Immunohisotchemical Mapping of Hypoxia in Ischemic Mouse Hindlimb Skeletal Muscle

Microcirculation and Tissue Repair Lab
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ABSTRACT

The study of blood vessel growth and remodeling is a complex endeavor. Hypoxia, the lack of oxygen in a tissue, is known to stimulate angiogenesis (the growth of new blood vessels), and have little effect on arteriogenesis (the enlargement of existing blood vessels). However, the role of hypoxia in vessel function is unknown, but may be determined using the results and methods developed in this experiment.

Supplied by the bloodstream, oxygen is required by all cells and tissues to remain healthy. If the bloodstream supplying a certain tissue with blood is disrupted, the tissue becomes ischemic, often leading to hypoxia. Hypoxia is the lack of oxygen in living tissue; specifically tissue oxygen levels less than 10 mmHg (hypoxyprobe.com).

When a tissue is ischemic, and therefore hypoxic, angiogenesis is known to occur, which expands the nutrient and waste exchange capacity of the microcirculation. Hypoxia-inducible factor (HIF) is a transcriptional regulator of angiogenic molecular pathways and is regulated according to the oxygen availability in a given tissue (Pugh, Ratcliffe).

By studying the locality of hypoxia within a given tissue, it is possible to predict the behavior of the growing blood vessel. Additionally, changes in vessel function may be understood by comparing the location of hypoxia with the location of the altered function. Cal Poly’s microcirculation laboratory is interested in investigating the effects of hypoxia, either prior or sustained, on vascular reactivity.
In order to carry out this investigation, a method for assessing the level of hypoxia throughout the tissue is needed.

In this study, immunohistochemical staining techniques were used to visually map hypoxia in ischemic mouse hindlimb skeletal muscle. The technique, which was developed into a repeatable protocol, may be useful in further investigating hypoxia induced vascular growth and changes in function.
INTRODUCTION

Many physiological processes involve the control of tissue blood flow. Blood flow control mechanisms act to maintain the functionality of blood, including transportation of nutrients, regulation of pH levels, and defense against pathogens (Marieb). Because of the wide oxygen demand (due to the wide metabolic range) of skeletal muscle, the health of the tissue is critically dependent on acute blood flow control. (Jain).

In cases of pathology, blood vessels can become occluded, affecting vascular reactivity and blood flow control. Many things can occlude vessels, disrupting normal blood flow. Atherosclerosis is a condition where the diameter of a blood vessel is dramatically lessened due to a build up of fatty substances in the vessel lumen (Maton). Ligation of the vessel prevents blood flow through the pathway to some degree. Occlusion, due to atherosclerosis, ligation, or other causes, lessens or eliminates blood flow through a vessel, causing the tissues normally supplied with blood from those vessels to be ischemic. The ligation treatment experimentally models ischemic conditions that would be found in response to pathologic arterial occlusion, such as atherosclerosis.

Tissues respond complexly to arterial occlusion. If the occlusion causes injury, inflammation can occur. Inflammation and hypoxia together may cause myofiber death and regeneration, in which muscle fibers re-grow to repair the damage that caused inflammation in the first place (Marieb). In addition to these possibilities, injury or any other disruption to the flowing capacity of blood vessels has two main results. First, arterial occlusion increases angiogenesis, the growth of
new vessels from existing vessels, and may increase arteriogenesis, and the outward
remodeling of existing collateral vessels. These changes in microvascular
architecture help maintain sufficient oxygen delivery. Secondly, occlusion decreases
vessel wall function, in terms of the ability of the vessel to constrict or dilate
(Marieb). When vessel wall function is impaired, specifically by injury, the vessel
has less control over blood flow, thereby decreasing the microvascular function of
the vessel, and the health of the affected tissue.

Because vessel injury usually leads to ischemia, which tends to cause
hypoxia, hypoxia can be viewed as the catalyst for increased angiogenesis, and
decreased vessel wall function. The goal of Cal Poly’s Microcirculation and Tissue
Repair lab is to investigate how changes in vessel architecture, in terms of
angiogenesis, usually catalyzed by injury, affect blood flow, and why vessel injury
affects vessel wall function.

A common consequence of ischemia is hypoxia, where a tissue is deprived of
a certain level of oxygen (Marieb). Hypoxia, as a consistent consequence to blood
vessel injury, affects angiogenesis and perhaps vessel wall function. However, the
exact impact of hypoxia on vessel wall function is unknown. In order to understand
how hypoxia affects vessel wall function, the physical location of hypoxia needs to
be correlated to the physical locations of dysfunctional resistance vessels. This
correlation may reveal more specifically how hypoxia impacts vascular reactivity.
The focus of this study is to determine the geographical behavior of hypoxia in
ischemic tissues of the mouse hindlimb, which can further be used to understand
how hypoxia impacts vascular reactivity.
METHODS

Overview

The purpose of this study was to develop a protocol for hypoxia mapping in ischemic mouse hindlimb skeletal muscle that the microcirculation lab could use in the future to examine the correlation between hypoxia and vascular function.

In this study, mice were used to map hypoxia in the calf muscle under ischemic conditions. Pimonidazole was injected into the anesthetized mouse. Next, the femoral and saphenous artery-vein pair were resected, such that the hindlimb skeletal muscle became ischemic. The tissues were dissected, fixed in Histochoice, processed, and embedded for histological use. Immunohistochemistry was then used to stain cross sections of the ischemic hindlimb tissue for areas of hypoxia.

Three surgeries were completed, each of which differed slightly from the others, in the attempt to achieve successful hypoxia maps. As was the staining protocol, the injection and surgical protocols were developed during execution. Process parameters were modified in the attempt to optimize the protocols.

Injection

Pimonidazole hydrochloride (Hypoxyprobe) was used as a hypoxia marker. Once an intraperitoneal injection into the mouse was made, Hypoxyprobe bound to hypoxic cells, or those cells with the partial pressure of oxygen less than 10 mmHg at 37 deg C. However, the pimonidazole required time to diffuse into the bloodstream and circulate throughout the body before it was able to even reach all
hypoxic cells. In this sense, the injection timing was critical. In surgery 1, 25 minutes were allowed between injection and the first ligation of the resection surgery. For surgery 2, 55 minutes were allowed between injection and initial ligation. For surgery 3, 20 minutes were allowed for uptake and circulation of pimonidazole by the blood after injection and before ligation.

Resection

In the next step of the surgery, the femoral artery-vein pair and saphenous artery-vein pair were resected from the left hindlimb, according to the established protocol developed by the microcirculation and tissue repair lab. To do this, the fur, skin, connective tissue, and fat pad were removed so that the vasculature was exposed on the medial aspect of the hindlimb. Two ligations were made, one between the knee and ankle, and the other just upstream of the muscular branch. Once ligated, the vessels between each suture were removed. This step, from the first ligation to the second, took different amounts of time. For surgery 1, it took 4 minutes, for surgery 2, it took 25 minutes, and for surgery 3, it took 20 minutes. The right hindlimb in all three surgeries was left untouched, so that ischemic conditions were only induced in the left hindlimb skeletal muscle.

Perfusion Fixation

Perfusion fixation is a step, paired with a vasodilator cocktail, intended to dilate, remove the blood from, and fix tissues throughout the entire body. The purpose of fixation is to disable damaging biomolecules, protect the sample from
foreign microorganisms, and increase the mechanical strength of the samples to maintain the morphology of the tissue. The benefit of fixation is that it preserves the vessels in a natural state, and allows them to be easily analyzed during histology.

Perfusion fixation is a method of fixation where the fixative is administered through the blood stream. Enough fixative to enter every vessel was administered into the heart and allowed to spread through the body. The tissues remained alive until the moment the fixative reached them. In this way, tissue integrity was maintained more than it would be with immersion fixation (where dissected tissues are placed in a fixative). With immersion fixation, no administration of fixative occurred through the bloodstream, and the fixative diffused throughout the organ.

**Stimulation**

Tungsten microelectrodes were used to stimulate muscle contraction. Electrodes were impaled into to the calf muscle and used to deliver current for 90 seconds at 8 Hz, 0.5 ms, 2 mA. The resulting muscle contraction required ATP to support actin-myosin cross bridge cycling. Oxygen in the calf skeletal muscle, which was used in the production of ATP, was depleted by the induced muscular contraction, and could not be restored by the bloodstream because of the resection. Therefore, stimulation of skeletal hindlimb muscle after resection of the femoral and saphenous artery-vein pair should have quickly depleted the tissue of oxygen, leaving it highly hypoxic.

Following the resection step, the procedure varied greatly between surgeries. For surgery 1, another period of waiting was implemented, this time 40 minutes, in
order to allow the circulating Hypoxyprobe to bind to newly hypoxic cells. After this period of waiting, a perfusion fixation was implemented, directly followed by tissue harvest. In surgery 2, 35 minutes if wait-time was allowed after resection and before tissue dissection, with no perfusion fixation step. Instead, tissues were fixed by immersion directly following dissection. In surgery 3, stimulation was used in the left calf only in an attempt to increase the level of hypoxia in the tissue. After this, 20 minutes of rest were used to allow the Hypoxyprobe to bind to newly hypoxic cells before dissection and immersion fixation.

Dissection

After either a period of waiting, or in the case of surgery 1, perfusion fixation, tissues were dissected from the euthanized animal. The main tissues of interest for the dissection were the calf and the gracilis muscle. These tissues were dissected from the ischemic left, and non-ischemic right hindlimb, and immersed directly into fixative. In surgery 1 and 2, Histochoice was used as a fixative. In surgery 3, the tissue was fixed with formalin. All three sets of tissues were left immersed in fixative for 24 hours before processing. The general injection protocol, subject to the mentioned alterations, is attached in Appendix A. Below is a summary of the differences between surgeries.
<table>
<thead>
<tr>
<th></th>
<th>Surgery 1</th>
<th>Surgery 2</th>
<th>Surgery 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time from injection to resection start</td>
<td>26 min.</td>
<td>57 min.</td>
<td>21 min.</td>
</tr>
<tr>
<td>Time in resection</td>
<td>4 min.</td>
<td>26 min.</td>
<td>19 min.</td>
</tr>
<tr>
<td>Time from resection end to dissection start</td>
<td>40 min.</td>
<td>35 min.</td>
<td>34 min.</td>
</tr>
<tr>
<td>Total time</td>
<td>70 min.</td>
<td>118 min.</td>
<td>74 min.</td>
</tr>
<tr>
<td>Stimulation?</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Perfusion/fixation?</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Fixative</td>
<td>Histochoice</td>
<td>Histochoice</td>
<td>Formalin</td>
</tr>
<tr>
<td>Fixative temperature</td>
<td>Room temperature</td>
<td>4 deg C</td>
<td>4 deg C</td>
</tr>
</tbody>
</table>

Processing

Sample processing was required before the tissues could be embedded for histological purposes. Processing involved two main steps: dehydration and clearing. Tissues were placed in labeled cassettes and in an automatic processor. The processing was done automatically over night. Dehydration involved removing water from the samples, and consisted of a series of steps where the samples were rinsed with increasing concentrations of alcohol. After dehydration, the tissue underwent clearing. The purpose of clearing is to remove the dehydrating reagents from the previous step, usually with xylene. Additionally, the processor infiltrated the tissue with paraffin wax.

Embedding

Once the samples were dehydrated and cleared in the automatic processor, they were embedded in paraffin wax. The tissues were cut in half along their cross-section, placed in hot wax and aligned in a plastic cassette. The wax was allowed to cool and harden into a block ready for sectioning. To map hypoxia in the dissected,
ischemic tissues, histological analysis was used. Eight μm sections of tissue were taken with a microtome and placed on microscope slides.

**Staining**

In this study, immunohistochemistry (IHC) was used to stain for hypoxia. Pimonidazole, which was injected into the animal before dissection, formed adducts when bound to proteins in hypoxic cells, which were the antigen source for the IHC stain. Therefore, a series of antibodies specific to pimonidazole-protein adducts produced a visual representation of the hypoxic gradient when added to the sectioned hypoxic tissues that were injected with pimonidazole.

The primary reagents in the immunohistochemical stain were the blocking serum, primary antibody, secondary antibody, avidin-biotin enzyme, and peroxidase substrate. All of these reagents had crucial roles that contributed to the resulting stain of hypoxic regions.

In this study, pimonidazole that was bound to protein in hypoxic environments acted as the antigen. The level of pimonidazole binding was expected to increase as the level of hypoxia increased. Pimonidazole labeled all hypoxic tissue by binding proportionately to each one, resulting in darker staining where more hypoxia existed. Non-specific binding of antibodies or enzymes to cells without pimonidazole adducts was prevented by the blocking solution.

The next key factor in the general IHC staining process was the primary antibody. The Hypoxyprobe kit included a mouse immunoglobulin monoclonal antibody (MAb IgG1), which detected and binds only to pimonidazole antigens in the
tissue. Next, a biotinylated secondary antibody was introduced to the tissue, which bound with the primary antibody. Biotin, a B-complex vitamin, which was conjugated to the secondary antibody, facilitated signal amplification in later steps.

After the biotinylated secondary antibody had bound to the primary antibody (which was bound to the antigen), avidin was added. Avidin and biotin form strong covalent bonds. In this case, avidin bound to the biotin molecule that was conjugated to the secondary antibody, at the hypoxic site. Finally, biotinylated peroxidase was added to the tissue. Each avidin molecule bound four biotin molecules, so for each avidin bound to each biotinylated secondary antibody, there were three biotinylated peroxidase molecules that attached to the complex. This mechanism lead to signal amplification, because more peroxidase molecules were present than were pimonidazole adducts. The peroxidase developed an added reagent, diaminobenzidne (DAB), into a brownish precipitate. When DAB was added, it should only turn brown in the presence of peroxidase. In this way, the more hypoxic the cell was, the darker brown it appeared. The results of the process were stained tissues on microscope slides, appearing in shades of brown. If successful, the degree of shading throughout each tissue cross-section correlated to the amount of hypoxia present there at the time of harvest.

In this study, an ABC kit from Santa Cruz Biotechnology was used (ABC-2017). The kit provided most of the undiluted reagents, such as blocking serum stock, secondary antibody stock, avidin, biotinylated horse radish peroxidase, substrate buffer, DAB chromagen, and peroxidase substrate. These reagents were combined into steps and diluted in PBS or deionized water. For example, blocking
serum stock and secondary antibody stock were provided components but were separately diluted in PBS. The following are the consolidated IHC steps provided by the ABC kit: blocking solution, secondary antibody, avidin-biotin enzyme, and peroxidase substrate. Hypoxyprobe provided the primary antibody.

Just as the vague injection protocol provided by the Hypoxyprobe manufacturer was interpreted several times to fit the needs of this study, so was the existing IHC protocol. Originally, the Immunohistochemistry protocol was used for staining alpha smooth muscle actin, with the ABC kit. The steps were altered before and during trials to mold the protocol into one that produced successful hypoxia maps. Several of the main alterations are as follows: lessening time in dehydration steps, and changing the primary antibody (depending on the antigen stained for). The most updated version of the staining protocol can be viewed in Appendix B. Another IHC staining protocol, provided by Hypoxyprobe, was interpreted and used for one run in this study (Appendix C). However, the modified alpha smooth muscle actin protocol (Appendix B) was used for most runs, because the technique was more straightforward, and the protocol had already produced successful results with the smooth muscle actin antibody.

In this study, two different experimental conditions were implemented, so that four different groups existed for each trial. The only group that should have had positive staining was the ischemic primary group. This is because pimonidazole IHC peroxidase staining only stains for hypoxia, which should only be present in significant quantities in the ischemic tissue. Moreover, the success of the whole IHC process depended on the presence of the primary antibody. Without the primary
antibody, the rest of the reagents (secondary antibody, avidin, biotin, peroxidase, and DAB) would not bind to or be developed near hypoxic cells, thereby failing to create a dark shade and more importantly, a hypoxia map. Therefore, hypoxic staining was only expected in ischemic tissues treated with primary antibody. The conditions and groups used in this study are summarized below:

<table>
<thead>
<tr>
<th>Table 2. Experimental Setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibody</td>
</tr>
<tr>
<td>Ischemic, Primary Group</td>
</tr>
<tr>
<td>No Primary Antibody</td>
</tr>
</tbody>
</table>

Repetition is a useful way of increasing the percentage of success in scientific processes. Although some factors were changed between runs, many factors remained unchanged. Repeated processes became more robust because of the need for the scientist to improve their technique. The repetition of the technique did seem to yield better results; proportional to the number of times the process was performed.

**RESULTS**

Some success was achieved from the modified alpha smooth muscle actin IHC staining protocol. As listed in the table below, several staining outcomes were
possible. Often, either the sectioning process or staining itself lead to too much tissue loss to decipher the results of the stain. This issue was addressed in different ways throughout the trials. For example, less time was spent in the dehydration steps, and an air-dry step was added into the beginning of the stain. Also, sections were more carefully chosen, so that the tissue started out with more integrity. Additionally, extra care was taken when moving the slides vertically into or out of reagents. The best procedure for maintaining tissue integrity has been included in the optimized staining protocol (Appendix B).

Another common result of this study was “no distinction” between the control and experimental groups. In other words, the tissues on the slides looked about the same in shade and shade pattern, regardless of the presence or absence of the primary antibody. These results are poor because, as described previously, the chromagen should only have been developed where primary antibody had attached. When the same brown coloring occurred in groups that were treated with primary antibody and those that weren’t, the validity of the results in the experimental group were questionable. That is, the brown shaded hypoxia maps obtained from tissues treated with primary were probably not accurate, because the same hypoxia maps were produced without the primary antibody. This suggested that non-specific binding, or some factor other than hypoxia and the primary antibody, was playing a role in staining the tissue, which lead to false positives and incorrect maps.

On the other hand, “no distinction” could also indicate false negative staining. It was actually difficult to tell whether false positive or false negative staining had occurred if ischemic control and ischemic primary tissues stained the same, because
this study is based on relative shading (between primary and control groups).

Either way, if both the primary and control ischemic tissues came out the same, the results were not accurate. In the results table, below, this outcome is simply named, “no distinction”. Another form of “no distinction” was where, within primary antibody treated groups, there was no difference in shading of tissue between ischemic and non-ischemic tissues. This result probably did not reflect reality because tissues that underwent induced ischemia should certainly have been more hypoxic than tissues that did not. However, this distinction was absent in some results. This disconnect indicated that either the ischemic tissues were not stained in a way that properly represented their hypoxia, or that non-ischemic tissues were stained such that more hypoxia was represented than actually existed. Either way, the results were not reliable.

The more successful results are denoted in the results table as “distinction in one slide”, “distinction in two slides”, or “distinction in some slides”. In these cases, there were expected differences in shading between control groups and experimental groups. Again, positive staining was only expected in one of four scenarios: the tissue was ischemic and treated with primary antibody. When there was clearly more shading (either in area or depth) in tissues that met this criteria than tissues either without ischemia or without primary antibody, the stain was successful. Never were the results clear across all slides in the run (usually about eight slides total). As stated in the staining results table below, distinction either occurred in one slide or some slides, but never in all slides. Modification of the
injection and staining protocol and repetition of both techniques could increase the percentage of success (from about 15% now).

Below is a summary of each stain and its results. The results for stain 2 that read, "non-ischemic distinctions" refer to the specific issue of false positive staining in tissue that should not have great amounts of hypoxia, because the vessel in the hindlimb from which the tissue was not resected, and thus, ischemia was not induced.
<table>
<thead>
<tr>
<th>Stain</th>
<th>Surgery</th>
<th>Tissue</th>
<th>Protocol</th>
<th>Oven setting</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Calf</td>
<td>Appendix C</td>
<td>7</td>
<td></td>
<td>No Distinction.</td>
</tr>
<tr>
<td>2</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Distinction in some slides. Non-ischemic distinctions.</td>
</tr>
<tr>
<td>3a</td>
<td>Calf</td>
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<td>7</td>
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</tr>
<tr>
<td>3b</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
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</tr>
<tr>
<td>4a</td>
<td>Calf</td>
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<td>7</td>
<td></td>
<td>Too much tissue loss.</td>
</tr>
<tr>
<td>4b</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Too much tissue loss.</td>
</tr>
<tr>
<td>5a</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Too much tissue loss.</td>
</tr>
<tr>
<td>5b</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Too much tissue loss.</td>
</tr>
<tr>
<td>6</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Distinction in two slides.</td>
</tr>
<tr>
<td>7a</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Distinction in some slides.</td>
</tr>
<tr>
<td>7b</td>
<td>Gracilis</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>No distinction.</td>
</tr>
<tr>
<td>7c</td>
<td>Calf</td>
<td>Appendix B</td>
<td>7</td>
<td></td>
<td>Distinction in some slides.</td>
</tr>
<tr>
<td>8a</td>
<td>Calf</td>
<td>Appendix B</td>
<td>3</td>
<td></td>
<td>Distinction in one slide.</td>
</tr>
<tr>
<td>8b</td>
<td>Calf</td>
<td>Appendix B</td>
<td>3</td>
<td></td>
<td>No distinction.</td>
</tr>
</tbody>
</table>

Note: Oven setting refers to the oven in Bonderson room 209. Setting 7 correlates to over 100 °C, whereas setting usually results in 30-40 deg C.

The results listed in the table above were observed visually using a white-light microscope. The photomicrographs below show pairs of slides representing various outcomes. Only the same tissue was compared between control and experimental treatments, because the skeletal muscle architecture was so different from tissue to tissue. No adjustments to the lighting, white balance, or zoom were made on the microscope between the primary and control of each pair of photos. Thus, if there was a difference in appearance between a pair of the same tissue, it was due to the stain.
Successful Stains

Figure 1 shows successful results in non-ischemic tissue. There is no difference in shading between the control and primary groups in the whole tissue. This was expected because the tissue was non-ischemic, and should not have been hypoxic. Therefore, both control and primary groups should be about equally unshaded.

Figure 1a. Control

Figure 1b. Primary
Figure 2 also shows successful results in non-ischemic tissue. As seen in the zoomed image, there is very little difference in contrast between the control and primary groups, as expected for non-hypoxic tissue.
Figure 3 shows successful results in ischemic tissue. There is an obvious difference in overall shading between control and primary groups, correctly representing hypoxia in the primary group, as stained for using IHC. This shading seems not to be regional, but rather, evenly spread through all tissue areas of the experimental group. Although the tissue in figure 3a and 3b is the same, and should have similar hypoxia levels in its sections, hypoxia shouldn’t be depicted unless the tissue was treated with primary antibody.
Figure 4 features the same tissue sample as figure 3, however figure 4’s image is magnified. Again, it is clear that the tissue treated with primary antibody is generally darker than the control. Additionally, the increased contrast seems to be located evenly throughout the experimental tissue, not just in certain areas.
Figure 5 shows a more localized form of success in an ischemic tissue. Here, the tissue treated with primary antibody shows dark staining as compared to the control tissue in individual cells rather than in overall shade. This localized response may be closer to the expected hypoxic response of the tissue to ischemia. It is possible that ischemia does cause a locally variable hypoxic response.
Unsuccessful Stains

**Figure 6** shows no distinction between control and primary groups in an ischemic tissue. Because the tissue is ischemic, dark brown staining was expected for the primary group only, thereby creating a difference in staining between control and primary groups. The dark brown areas seen in the primary group are simply the result of tissue folding, not successful staining. Therefore, this stain was unsuccessful.
Figure 7 shows a magnified view of the tissue from figure 6. Again, this slide was unsuccessful because, unexpectedly, there is very little difference in shading between control and primary groups. Because there is no difference between groups, it cannot be determined whether false positive or false negative staining has occurred.
Figure 8 shows another unsuccessful stain in an ischemic tissue. Again, as seen from the image of the whole calf cross-section, there is very little difference in contrast between the control and primary treated tissue, even though the tissue is expected to be hypoxic. In fact, there seems to be slightly more staining in the control tissue than in the primary, which is opposite of the expected hypoxic reaction in these two tissues. The stain was unsuccessful because the primary treated tissue is not shaded more than the control tissue.
Figure 9 shows a magnified view of the ischemic tissue from figure 8. Here, it is clear that the control group’s shading is slightly more defined than is the experimental group’s. In this case, the control group may be falsely positive and the primary group may be falsely negative.
Figure 10 shows more contrast in the primary tissue than in the control tissue. This result would be expected had the tissue been ischemic, but the tissue is non-ischemic. Because of this, little hypoxia is expected, and therefore there would be little staining, regardless of whether the sample was treated with primary antibody or not. This result is unsuccessful at accurately displaying the location of hypoxia.
Figure 11 displays a magnified view of the tissue in figure 10. Although it is slight, the shading in the experimental group may be more defined than the shading in the control group. Again, this stain is unsuccessful. Both groups are expected to have little to no shading due to the lack of ischemia in the tissue.
Figure 12 shows a sample that has had too much tissue loss for staining analysis.
DISCUSSION

There were some successful results of this study, supporting the proof-of-concept for mapping hypoxia in mouse hindlimb skeletal muscle. Although there were some false positives in every batch, the validity of the successful slides is not undermined. However, instead of using successful slides as definitive victories, they must be looked at as possibilities of future success. Although it can’t be told if the successful-looking slides represent accurate maps of hypoxia in the tissue used, the more frequently these successful results occur, the more likely this is the case.

There were also unsuccessful slides. In general, “no distinction where there should have been”, and “distinction where there should not have been” were the two incorrect outcomes that occurred. These false positives or false negatives indicate either over-staining of non-hypoxic cells or under-staining of hypoxic cells. The incorrect stains could be the result of mistakes in technique or logic in injection, surgery, processing and embedding, sectioning, or staining.

The lack of staining where there should be (false negative) or the presence of staining where there should not have been (false positive) are both logical possibilities for results that have no distinction in tissue shading between control and experimental groups. However, it is difficult to distinguish which scenario has played out, because success was measured in this study by comparison between samples. Therefore, if there was no distinction between an ischemic control tissue and an ischemic experimental tissue, it was deducible that the run was unsuccessful, but it was hard to discern more than that. Postulations have been made, however,
as to what has caused some of the poor results. No distinction would be apparent
where expected if the surgery did not cause significant hypoxia. Many things could
go wrong with the injection, resection, and dissection technique and timeline,
resulting in an unnoticeable change in hypoxia levels. In this case, the negative
would not be false, but unexpected. Moreover, because the blocking serum bound to
and blocked open binding sites, if the tissue was treated with blocking serum out of
sequence, false positive staining would have occurred. Finally, underexposure to
DAB is a condition that could possibly lead to false negatives. DAB needed time to
develop into a brown hue, and if this time was not allowed, little or no staining may
have been seen. On the other hand, false positive results may have been caused by
non-specific binding of antibodies. The technique of immunohistochemistry
depends on specificity between antigens and antibodies. For example, if the binding
of primary antibody was not specific to pimonidazole, not only hypoxic cells would
be stained. Additionally, false positive staining could be caused by over-exposure to
DAB. Because both false positive and false negative staining occurred in most trials,
a combination of the issues mentioned probably occurred.

More successful slides per run and the limitation of false positive and false
negative slides are desirable goals. To do this, there are many avenues for protocol
modifications. It is the hope that one or a combination of some of the following
suggestions may help to increase the ratio of good slides to bad slides. The
suggestions for the future are as follows:
Table 4. Future Suggestions

<table>
<thead>
<tr>
<th>Suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alter the surgery timing to allow for better circulation of pimonidazole, more hypoxia, or better binding of pimonidazole to hypoxic cells</td>
</tr>
<tr>
<td>Alter the exposure time of the samples to the DAB chromagen</td>
</tr>
<tr>
<td>Use the staining protocol provided by Hypoxyprobe (Appendix C)</td>
</tr>
<tr>
<td>Keep the oven setting as low as 3 or 4 (so as not to damage the tissue)</td>
</tr>
<tr>
<td>Use other hindlimb tissues</td>
</tr>
<tr>
<td>Use more repetition and larger sample sizes to override technique problems</td>
</tr>
</tbody>
</table>

The successes achieved in this study have validated, in part, the developed protocol for hypoxia mapping in ischemic mouse hindlimb skeletal muscle. It is true that the protocol, which produces consistent true positive results, needs to be further developed, but that protocol could be largely based on the one used in this study. Much has been discovered about the nature of immunohistochemical staining in hypoxic mouse tissue, and these discoveries can be used to further perfect the hypoxia mapping technique.

The developed injection and staining technique can be used by the microcirculation lab to assess the presence of hypoxia near the muscular branch artery. Vascular reactivity measurements have already been, and continue to be, collected from this area, so adding the developed procedure for hypoxia mapping may help draw connections between regional oxygen levels and vascular function. Hypoxia's role in vascular reactivity, whether it be in vessel growth, or in vessel wall functionality, may be established with this or subsequent modifications of this protocol.
REFERENCES


Hypoxyprobe™. <hypoxyprobe.com> (2010).

Santa Cruz Biotechnology, Inc. <scbt.com> (2010).
APPENDIX

Appendix A: Injection Protocol

Injection Protocol
Interpreted by Emily Deckert, August, 2010

This protocol can be altered to achieve better results. What is written has not yet been verified to be the best injection method. Things to alter include timing, fixatives, and temperatures. Additionally, after the injection and staining protocol have been optimized for use in the lab as a method to map hypoxia, this injection protocol can be altered to work in conjunction with other studies involving the animal.

- **Timing:**
  - Injection
  - Resection
  - Stimulation
  - Dissection
  - Fixation

- **Injection:**
  - Intraperitoneal
  - Solution: 250 µL PBS, 1.8 mg pimonidazole per mouse
  - Hypoxyprobe injection at room temp, store in fridge, but do not freeze.

- **Resection:**
  - Following current lab protocol
  - Only resect one limb. The other will serve as a control.

- **Stimulation:**
  - Optional
  - Using the lab’s electrodes, stimulate the calf for 90 seconds: 8 Hz, 0.5 ms, 2 mA

- **Dissection:**
  - 15 to 90 minutes (or 1-2 hours) after injection (Hypoxyprobe will be “frozen” in place at the time of harvest).
  - Actual harvest time as quick as possible.

- **Fixation:**
  - Immersion fixation in formalin
  - Let sit in fixative over night at room temp
Appendix B: Staining Protocol

Immunohistochemical Hypoxia Staining Protocol
Emily Deckert, August 2010
Adapted from protocols from Chris Miracle and Santa Cruz Biotechnology

1. Incubate slides for 5 – 10 minutes in oven after sectioning
      i. 75 µL normal goat blocking serum stock
      ii. 5 mL PBS

2. Dip with Xylene – 10 min

3. Air dry – 30 sec

4. Dip with 100% ethanol – 2 min

5. Air dry – 30 sec

6. Dip with 95% ethanol – 1 min

7. Incubate slides (room temperature) with blocking serum – 1 hour*
      i. Pipette 30 µL Hypoxyprobe IgG mouse monoclonal antibody (with an antibody solution concentration of 70 µg/mL)
      ii. Pipette 970 µL blocking serum
      (The resulting antibody solution concentration is 2 µg/mL)
   b. Suction reagent (blocking serum) after 1 hour**
      i. Suction 1 slide at a time, proceed to step 8 and repeat for each slide

8. Incubate slides (room temperature) with primary antibody – 30 min

9. Wash with 3 changes of PBS – 5 min each
   a. During these washes, prepare secondary antibody. Mix in green bottle.
      i. 75 µL normal blocking serum stock
      ii. 5 mL PBS
iii. 25 µL secondary antibody stock

10. **Incubate slides (room temperature) with secondary antibody solution – 30 min**
   a. During this step, prepare AB enzyme reagent. Mix in purple bottle.
      i. 50 µL avidin
      ii. 50 µL biotinylated horse radish peroxidase (HRP)
      iii. 2.5 mL PBS

11. **Wash with 3 changes of PBS – 5 min each**

12. **Incubate slides (room temperature) with AB enzyme (having allowed the AB enzyme to sit for 30 minutes)– 30 min**

13. **Wash with 3 changes of PBS – 5 min each**
   a. During these washes, prepare peroxidase substrate. Mix in yellow bottle.
      i. 1.6 mL distilled water
      ii. 5 drops 10X substrate buffer
      iii. 1 drop 50X DAB chromogen
      iv. 1 drop 50X peroxidase substrate

14. **Incubate with peroxidase substrate – 5 min**

15. **Wash in distilled water – 4 min**

16. **95% ethanol – 10 sec**

17. **100% ethanol – 10 sec**

18. **Xylene - 25 sec**

19. Coverslip each slide individually and prior directly from xylene

*Note: Use a food container dish with a moist paper towel on the bottom to create a moist chamber environment for incubating both inside and outside of the hood for all incubation steps.

**Note: Use suction pump/vacuum to remove reagents one at a time between all incubation steps**
Appendix C: Alternate Staining Protocol

Immunohistochemical Hypoxia Staining Protocol (Alternate)
Interpreted by Emily Deckert, August, 2010
Provided by Hypoxyprobe Inc.

1. Warm paraffinized tissue section. 40°C, 20 min.
2. Dip and blot 10 times (slides held vertically): xylene, 2 min.
3. Dip and blot 10 times (slides held vertically): 100% ethanol, 2 min.
4. Dip and blot 10 times (slides held vertically): 95% aqueous ethanol, 2 min.
5. Dip and blot 10 times (slides held vertically): 80% aqueous ethanol, 2 min.
6. Dip and blot 10 times (slides held vertically): D.I. H₂O + 0.2% Brij 35, 2 min.
7. Dip and blot 10 times (slides held vertically): PBS + 0.2% Brij 35, 2 min.
8. Incubate (slides held vertically): 3% H₂O₂ in distilled water, 5 min.
9. Choose 1, 2, or 3:
   1) “Heat treatment (recommended method): Place slides in a container and cover with 10 mM sodium citrate buffer, pH 6.0; or with 50 mM glycine-HCl buffer (glycine: sc-29096), pH 3.5, with 0.01% (w/v) EDTA (EDTA: sc-29092). Heat at 95°C for 5 minutes. Top off with fresh buffer and heat at 95°C for 5 minutes (optimal incubation time may vary for each tissue type). Allow slides to cool in the buffer for approximately 20 minutes. Wash in deionized H₂O three times for 2 minutes each. Aspirate excess liquid from slides.”
   2) “Pepsin: Incubate sections for 10–20 minutes in 0.1% pepsin in 0.01 N HCl at room temperature. Wash slides several times in deionized H₂O. Aspirate excess liquid from slides.”
   3) “Saponin: Incubate sections for 30 minutes in 0.05% saponin in deionized H₂O at room temperature. Wash at least three times in PBS. Aspirate excess liquid from slides.”
   (Santa Cruz Biotechnology, 2010)
10. Wash: PBS + 0.2% Brij 35, 0°C, 2 min. Reagent magnetically stirred in a Coplin jar held on ice.
11. Incubate (slides held horizontally): Blocking solution, 5 min. (ABC Kit)
12. Incubate (slides held horizontally): 1/50 Primary Antibody (for Hypoxyprobe, see reagent directions), 40 min.
13. Wash (slides held horizontally): PBS + 0.2% Brij 35, 0°C, 2 min. Reagent magnetically stirred in a Coplin jar held on ice.
14. Incubate(slides held horizontally): 1/500 secondary antibody in 10 mM PBS, with 0.2% Brij 35 and 1 drop protein blocker /mL, 10 min. (ABC Kit)
15. Wash (slides held horizontally): PBS + 0.2% Brij 35, 0°C, 2 min. Reagent magnetically stirred in a Coplin jar held on ice.
16. Incubate (slides held horizontally): Peroxidase substrate, 10 min. (ABC Kit)
17. Wash (slides held horizontally): PBS + 0.2% Brij 35, 0°C, 2 min. Reagent magnetically stirred in a Coplin jar held on ice.
18. Incubate (slides held horizontally): Peroxidase chromagen (DAB), 10 min. (ABC Kit)

20. Incubate: Hematoxylin, 30 seconds.


22. Crystal/Mount, 20 min.

(Hypoxyprobe, 2010)

Purposes:
- 2-7: deparaffinization, hydration
- 8: quench tissue peroxidase
- 9: antigen retrieval
- 11: Block non-specific binding
- 16, 18: color
- 20: counterstain
- 22: mount and dry slides
## Appendix D: Staining Log

<table>
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<th>Tissue</th>
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Blocks denoted by an “(i)” contain ischemic tissues.
Appendix E: Hypoxyprobe™-1 Kit Information

Hypoxyprobe™-1 Kit

Kit contents:

Solid p-hydroxybenzoate HCL (Hypoxyprobe™-1)
Mouse IgG1 monoclonal antibody (MAb1)

Applications: Immunohistochemical detection of cell and tissue hypoxia including immunofluorescence, immunoperoxidase or flow cytometry. Use low background in mouse tissues when the Hypoxyprobe™-1 Kit is combined with an Peroxidase 2 antihistone secondary reagent.

Quantities: 1. Hypoxyprobe™-1 Plus Kits contain 100 mg, 200 mg or 1000 mg of p-hydroxybenzoate HCL. Typically a dosage of 50-100 ng body weight is used for animal studies.

2. 1.0 or 2.0 ml of an enhanced peroxidase-stained antibody, clone 4.3.11.3 containing 70 nanograms/ml of an IgG1 mouse monoclonal antibody and 0.15% sodium azide. Optimal dilution of MAb1 is to be determined by the investigator but a 1:50 dilution has been found to give strong immunostaining in mouse tumor tissue when combined with a peroxidase conjugated trastuzumab anti-human secondary reagent.

Not supplied: Standard reagents used for immunohistochemical analysis including the recommended Peroxidase 2 antihistone secondary reagent.

Storage: 1. Store Hypoxyprobe™-1 solid at room temperature or 2-8 degrees C in the dark.
2. Store MAb1 frozen.
Hypoxyprobe™-1 Kit components

Hypoxyprobe™-1 is a substituted 2-nitroimidazole whose chemical name and only ingredient is phenytoin hydrate. Hypoxyprobe™-1 has a molecular weight of 299.4, a water solubility of 400 millimolar equivalent to 116 mg/mL and ultraviolet absorbance at 324 nm with an extinction coefficient of 7600 at 0.9% saline. The free base, phynitroimidazole, has a molecular weight of 254.3, a pKa of 8.7 and an octanol-water partition coefficient of 8.5. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications for Hypoxyprobe™-1 kit.

Hypoxyprobe™-1 is chemically stable in both solid form and aqueous solution. For example, solid Hypoxyprobe-1 has been stored for two years at room temperature in subdued light without detectable degradation as assessed by UV and HPLC analyses. Hypoxyprobe™-1 solutions in 0.9% saline have been stored at a concentration of 100 pmol liter at -20°C for 4.5 years without detectable degradation (UV and HPLC analyses).

2) MAAB is a mouse IgG1 monoclonal antibody (MAb) clone 3.1.1.3.1 that binds to protein adducts of Hypoxyprobe™-1 in hypoxic cells. MAAB is supplied as a filter sterilized, endotoxin-free supernatant from hybridoma clone 3.1.1.3.1 containing 70 micrograms/mL of MAb and 0.15% sodium azide for added stability. MAAB primarily detects protein adducts of Hypoxyprobe™-1 in hypoxic tissue. Tissue of interest can be studied by immunohistochemistry of tissue fixed sections or formalin fixed paraffin embedded sections or by flow cytometry following tissue disaggregation. Typically, 100 microliters of a 1:50 dilution of MAAB is added to tissue sections. A chromogenic secondary anti-mouse (Fab')2 fragment is then applied to reveal Hypoxyprobe™-1 adducts. The peroxidase-conjugated anti-mouse (Fab')2 secondary protocol described below provides strong immunostaining with very low background in formalin fixed, paraffin embedded mouse tumor tissue.

Assay Instructions

1. Investigations of normal or tumor tissue hypoxia begin with the intravenous infusion, intraperitoneal injection or oral ingestion of a Hypoxyprobe™-1 solution at a dosage of 60 mg/kg body weight. For a 25 gram mouse this amounts to 1.5 mg mouse. Dosages up to 400 mg/kg have been used in mice

121 Middlesex Ave. - Burlington, MA 01803 USA - Tel 781-272-6588 Fax 781-272-9290 See www.natural-phenol.com
without detectable toxicity or change in tissue hypoxia but 60 mg/kg given i.v. immunostaining at a minimal cost. The solubility of Hypoxprobe™-1 in saline is 116 mg/mL so that very small volumes can be used to immunostain Hypoxprobe™-1.

Following injection or instillation, Hypoxprobe™-1 distributes to all tissues including brain but it forms adducts with thiol-containing proteins only in those cells that have a oxygen concentration less than 14 nanomolar — equivalent to a partial pressure pO₂ of 10 mm Hg at 37°C. In addition to tumors, normal tissues such as liver, kidney and skin possess cells at or below a pO₂ of 10 mm Hg. These normal tissues bind Hypoxprobe™-1.

The plasma half-life of Hypoxprobe™-1 in mice is approximately 25 minutes (see the FAQ link at www.hypoxprobe.com for references). For comparison, plasma half-lives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Normal tissues of interest may be harvested 15 to 90 minutes after Hypoxprobe™-1 administration. Hypoxprobe™-1 residual in tissues at the time of harvest will be bound when dissected tissues go unviable. However, the amount of residual Hypoxprobe™-1 is very small compared to the amount that tissues are exposed to during a 15 to 90 minute experiment so that any non-specific binding due to residual Hypoxprobe™-1 is undetectable.

In addition to animal studies, Hypoxprobe™-1 has been used for cells in tissue culture free Applications link at www.hypoxprobe.com. Typically, cell suspensions are incubated under hypoxia for 1 to 2 hours in the presence of 100 to 200 nanomolar Hypoxprobe™-1. The cells are harvested by cytopin, fixed and immunostained with MAb1 and a chromogenic or fluorescent secondary reagent.

121 Middlesex Ave., Burlington, MA - 01803 USA - Tel: 781 272 6868 1-800 781 272 6500 See www.hypoxprobe.com.
### PRODUCT INSERT

Recommended procedure for immunostaining using monoclonal antibodies in formalin-fixed, paraffin-embedded tissues using a Flash™ secondary strategy.


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**Technical Notes**

1. For step 11, 1X sodium citrate buffer pH 6.0 is a good choice; 1X sodium citrate buffer pH 5.0 is also available from all commercial manufacturers.

2. 1X sodium citrate buffer pH 6.0 is available from Dako Corporation (CA) or BioGenex Laboratories (CA) and is a good substitute for the original 1X sodium citrate buffer pH 6.0.

3. 1X sodium citrate buffer pH 5.0 is available from BioGenex Laboratories (CA) or Dako Corporation (CA).

4. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

5. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

6. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

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11. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

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13. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

14. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

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18. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

19. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

20. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

21. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.

22. 1X sodium citrate buffer pH 5.0 is a good substitute for the original 1X sodium citrate buffer pH 5.0.
PRODUCT INSERT

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For example, see fluorescent protein blocking from DAKO Corp. (a product of Agilent Technologies, CA) N0117.

1. Sodium bicarbonate is used to keep the pH of the sections neutral.

10. Paraformaldehyde, containing 5% in 10 mM PIPES containing 0.1% Brij 35 and 1 drop of DAKO protein blocker #1. Typically, 250 ml of a 10% paraformaldehyde solution is applied to each tissue section.

11. For example, allow the antibodies to diffuse into the antibody solution containing 2% Brij 35 and 1 drop of DAKO protein blocker #1.

Secondary antibody to other than the Fab3 approach can be used. For example, DAKO EnVision System Amplification for mouse antibodies (K80150) is used routinely in our laboratory for clinical samples.

12. Peroxidase conjugated goat anti-rabbit from DAKO (clone K400).

13. Liquid 5-aminofluorescein reagent (DIH from DAKO) # K30051

14. Nuclei washed in ultrapure water at R1 in a centrifuge.

15. Any commercially available peroxidase-conjugated reagent is suitable including a commercial universal Bioconjugate #2664.


Procedure for immunostaining hypoxia-prox1 adducts in frozen, fixed tissues.

Most of the published work reporting fluorescent immunochemistry detection of pimonidazole adducts is based on frozen sections and comes from Dr. A.J. van der Kogel's laboratory in Nijmegen. The tissue or tissue specimen is collected and directly frozen in liquid nitrogen and cryosectioned into 4 mm sections. Consecutive sections are cut at the largest circumference of the tissue. The sections are then stored at -80°C until stained. After thawing, the sections are fixed in cold acetone (4°C) for 10 minutes. The sections are rinsed and incubated overnight at 4°C with mouse monoclonal anti-pimonidazole antibody (clone 4.511.130/MA61) diluted 1:10 in PBS containing 0.1% bovine serum albumin and 0.1% Tween 20. The sections are then incubated for 90 min with Cy-3-conjugated goat anti-mouse antibody 1:150 (Jackson Immuno Research Laboratories). Between all steps of the staining procedure, the sections are rinsed three times with for 2 min in PBS.

121 Midlaser, Inc. Burlington, MA 01803 USA. Tel 781 272 4188 Fax 781 272 4500 See www.midlaser.com