Whole Mount Immunostaining: A Comprehensive View of Tissue Architecture

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Introduction

Ischemia encompasses a number of disorders and diseases resulting from the restriction of blood flow to a tissue. Some of the common complications that stem from ischemia include coronary artery disease (CAD), peripheral artery disease (PAD), and stroke. In each of these conditions, occlusion of an artery due to atherosclerotic plaque build-up leads to restricted blood flow downstream to skeletal muscle, cardiac muscle, or brain tissue, respectively. As a result, oxygen and nutrient delivery to and waste removal from the affected tissue becomes scarce or absent. This often leads to tissue necrosis due to the inability of the tissue to support metabolism. An estimated 32 million Americans suffer from these ischemic diseases, collectively (American Heart Association, 2010). Due to these overwhelming statistics, a plethora of research into various treatments continues to progress and evolve.

A couple of commonly used treatments for CAD, the most prevalent of the ischemic diseases, include angioplasty and coronary artery bypass surgery. Angioplasty involves threading a catheter through the arteries to the site of blockage (NIH, 2011). The catheter contains a deflated balloon at one end with a metal stent surrounding it and once at the proper site the balloon is inflated and the stent deployed. This acts to push the atherosclerotic plaque against the artery walls and the stent supports the restoration of blood flow by maintaining an open diameter of the arterial lumen. Coronary artery bypass surgery seeks a comparable result through a different means. This treatment method involves grafting either a piece of an artery or vein from another site within the body or a synthetic polymer to the area of blockage in a manner that serves to reroute blood flow around the blockage (NIH, 2011).
Although angioplasty and coronary artery bypass surgery represent two widely used treatment options for CAD, a majority of patients are not ideal candidates to undergo these procedures. Angioplasty remains the preferred preliminary method of intervention because it is less invasive than coronary artery bypass surgery. However, a number of patients cannot benefit from this treatment due to the extent of the atherosclerotic plaque build-up, in the coronary arteries particularly (NIH, 2011). Furthermore, restenosis or the reformation of plaque and re-narrowing of the blood vessel frequently occurs in patients with severe coronary artery disease even with the use of stenting during angioplasty. Similarly, many patients cannot undergo coronary artery bypass grafting procedures due to factors such as age or the occurrence of other diseases such as diabetes, kidney or lung disease, and PAD (NIH, 2011). These procedures are too risky in these patients and therefore do not represent a viable option for treatment. Additionally, in most cases the severity of CAD limits the availability of viable vessels to use for a graft.

Some endogenous revascularization mechanisms exist as a physiological response to changes in blood flow including arteriogenesis and angiogenesis (Simons, 2005). Arteriogenesis refers to the remodeling of existing arteries, known as collateral enlargement, or the de novo synthesis of arteries resulting from physical forces such as shear stress that ensues from a partial occlusion upstream (Simons, 2005; Heil et. al, 2006; Peirce and Skalak, 2003). However, this natural mechanism becomes initiated more as a response to shear stress rather than hypoxia resulting from ischemia. Angiogenesis, on the other hand, refers to expansion of the capillary network via growth of new capillaries from existing capillaries (Simons, 2005; Peirce and Skalak, 2003).
Ischemia initiates new capillary growth by inhibiting the degradation of hypoxia-inducible factor-1α (HIF1α). Subsequently, HIF1α leads to the activation of transcriptional programs, which result in production of factors such as vascular endothelial growth factor (VEGF) that stimulate angiogenesis (Simons, 2005). Activation of angiogenesis leads to increased perfusion rates due to the presence of new capillaries thereby increasing oxygen delivery to hypoxic tissues.

Though natural machinery exists to combat ischemic diseases, these mechanisms are not adequate to counteract the insufficient blood flow in patients most severely affected. Therefore, studies seek to promote angiogenesis therapeutically in order to supplement and mimic natural healing within the body. Primarily, these studies aim to promote growth of new capillary networks by applying factors such as VEGF, a known growth factor responsible for vascular network expansion (Simons, 2005; Emanueli, 2003.) This factor leads to de novo network formation, growth, and maturation. For therapeutic use, the maturation step proves most important in creating a functional mode of restoring blood flow. However, clinical trials utilizing this methodology as a means of promoting blood flow and vascular remodeling fail (Jain, 2003; Simons et. al., 2000).

A number of ideas arise regarding the reasons for failure of clinical trials. One such thought relates to the group of patients selected for clinical trials. Simons et. al. (2000) suggests that the patients chosen represent a group that is least likely to respond to treatment. Unsurprisingly, many pre-clinical trials are performed in healthy animals and therefore do not accurately recapitulate a human model. It follows that selectively choosing patients seemingly more likely to respond to treatment does not accurately recapitulate the majority of the population suffering from these diseases. Another
proposal for failure of exogenous stimulation of angiogenesis stems from the idea that although administration of factors such as VEGF might lead to capillary network remodeling, the organization of these networks becomes abnormal (Jain, 2003). As a result, the restoration of blood flow is incomplete. Sullivan et. al (2002) demonstrated that ischemia promotes increases in capillary density, but that density levels off and blood flow is not restored. Formation of an irregular, chaotic vascular network might explain this trend.

Based on this information, novel therapeutic approaches should aim to promote revascularization though exogenous means that lead to the formation of an organized network. Clearly, angiogenesis represents an important mode of restoring blood flow to a hypoxic region, but without control of the network expansion this therapy will undoubtedly fail. So, in an effort to explore this new approach creation of tools to assess network expansion become increasingly important. This study aims to develop a research tool to assess tissue architecture and provide a comprehensive view of the vasculature pre- and post-ischemia. Development and optimization of a whole mount immunostaining protocol provides the basis for this comprehensive analysis tool.

Methods

Tissue Harvest and Fixation

The Animal Care and Use Committee of California Polytechnic State University, San Luis Obispo approved all surgical procedures. Four C57B1/6 mice were used in this study. One of the mice was perfusion fixed with 10% formalin via delivery through the left ventricle. In situ fixation was performed on the remaining mice by exposing the
muscles and generously applying fixative to the area. The exposed tissue was covered with saran wrap and allowed to fix for 30 minutes. Following fixation, the right and left gracilis anterior muscles were excised from all animals. Additionally, the right and left spinotrapezius, right and left tibia, and right and left extensor digitorum longus muscles were excised from the perfusion fixed animal and one of the in situ fixed animals. Tissues were stored in PBS at 4°C until ready for use. (See A1 for detailed protocol).

**Staining of Whole Mount Tissues**

Spinotrapezius muscles from a perfusion fixed (10% formalin) and in situ fixed (10% formalin) animal were permeabilized and stained with α-smooth muscle actin (α-SMA; IA4-Cy3, Sigma 1 mg/mL) overnight at 4°C or at room temperature for 1 hour. The tissues were washed and mounted on slides for whole-tissue fluorescent imaging. Seven gracilis anterior muscles (right from 10% formalin perfusion fixed animal, right and left from three 10% in situ fixed animals) were permeabilized and stained with α-smooth muscle actin (α-SMA; IA4-Cy3, Sigma 1 mg/mL) for 72 hours at 4°C. All tissues were washed and mounted on slides for whole-tissue fluorescent imaging. (See A2 for detailed protocol; adapted from MacGabhann and Peirce, 2010).

**Image Analysis**

Tissues were imaged using a standard fluorescent microscope at 4X and 10X magnifications. Images were acquired from the entire muscle and Adobe® Photoshop® CS5 was used to collage the images for a comprehensive view of the muscle vasculature.
**Results**

The right spinotrapezius muscle from a perfusion and *in situ* fixed animal was stained with $\alpha$-smooth muscle actin ($\alpha$-SMA) overnight at 4°C (Figure 1). This served as a preliminary experiment to assess proper antibody concentration and specificity as well as evaluate the differences in staining quality between the two fixation methods. Additionally, the left spinotrapezius muscle from a perfusion and in situ fixed animal was stained with $\alpha$-SMA at room temperature for an hour to determine appropriate incubation time for a thinner tissue. The left spinotrapezius muscles just described were also stained with lectin (Isolectin GS-IB₄, Invitrogen), but no staining was observed in either sample and therefore results are not pictured here.

Based on these data, the right gracilis anterior muscle from a perfusion and *in situ* fixed animal was stained with the same $\alpha$-SMA antibody solution and concentration as the right spinotrapezius muscles. The perfusion fixed right anterior gracilis muscle was shaken on a rotating plate during wash steps. Conversely, the in situ fixed right anterior gracilis muscle experienced static washes. Each muscle exhibited similar staining specificity and quality.

From these results, staining experiments with $\alpha$-SMA were performed on the right and left gracilis anterior muscles of three *in situ* fixed animals. These six replicates yielded similar fluorescent images displaying high antibody specificity and staining quality. Figure 2 depicts a representative image of one of these replicates.
Figure 1. Representative image of right spinotrapezius muscle of in situ fixed animal stained with α-SMA. Image was taken using a standard fluorescent microscope. Antibody specificity and quality for α-SMA is very high in these samples. Perfusion fixed muscles produced similar images.
Figure 2. Right gracilis anterior muscle from in situ fixed mouse stained with α-SMA. Multiple images across the length of the muscle were taken using a fluorescent microscope at 4X magnification. Images were collaged to provide a comprehensive view of tissue structure. The white arrow indicates the area magnified (10X) within the white box.
**Discussion**

Ischemic disease affects an overwhelming number of individuals in the United States. Current therapies include angioplasty with stenting and coronary artery bypass surgery. However, these therapies are insufficient in many cases due to the severity of the disease in some patients. As a result, a need exists for the development of novel therapeutic approaches. Taking queues from the natural healing mechanisms, many of these new therapies seek to promote arteriogenesis and/or angiogenesis. These two processes, collateral enlargement of existing arteries and de novo synthesis of capillaries from existing capillaries, respectively, lead to increases in blood flow and consequently oxygen delivery to hypoxic tissues. Although these physiologic mechanisms are not always sufficient to completely restore blood flow to an injured or ischemic tissue, they provide an important model from which to base new methods of therapy which seek to increase perfusion.

Previous studies demonstrate that exogenous administration of growth factors that promote angiogenesis lead to an increase in capillary density. Despite this revascularization, blood flow rarely returns to the original state. Perhaps this lack of complete restoration of perfusion in the presence of increased capillary density results from development of an irregular and chaotic vascular network. The capillaries remodel, but not in a manner that supports perfusion. Based on this idea, novel therapies might explore modes through which stimulation of angiogenesis leads to an organized network. With this approach, the need arises for a method to assess overall changes in tissue architecture in terms of the vasculature. In other words, without a technique for looking
at overall topological changes in tissue architecture organization of the remodeling network cannot be assessed.

Currently, two widely accepted methods for assessing capillary density exist. The first method employs tissue sectioning of muscles of interest. Within each section, capillaries are counted leading to a density determination. But, this method does not provide an overall view of tissue architecture. The second commonly used method, vascular casting, presents a more comprehensive view of the overall vascular network. In this method, a dye or paint is perfused throughout the animal following a perfused fixative. Though vascular casting paints a more complete picture of the vascular network, it contains a couple of flaws. First, this is a labor-intensive method and therefore less desirable. Second, ischemic models suffered from decreased perfusion and as a result the dye might not completely penetrate the entire vasculature leading to incomplete views of the entire system. The downfalls of the current assessment tools for viewing tissue architecture provide the basis for this study. A whole mount immunostaining protocol was successfully developed and optimized as a tool for obtaining a comprehensive view of tissue architecture pre- and post-ischemia.

Based on these data, whole mount immunostaining provides a valuable method for looking at capillary networks. The experiments here demonstrate the specificity and quality of immunostaining. Also, the ease of use makes the protocol a desirable mode for large-scale experiments. Perfusion fixation and rotating wash steps proved unnecessary for producing useful images. Therefore, this whole mount immunostaining protocol will greatly contribute to the toolbox and provide a basis for comparing novel therapeutic strategies that aim to promote angiogenesis in a directed, organized fashion.
Appendix

A1. Tissue Dissection—In Situ Fixation for Whole Mount Immunostaining

Materials
Forceps (1)
Fine forceps (2)
Iris scissors (1)
Cauterizer
Cotton Swabs
Heat pad
Depilatory Cream
Bench cover
Non-sterile saline
Microcentrifuge tubes
PBS

Preparation
1. Heat heating pad in microwave.
2. Label microcentrifuge tubes.
3. Anesthetize animal using 4% isoflurane.
4. Shave both medial hindlimbs and use depilatory cream to remove all hair.
5. Lay animal on bench cover, over heating pad, and tape hindlimbs in adducted position.

Dissection
6. Remove the skin overlying the medial thigh to expose the musculature.
7. Apply saline to prevent desiccation and place saran wrap over the exposed muscle.
8. Perform cervical dislocation to euthanize the animal.
9. Apply 10% formalin fixative generously over tissue of both hindlimbs and place saran wrap over the exposed muscle to prevent desiccation.
10. Allow fixative to sit for 30 minutes.
11. Dissect the left gracilis anterior and place tissue in a pre-labeled microcentrifuge tube containing PBS.
12. Repeat step 11 for right gracilis anterior.

Post-Dissection
13. Store tissues at 4°C until ready for use. (If you plan to perform staining the same day as dissection, wash tissue in PBS for 30 minutes).
A2. Whole Mount Immunostaining

Materials

Sylgard® 184 Silicon Elastomer (Cat #: NC0004554, Fisher Scientific)
24-well culture plates (Cat#: 3738, Corning Incorporated)
PBS
0.1% Saponin (Cat#: 47036, Sigma-Aldrich)
2% Bovine Serum Albumin (Cat# B6917, Sigma Aldrich)
Monoclonal Anti-Alpha Smooth Muscle Actin, Cy3 Conjugate (Cat#: C6198, Sigma)
Fluoroshield Mounting Medium with DAPI (Cat#: ab104139, Abcam)
Slides
Coverslips
Parafilm
Aluminum foil

Sylgard-coated Plate Preparation
1. Prepare sylgard-coated plates at least 3 days prior to staining.
2. Mix 10:1 sylgard elastomer to curing agent in a 50 mL conical tube. (Note: sylgard is extremely viscous. Cut tip of pipette to create a larger opening and pipette very slowly, changing tips often).
3. Add 500-750 uL of sylgard mix to each well of a 24 well plate.
4. Allow sylgard to harden at room temperature for 48 hours.

Staining
5. Using forceps, remove muscle from PBS (stored in microcentrifuge tube at 4°C) and place in a single well of a 24-well sylgard-coated plate.
6. Prepare antibody solution containing 1:200 1A4 clone (alpha-smooth muscle actin, Cy3 conjugate) in 0.1% saponin (reconstituted in PBS), 2% BSA (reconstituted in PBS) in PBS.
7. Incubate muscle in antibody solution for 3 nights (72 hours) at 4°C. (Note: Critical step—3 nights crucial for bright staining by gently pipetting solution over muscle.)
8. Wash in 0.1% saponin in PBS 3x for 20 minutes at room temperature. Wrap plate in parafilm and cover with foil during each wash.
9. Wash in plain PBS for 30 minutes. Wrap plate in parafilm and cover with foil during each wash.
10. Remove muscle from well using forceps and place on a slide.
11. Add 1-2 drops of mounting media with DAPI and place coverslip over muscle.
12. Paint edges of coverslip with clear nail polish to create a seal and prevent tissue desiccation.
13. Store slides at 4°C wrapped in foil between imaging.

Imaging
References

American Heart Association. Heart Disease and Stroke Statistics – 2010 Update. Dallas,


