Quantification of Microvessel Fragments from Primary Isolation using 3-D Confocal Microscopy

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Faculty Members

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Introduction

The field of regenerative medicine is one that has been progressing for many years now. However, while it shows great promise, the full potential has not yet been realized due to its complexity. Skeletal development and fracture repair includes the coordination of multiple events such as migration, differentiation, and activation of multiple cell types and tissues. The development of a microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die. Seeding constructs with micro-scale blood vessel fragments has shown success in promoting the formation of an interconnected vascular network that can integrate with host vasculature when implanted.

These vascular fragments can be harvested from adipose tissue and isolated with chemical digestion and selective filtration. Additionally, further digestion of adipose tissue yields a single-cell level heterogeneous cell population referred to as stromal vascular fraction (SVF) that also has revascularization potential. The different cell populations in the SVF have been identified to be mesenchymal stem cells (MSCs), endothelial progenitor cells, T and B cells, and many others. Characterizations of the cell subpopulations constituting the SVF often revolves around the examination of CD 34 cells, which represent 50-80% of the group. While data suggests that the majority of SVF adherent CD 34 cells may represent resident pericytes who play a role in vascular stabilization by mutual structural and functional interactions with endothelial cells, the importance of completing standardization of protocols becomes vastly greater as use of adult stem/stromal cells increases, both in research and clinical studies.

Overall, this project seeks to characterize the distribution of various cell types within the stromal vascular fraction (SVF), to understand their relative importance in the revascularization process in vitro, and to assess their structural fates within the vascular networks they form. A series of immunohistochemistry staining and confocal microscopy will be done to identify relevant cell subpopulations in the SVF, determine the relative importance of each cell type in neovascularization, and correlate cell types observed in the SVF to their structural roles, respectively. Comparisons of SVF and MVF derived vasculature will be done as well. The results of this project will identify the SVF compositions with the greatest therapeutic revascularization potential. Further predictive models for bone growth based on the quantitative analysis of both the MVF and SVF can be composed once enough data is collected.
**Literature Review**

The human skeleton has the ability to regenerate itself after injury. This restorative ability allows bones to heal at shapes, sizes, and strengths that were characteristic of the bone before injury. However, conditions for ideal bone regeneration are not always met. While immobilization has for a long time been deemed important in fracture healing, infection, poor vascularization, bone or soft tissue loss, and critically sized defects can all act as deterrents for bone healing\(^4\). Bone is a feedback controlled composite organ that fulfills several interconnected functions including locomotion, involvement in phosphate and calcium metabolism, synthesis of endocrine molecules and hematopoiesis, with bone vascularization being at the interface of these functions\(^6\). As such, the development of a new vascular network is a critical step in wound healing and represents a primary limiting factor in functional tissue regeneration\(^7\).

Quantification of vascularization lends itself to many parameters including percentage vascular volume per tissue volume, mean vessel thickness (µm), or separation and vessel number per mm. Expectedly, numerical results depend on the quality of the image, which itself is contingent upon staining conditions, leading to high variability, and consequently, pronounced heterogeneity of the results among the various publications\(^4\). Still, fluorescence microscopy allows for the range of quantification parameters to all be measured at once.

In quantitative fluorescence microscopy, the goal is to measure the signal coming from the fluorophores used to label the object of interest in the specimen\(^8\). Background, which comes from a variety of sources, adds to the signal of interest, such that the intensity values in the digital image are equal to the signal plus the background. Although background fluorescence must be subtracted from the quantitative measurements, it is important to remove as much background intensity as possible before imaging as it effectively reduces both the dynamic range and the signal-to-noise ratio (SNR)\(^8\). Along with background, noise can cause variance in the intensity values above and below the real intensity value (signal + background). To detect the presence of a signal, the signal must be significantly higher than the noise level of the digital image or else the intensity caused by the noise will make the signal indistinguishable from the noise. The precision of quantitative microscopy measurements is therefore limited by the SNR of the digital image. SNR is a parameter that controls the sharpness of an image result. The higher this value, the sharper the image the lower the error in intensity. The correct method to estimate the SNR must find the standard deviation or the noise around the maximum level, which is defined to be the signal. Ideally, one would introduce in the sample a large homogeneous high intensity plateau, where because of the present noise, some standard deviation would be measured\(^8\).

Unfortunately this is experimentally difficult, and hardly ever are there large volumes with high constant intensities. Following the intrinsic Poisson statistics of the photon-electron events, the standard deviation is proportional to the number of photons, leading to the conclusion that the SNR is the square root of the number of photons\(^9\). The value can be calculated differently for images with high or low noise. For the high noise case the SNR is calculated to be the square root of the max intensity divided by the intensity of a single photon hit, while for the low noise case, comparisons are made between energies in and outside the band limit. For good quality confocal images, there is usually an SNR ranging from 20 to 40\(^9\).
For extracting information from 3-D images of the vasculature, a four-step process has been experimented with and has shown results useful in medical image analysis\(^1\). For the purposes of this research, step one of the proposed method, or pre-processing, will play an important role. First, the images are pre-processed for noise suppression and converted to binary images for further processing. Since the quality of a confocal microscopy image depends on its distance from the central slice, the noise level is higher in the images that are farther from the central image. To combat this, adaptive thresholds were used. Initially, a fixed threshold is used to segment most of the vessels. Assuming that the image pixels are either from a vessel (signal) or background (noise), the background average and standard deviation are calculated. Then the final threshold is set to the noise average plus two times of its standard deviation. This threshold ensures that more than 94% of noisy pixels are eliminated from the image\(^1\). This along with accounting for SNR allows for accurate images to be taken and therefore accurate quantification.

### Work Plan

<table>
<thead>
<tr>
<th>Low Aim (2-3 months)</th>
<th>Target Aim (5-6 months)</th>
<th>Ideal Aim (9+ months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Develop confocal quantification procedure</td>
<td>Determine bone growth quantitative parameters</td>
<td>Publish paper with results</td>
</tr>
<tr>
<td>Participate in endothelial isolations</td>
<td>Determine relationship between quantitative MVF and bone growth parameters</td>
<td>Develop predictive models for bone regeneration based on quantitative parameters</td>
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<tr>
<td>Develop experimental groups from isolations</td>
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<tr>
<td>Develop quantification parameters for the microvessel fragments (MVF)</td>
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<tr>
<td>Produce 3-D images of the vascular structures grown</td>
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### Materials and Methods

**Background:** Amira is an imaging software that allows for quantitative and histomorphometric analysis of neovascularization in a region of interest (ROI) of a multichannel z-series stack. Its purpose in this protocol is to evaluate the characteristics of the blood vessels over their in-vitro life term and ultimately to use that data to map these characteristics to predictable long-term bone growth.

**Experimental Goals/Possibilities**
- Finding a relative effect associated with different conditions rather than providing some absolute quantification.
- To segment features of interest in confocal images for analysis on their 3-D organization

**Materials Needed:**
1) Z – series stack of vessels grown in vitro (Vessels isolated from breeding rat)
2) Amira Software w/ appropriate plug-ins (Image J, Fiji)
Density Based Segmentation

- Amira offers segmentation software according to inputted thresholds.

4 – Step Analysis of Vessels Using Amira

1. Orthogonal Slice Analysis
   a. *Bounding box --> OrthoSlice*
   b. Choose orientations for each slice (coronal, sagittal)
   c. Rotate in viewer for a more general view
   d. *if an arbitrary slice is needed, use ObliqueSlice*
   e. Use data window to determine a threshold for later segmentation
   f. Set mapping type to linear

2. Isosurface Analysis (encloses all parts of a volume that are brighter than a defined threshold)
   a. Resample data for decreased computation and improved rendering performance
b. Connect resample module to data

c. Enter x,y,z values for a coarser resolution

d. Turn off viewer toggle of OrthoSlice

e. Connect Isosurface module to data and adjust threshold port (~85)

f. *For cropping part of the image field, remove resample data and active OrthoSlice display

g. *For volume rendering without segmentation use the Voltex module and enter data range values into Colormap port. Lower bound (40-60) for visible influence of the transfer function. For maximum intensity projections, use the mip option.

Reconstruction w/ Segmentation

a. Image Segmentation (assigns each pixel a value depending on the region)
   i. LabelField (right click green icon from Labelling section)
   ii. Expand viewer if current frame constraints don’t include the entire sample
   iii. Use slider on bottom right to scroll through slices
   iv. Choose the brush under Tools label
   v. Assign pixels to a structure

b. Volume Measurement
   i. LabelField (right click green icon from Labelling section)
   ii. Measure/MaterialStatistics
   iii. Units in voxel dimensions cubes (usually µm)

c. Surface Reconstruction
   i. Connect SurfaceGen module to the data and apply
   ii. *Reduce number of grid triangles if needed using the Surface simplification editor
   iii. Through Simplify port, set the desired number of faces and apply

*Amira offers manual tracing of features as well

d. Manual Tracing
i. Trace the border of the feature of interest (paintbrush tool) and fill in (“f”).
ii. Generate surface rendering (ObjectPool → SurfaceGen)
   • Surface properties → Apply
   • Use SurfaceView to visualize segmented volume.

4. **Network Skeletonization** (ordered set of points that define vessel centerlines)
   a. Image data should be in the form of stacked 2-D images, with the top slice as #1
      i. TIFF format is good (lossless compression/readable by many systems)
   b. Load slices and enter position and voxel size
   c. Copy files to actually work with, preserving the original data
   d. Create/Skeleton → Mosaic
   e. Add files (bricks) to the Mosaic, then display and save it.
   f. Apply a digital filter to all blocks in the brick

*Manual tracing can be used as a substitute for skeletonization*
**Figure 1.** Rhodamine + GFP Stained gels. Front view. Gels are ordered 1-4 and represent different samples of the same experimental group.
Figure 2. Lateral views of the gels in the MVF + GFP experimental group

<table>
<thead>
<tr>
<th>Gel 1</th>
<th>Gel 2</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
<th>Gel 3</th>
<th>Gel 4</th>
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<tbody>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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Figure 3. 3-D Skeleton view of MVF groups. Samples are in the same order as Figures 1&2.

Figure 4. Sample Orientation View for Determining Growth Angle
Figure 5. Vessel length ranges for MVF groups 1-4.
Table 1. Threshold ranges calculated for an initial study of 4 MVF Samples. These threshold ranges were calculated using default local threshold analysis.

<table>
<thead>
<tr>
<th>MVF Sample</th>
<th>Threshold Range</th>
<th>% of material displayed</th>
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<tbody>
<tr>
<td>1</td>
<td>0-66</td>
<td>93%</td>
</tr>
<tr>
<td>2</td>
<td>0-42</td>
<td>95%</td>
</tr>
<tr>
<td>3</td>
<td>0-63</td>
<td>96%</td>
</tr>
<tr>
<td>4</td>
<td>0-68</td>
<td>94%</td>
</tr>
</tbody>
</table>

Table 2. Microvessels were quantified by both orientation standard deviation and total area. Microvessels that were oriented more similarly were shown to also have a greater total vessel area.

<table>
<thead>
<tr>
<th>MVF Sample</th>
<th>Orientation Std. Deviation (degrees)</th>
<th>Vessel Area (um^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.83</td>
<td>1.68E6</td>
</tr>
<tr>
<td>2</td>
<td>29.45</td>
<td>1.42E6</td>
</tr>
<tr>
<td>3</td>
<td>25.36</td>
<td>1.65E6</td>
</tr>
<tr>
<td>4</td>
<td>26.13</td>
<td>1.72E6</td>
</tr>
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**Discussion**

Considering the parameters used to measure microvessel morphological characteristics, relationships can be found between morphological characteristics and angiogenesis confirming the methods listed above are indeed valid ways to analyze and quantify these characteristics. First, a threshold range is determined so that the software only picks up the microvessel structure and leaves out other materials such as cell debris which constitutes a certain percentage of each sample. Figure 2 shows a sample of each image once a threshold has been determined. Once a pixel threshold has been defined, morphological characteristics such as vessel length, area, and orientation are able to be determined. Figure 3 shows a skeletal view, which allows one to determine number of branches, junctions, and length of the branches. This data can help determine overall area and density of the vasculature. Figure 4 shows how vessel orientation angle is calculated once a threshold has been determined. While these individual characteristics are important to know when comparing different samples, we also want to determine if these characteristics form correlations between each other. Figure 5 displays a point for each vessel branch determined as well as its length in µm. Comparing the length ranges to the total area values listed in Table 2, the samples with less vessels, but with larger lengths lead to a greater overall volume than the samples that contain more vessels, but have lesser lengths.

Table 1 shows the correlation between threshold range and amount of total material analyzed. Looking at the results it can be inferred ~5% of each sample constitutes some form of cell debris or other structure not related to the vasculature. Table 2 displays a relationship between vessel orientation and total vessel area. Specifically, it measures compares the standard deviation of the vessel orientations to the vessel area. It is shown that as the standard deviation of the vessel orientation angle increases, the quantified area decreases. This shows that as the process of angiogenesis produces microvessels that share a more ordered pattern or orientation, they are also able to grow to a larger total area. As increased microvessel density is ideal for angiogenesis, more experimentation should be done to produce more ordered alignment of microvessels during angiogenesis. Due to time constraints of the project, the vascular characteristics measured were not able to be compared to overall bone growth. Future research should also look at even more morphological characteristics to determine more correlations as well as tying this data back to effectiveness of bone growth once the microvessels are implanted in a bone defect.
Works Cited


