined in the previous chapter, we imaged SCID mice at one and two weeks after scaffold implantation using three experimental cohorts. In the first cohort, we imaged ten mice at two weeks post-implantation. Five mice were implanted with scaffolds containing fibrin and hASC aggregates, while the other five, categorized as the control group, were implanted with scaffolds containing only a fibrin aggregate. We performed a second set of experiments on four mice at a one week time-point in which two implants contained fibrin, HUVECs and hASCs while the other two implants contained only fibrin. Typically, HUVECs are a common choice for co-implantation with stem cells for tubulogenesis assays [1], [2], [3], because they result in a higher tube formation rate than stem cells alone. Since we wanted to observe blood vessel growth within the first week after implantation, we used a 1:1 ratio of HUVECs and hASCs. For our third set of experiments, we wanted to assess if pre-vascularizing the scaffold matrices in vitro in a bioreactor for 18 days, would promote a higher rate of angiogenesis than in vivo vascularization alone. For this experiment, we used two mice with pre-vascularized scaffold matrices containing a fibrin, HUVEC and hASC aggregate while the other two mice were implanted with the same matrix but without pre-vascularization. Table 4.1, below, summarizes the specifics of our experiments.

Table 4.1: Description of the Three Cohorts

<table>
<thead>
<tr>
<th>Cohort #</th>
<th>Experiment Description</th>
<th>No. of Mice</th>
<th>No. of Imaging Time-Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Terminal experiment in which five mice were implanted with scaffolds containing a fibrin and hASC aggregate and the other five, labeled as controls, implanted with scaffolds containing only a fibrin aggregate. The modalities used were IOS</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Terminal experiment in which two mice were implanted with scaffolds containing a fibrin and hASC:HUVEC aggregate and the other two, labeled as controls, were implanted with scaffolds containing only a fibrin aggregate. The modalities used were IOS imaging, LSCI, carbogen gas challenge and fluorescence imaging.</td>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>1b</td>
<td>4 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Terminal experiment in which two mice were implanted with a scaffold containing a pre-vascularized aggregate and the other two mice were implanted with a scaffold containing a non-prevascularized hASC:HUVEC aggregate. The pre-vascularized set contained a hASC:HUVEC and fibrin gel matrix that was cultured in a bioreactor for 18 days. The modalities used were IOS imaging and LSCI.</td>
<td>4 1</td>
<td></td>
</tr>
</tbody>
</table>

4.1 Results

4.1.1 Results from Cohorts 1a and 1b

Fig. 4.1, below, depicts a morphological comparison of the degree of vascularization at one and two weeks after implantation. Fig. 4.1a shows a representative field of view at one week after implantation, and Fig. 4.1b corresponds to a two week time-point. Fig. 4.1c and d show a comparison of the total branch length in the scaffold area and the fraction of the scaffold area occupied by blood vessels in Cohorts 1a and 1b, respectively. The parameters were calculated using the image processing pipeline explained in Chapter 3.
Figure 4.1: IOS images that illustrate a morphological comparison between vascularization one (a) and two (b) weeks after scaffold matrix implantation. We quantified blood vessel growth by comparing total branch length in the scaffold area (c) and the fraction of the field of view occupied by vessels (d).

In order to analyze the degree of vessel branching, we also conducted a Sholl analysis on our images. Typically used to characterize neuronal dendritic morphology, Sholl profiles are the number of dendrite intersections on concentric radii from a defined center. Because angiogenesis has similar branching profiles to those of neuronal structures, Sholl analysis can be used to parameterize blood vessel ‘arborization’. We used a linear approach to conduct our analysis. We use Equation 4.1, below, to generate our plots on ImageJ.

\[ N = a + br + cr^2 + dr^3 + \ldots \]

Equation 5.1

Here, \( N \) is the number of vessel intersections with a concentric circle or radius \( r \), and \( a, b, c \) and \( d \) are constants belonging to the best-fitted polynomial equation. The ImageJ plugin outputs an optimized polynomial equation based on \( R^2 \) results. In our field of view, we normalize our calculations to 4 mm and the centerline of the middle
scaffold. **Fig. 4.2**, below, shows a comparison of Sholl analysis performed on samples from one (4.2a) and two (4.2b) week time points. As shown in the graphs, the number of average intersections, $N_{av}$, is approximately 2.7 fold greater, on average, at two weeks compared to one week after surgery.

![Graph](image)

**Figure 4.2**: A comparison of the degree of branching at one (a) and two (b) weeks after scaffold implantation. The Sholl analysis, conducted on ImageJ, was done using linear fits of 8th and 7th degree polynomials respectively. The results indicated approximately 2.7 times more branching ($N_{av}$) at two weeks compared to one week time points.

Next, we compared blood perfusion measured using LSCI between the two time points, **Fig. 4.3a** and 4.3b. We noticed a strong wound healing response at the one-week time point. In order to exclude granular tissue from our analysis, we cropped **Fig. 4.3a**. Although we could not eliminate noise arising from the scaffold grid as it directly covers the field of view, we can observe some blood flow around the scaffold. The perfusion signal in **Fig. 4.3a** was not resolved to individual blood vessels, indicative of either de novo angiogenic sprouting or lack of perfusion. In
contrast, we observed elevated perfusion two weeks after scaffold implantation in Fig. 4.3b.

**Figure 4.3:** Relative blood perfusion comparison using LSCI between one (a) and two (b) weeks after the scaffold matrix implantation. We observe greater and resolved blood flow, and less noise signal due to granular tissue formation at two weeks after surgery.

Furthermore, we observed changes in blood flow in response to a carbogen gas challenge. Figs. 4.4a and d below show the relative LS maps for weeks one and two respectively. We observed a high degree of granular tissue formation and therefore low SNR at week one. As shown in Fig. 4.4c, we did not observe a hemodynamic response during carbogen inhalation at week one. In contrast, as shown in Fig. 4.4b and e, we observed a large blood flow response to carbogen inhalation indicating a larger proportion of mature vessels in the FOV.

**Figure 4.4:** Comparison of percent change in blood flow maps for one (a) and two (d) week time-points. We observe more granular tissue and therefore a lower SNR at one
As another indicator of vessel development, we tracked the perfusion of dextran-FITC within the scaffold area via fluorescence imaging. Our dextran-FITC concentration vs. time data was fit with a gamma variate probability density function on a pixel-wise basis using AFNI. Fig. 4.5, below, shows a sample fitting for a pixel in a blood vessel. From these fits we generated parametric maps of the onset time, $t_0$, the time to peak, $t_{\text{max}}$, and the wash-in rate exponential term, $r$. Fig. 4.6a-c show representative parametric maps for a two-week time point sample. We do not have corresponding data for a one week time point because there were very few perfused blood vessels in the scaffold area and hence, negligible dextran-FITC signal.

![Figure 4.5: Sample gamma distribution function fitting](image)

Furthermore, in order to classify vessel segments into three categories of maturity, we combined data from our carbogen gas challenge and tracer images. First, we categorized pixels as either exhibiting a greater than or less than 10% change in blood flow in response to carbogen. This map is shown in Fig. 4.6d. Next, using a squared Euclidean distance k-means clustering approach on MATLAB, we incorporated our maximum signal maps in order to classify each vessel segment. We
defined three classes of decreasing vessel maturity: 1, 2 and 3. A cropped ROI illustrating this is shown in Fig. 4.6e.

Figure 4.6: time delay of response (a), maximum signal (b) and rise rate exponential term (c) maps for a two-week time point sample. Image (d) shows pixels categorized as either showing less than or greater than a 10% change in blood flow in response to carbogen gas. The map was multiplied by (b) and three vessel maturity classes, 1, 2 and 3, were created based on this result. Image (e) illustrated this classification for a cropped ROI.

4.1.2 Results from Cohort 2

Furthermore, Cohort 2 was imaged to investigate the differences in in vivo angiogenic response between pre-vascularized and non-prevascularized scaffold groups. As mentioned in Table 4.1, one group (n=2) of fibrin gel, HUVEC and hASC aggregate scaffolds were vascularized for 18 days in vitro using a supplementary HUVEC growth medium. The other group (n=2) did not have pre-vascularized scaffolds and was identical to that used in Cohorts 1a and 1b. The mice were imaged at one-week post-implantation. Fig. 4.7a-d, below, shows sample IOS and LSCI images from both groups.
Figure 4.7: IOS and LS images for both pre-vascularized (a and b) and non-prevascularized (c and d) groups

Fig. 4.8, below, illustrates the total branch length and fractional area differences between the two groups.

Figure 4.8: Difference in total branch length (a) and fractional area of field of view occupied by blood vessels (b) between pre-vascularized and non-prevascularized scaffolds at a one-week time point.
4.2 Discussion

To summarize, we had two aims for our experiments. First, we investigated differences *in vivo* angiogenesis between two weeks and one week of post-scaffold implantation surgery. Second, we assessed the effectiveness of pre-vascularizing scaffold matrices 18 days before implantation.

We extracted blood vessel mask binary images from our IOS and LS images in order to compare total branch length and fractional area covered by blood vessels in the field of view for both time points. We assessed morphological ‘arborization’ by performing a linear Sholl analysis on our data. We also calculated percent change in blood flow induced by a carbogen gas challenge. Finally, we conducted nonlinear regression analysis for our fluorescence imaging concentration vs. time series data for both cohorts as a means of categorizing vessel development. We investigated the same parameters for pre-vascularized and non-prevascularized scaffold matrices.

Although our sample size for cohorts 2 and 3 was small as these were preliminary studies, we did notice, through our multi-contrast images, significantly more vascularization at a two week time-point. We also noticed more angiogenesis with pre-vascularized scaffolds.

A limitation of our studies is that because they were terminal experiments, it was not possible to determine how many blood vessels were pre-existing to the scaffold implant and how many sprouted as a result of the scaffold and stem cell aggregates. Furthermore, results may differ based on a mouse’s age and body mass index. To resolve these issues, we proposed conducting a longitudinal study in which individual mice can be imaged every day through a chronic cranial window preparation. A longitudinal study would allow us to analyze trends in *in vivo* angiogenesis. The next chapter explores preliminary design of a novel optical imaging window and describes initial results for a trial longitudinal study.

4.3 References

CHAPTER 5: DEVELOPING A PROTOCOL FOR LONGITUDINAL IN VIVO IMAGING OF THE CALVARIAL DEFECT MODEL

The terminal experiments described in Chapter 4 provided us with insight on assessing in vivo vessel growth using our multi-contrast imaging system. Although we were successfully able to quantify in vivo angiogenesis with our imaging pipeline, a drawback of the experimental approach was that measurements were limited to a single post-implantation time-point. As a result, our next goal was to develop an in vivo imaging protocol that enabled the assessment of longitudinal changes (i.e. serially over days) in the tissue microenvironment following scaffold implantation.

Having a single time-point per animal limits precludes an understanding of the continuous dynamic remodeling of the tissue microenvironment. For example, the amount of pre-existing vessels, the degree of vessel growth induced by our assay and the rate of angiogenesis around the scaffold all change over time. Furthermore, since the calvarial defect is surgically exposed only once before the mouse is euthanized, it is challenging to distinguish pre-existing vessels from those that have been induced by the presence of hASCs.

This chapter describes a new approach for longitudinal imaging and demonstrates the utility of our model using the imaging pipeline described in Chapter 3. We conclude the chapter with a discussion of methods for improving our initial protocol.

5.1 Methods

For chronic imaging, we envision making a window for chronic optical access with a glass coverslip that gives us a direct view of the cranial area. The window (schematic shown in Fig. 5.1, below) is made from a well of UV dental cement that is leveled with a glass coverslip and a 3D printed disk. The layers are sealed together using Vetbond® Tissue Adhesive. The enclosed well is filled with sterile saline solution, so as to provide a moist window microenvironment.
Figure 5.1: Exterior shell of the chronic cranial window. The window has a diameter of 8mm and a thickness of 0.2mm. The layers of the window include UV dental cement, a glass cover slip and a 3D-printed disk, and are sealed together using Vetbond Tissue Adhesive. The well is enclosed with saline solution and permanently sealed for sterilization purposes.

For these experiments, we used a 1:1 ratio of HUVECs (i.e. human umbilical vein endothelial cells) and hASCs, with scaffold seeding densities of $2 \times 10^7$ cells mL$^{-1}$ and $1.2 \times 10^5$ cells/scaffold. HUVECs are derived from the endothelium lining of umbilical cord veins. Due to their low cost and relatively ease of isolation, they are typically used in many angiogenesis assays. We included HUVECs in this cohort because they promote blood vessel growth at a faster rate than just using hASCs. This would therefore accelerate angiogenesis at earlier time points. The HUVECs were also fluorescently tagged with RFP. Aside from this protocol modification, the isolation, culturing, seeding and the surgical procedures described in Chapter 4 remained identical for this series of experiments. We imaged the mice four days after scaffold implantation to give them time to recover from the surgical procedure. The mice were imaged every other day from Day 4 onwards.

5.2 Results

We performed multi-contrast imaging on a cohort of 5 mice for a period of two weeks. As mentioned above, our imaging pipeline was the same as that used in Chapter 4. As a primary indicator of angiogenesis, we first acquired IOS and LS images. Since we noticed granular tissue formation in these images (explained below), we also conducted a carbogen gas challenge and fluorescence imaging to investigate if there was vessel growth and stem cell survival.
Fig. 5.2, below, depicts a 16-day time course of the IOS and LS images for the same mouse. As shown in the series of images, we observed greater granular tissue formation than that in the terminal experiments (see Chapter 4). The LS images indicate minimal blood flow around the scaffold area. The blood vessels were not optically resolvable, perhaps due to the excessive granulation tissue within the FoV. As a result, our images had a very low SNR in comparison to our terminal cohorts. To confirm that there were stem cells present, we imaged the same mouse with RFP imaging. Fig. 5.3, below, shows positive RFP signal for the same mouse, indicating that although there were HUVECs present around the scaffold, they may not have differentiated into blood vessels under the newly formed granular tissue. Similarly, Figs. 5.4a and 5.4b, indicate a positive dextran-FITC response. Individual blood vessels, could not be resolved because of the presence of excess granular tissue.
Figure 5.3: RFP image (taken at Day 8 after surgery), corresponding to the series in Figure 5.2. We observed a positive RFP signal in this field of view, indicating hASC survival within the window.

Figure 5.4: Image (a) is a fluorescence image of the field of view. As shown, there is excess granular tissue in the region. There is, however, weak dextran-FITC signal (b).

5.3 Discussion

For our longitudinal study, we aimed to design a chronic cranial window and utilize our image processing pipeline on a small cohort of SCID mice. Our ultimate goal was to observe blood vessel growth and eventually osteogenesis around the scaffold area. The primary motive for translating our initial experimental protocol to a
longitudinal study was to be able to identify the dynamics of angiogenesis, osteogenesis and engraftment.

Our initial results indicated a strong inflammatory response within the imaging window, which led to excess granular tissue deposition in all the animals. As a result, the FOVs were obscured and individual blood vessels that may have grown under the granular tissue were not resolvable by any of the imaging methods. However, the RFP images displayed weak positive signal, indicating HUVEC and hASC survival. The weak dextran-FITC fluorescence indicated the possibility of some perfused blood vessels under the granular tissue.

Furthermore, we observed that the cranial region was continuously reabsorbing the saline solution under the cover slip. Hence, drying out of the implant site and preparation could be another reason why no blood vessel growth was resolvable optically. Since we had to reopen the cranial window to replenish the saline solution every 36 to 48 hours, it was possible that window disruption damaged the fragile angiogenic sprouts around the scaffold.

In order to circumvent these limitations, we designed an alternate cranial well using a silicone-based polydimethylsiloxane (PDMS) coverslip with a built-in port for replenishing saline. The design was inspired by a recent chronic imaging window reported by Heo et. al [1]. The advantage of using a PDMS coverslip was that it allows for injecting saline solution via a port, without exposing or disturbing the cranial window. Fig. 5.5, below, depicts a schematic of our proposed design. We aim to use this for our next cohort of animals.

![Figure 5.5: Self-healing PDMS cover slip port design. This design will keep the cranial region sterile from atmospheric impurities and will also resolve evaporation issues.](image)

To summarize, although our longitudinal cranial window design fostered stem cell survival, it did not lead to establishment of blood vessels. This was probably due
to inflammation, other wound healing responses and drying out of the cranial window. To circumvent these limitations we redesigned our optical window that will be used for future chronic in vivo imaging experiments.

5.4 References

Chapter 6: Conclusions and Future Directions

Our overarching goal is to recreate the gold standard of CMF surgery by using a paradigm-shifting TERM approach. Specifically, we aim to do this by the means of 3D-printed PCL scaffolds embedded with cells from human SVF suspended in fibrin gel. Many TERM approaches in pre-clinical literature today explore oxygenation, angiogenesis and osteogenesis in the tissue microenvironment using only in vitro protocols and imaging modalities. In order to investigate the same in vivo, we developed an economically efficient optical imaging pipeline on a calvarial defect model. From this, we extract quantitative morphological, flow and tracer kinetic parameters to quantify the degree of angiogenesis and maturity class of blood vessels.

We utilized our optical imaging pipeline in a pilot terminal study on a series of SCID mice that had one of the following: fibrin gel only, fibrin gel with hASC or/and HUVEC aggregates or fibrin gel with hASC aggregates after 18 days of in vitro vascular induction in supplementary HUVEC growth medium. A limitation in our study was that because it was cross-sectional, we cannot distinguish between pre-existing and fibrin aggregate induced blood vessels in the scaffold region. Additionally, a mouse’s age and body mass index can be a major factor in our results.

Consequently, we proposed building a longitudinal in vivo imaging protocol for the same pipeline. We aim to serially image over days using an optically accessible chronic cranial so that angiogenesis growth rates and vessels can be compared and classified for a same mouse. In the preliminary study, we noticed excessive granular tissue formation and inflammation in response to the mice’s wound healing response and window design flaws. For our next cohort, which is beyond the scope of this thesis timeline, we proposed a new surgical window design that uses self-healing PDMS coverslips that aims to resolve these issues. Once successful, a longitudinal protocol performed over a larger sample of mice can help us make claims on and validate our data trends.
Namrata Batra graduated with a BS in Chemical and Biomolecular Engineering from Johns Hopkins University in May 2016. Having a keen interest in exploring the interface between regenerative medicine and image processing, she continued on at Johns Hopkins to pursue a MSE within the same department. She was attached to the labs of Dr. Arvind Pathak in Radiology and Radiological Sciences and Dr. Warren Grayson in Biomedical Engineering for her thesis. She is set to defend her work in May 2017 and will join a Boston-based medical devices startup that specializes in radiotherapy and wound healing technologies post-graduation. In addition to her academic activities, Namrata is a passionate musician. Having pursued a minor in Music during her undergraduate career at Johns Hopkins, Namrata has been involved with singing and band groups on campus and has performed at piano recitals at the Peabody Conservatory in Baltimore, Maryland.