

Update #6 from the Lab Team

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Hello young aspiring scientists!

I am happy to report that the Lab Team has been hard at work and is making tremendous progress. We've been busy practicing our stains on the mouse muscle cross-sections that we prepared last month and are now getting ready to start cryosectioning the Weddell seal tissue.

Over the last couple of weeks I have been learning three different staining protocols: Lead ATPase to locate vasculature, Oil-Red-O to test for intramuscular fat and immunohistochemistry to look for myoglobin. Both Oil-Red-O and immunohistochemistry have been described in detail by Jesse and Peter in previous updates. In this update, I will concentrate mainly on Lead ATPase, while briefly touching on my experience with Oil-Red-O and immunohistochemistry.

Lead ATPase Vasculature

Lead ATPase is a fairly straight forward protocol that works similarly to Oil-Red-O. The first part of the protocol involves preparing several solutions that are later combined to make an incubation medium. The incubation medium is used to catalyze or accelerate a desired reaction. In our case it will activate ATPases within the muscle, an enzyme that breaks down ATP to produce energy for the cell. Before the slides can be placed in incubation medium they have to be warmed to room temperature. Also, they have to be placed in a fixative to guarantee the integrity of the muscle while we take it through different treatments. The slides are then put into a coplin jar with the incubation solution for one hour. This is where all the reactions are happening. In order to see the effects of the stain on the muscle tissue, the

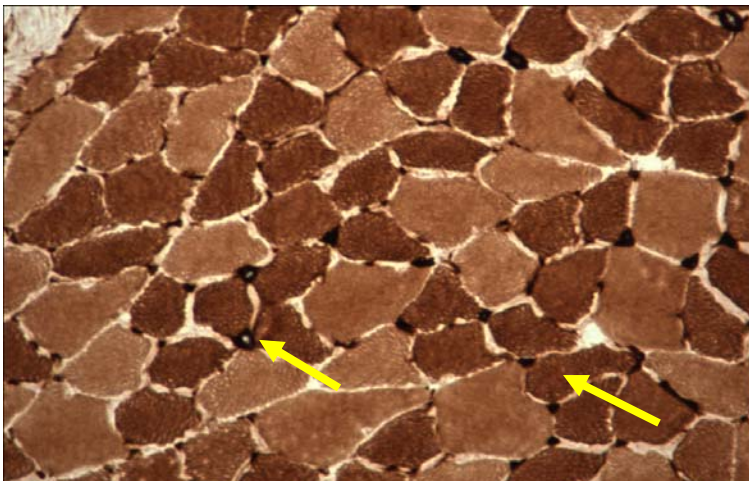


Figure 1. Mouse gastrocnemius cross-section stained with Lead ATPase. Capillaries are seen as small dark dots surrounding each muscle fibre. Some fibres have more surrounding vasculature due to an increased demand in oxygen supply.

slides have to be developed, much like in film photography. Developing takes no more than two minutes and it is possible to see a change in colour in seconds. *Figure 1* shows a mouse muscle stained with Lead ATPase.

As you know from our Update #3, vasculature extends throughout the entire body. It delivers oxygen and takes carbon dioxide away through tiny vessels called capillaries. Capillaries surround every tissue in our body in order to reduce the work tissues have to do to

exchange materials. This is easily seen in *Figure 1* as small dark circles around each

muscle fibre. Another, even more noticeable trend seen in *Figure 1* is the number of capillaries that surround the big pale fibres as opposed to the small dark fibres. We can hypothesize that the large pale fibres are Type II fibres and small dark fibres are Type I fibres, although we cannot state this with 100% certainty. This hypothesis is plausible because: (1) Type I fibres rely on a constant supply of oxygen to undergo cellular respiration and it is clear that the small fibres are surrounded by more capillaries than the large fibres and; (2) Type II fibres are generally larger than Type I fibres and don't depend on oxygen to produce ATP (*Figure 2*). This hypothesis can be proven or disproven with Jesse's Metachromatic stain, which tests specifically for fibre type.

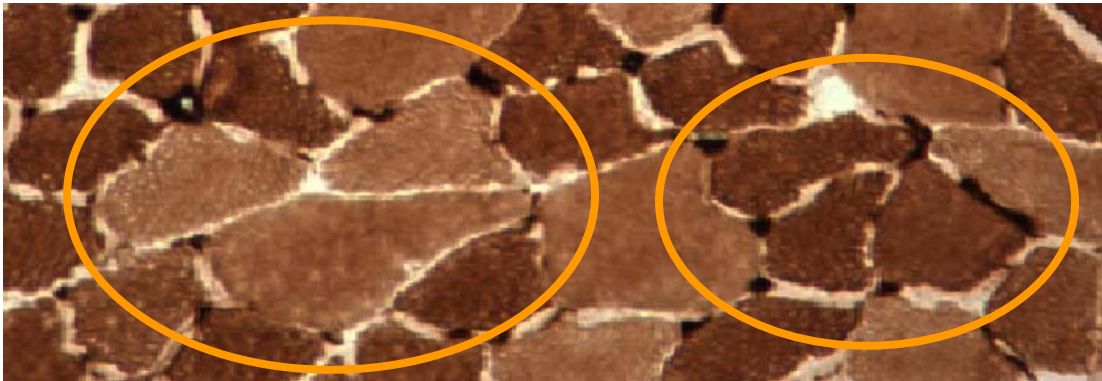
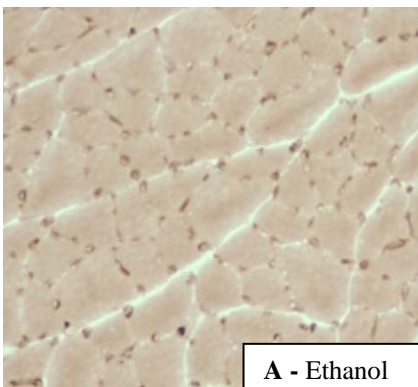


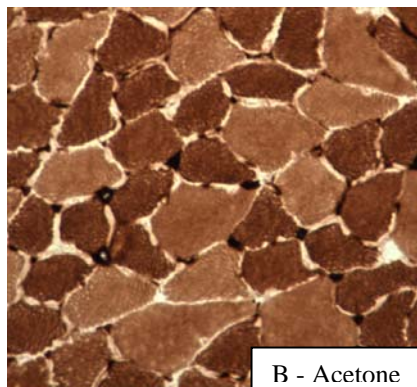
Figure 2. Mouse gastrocnemius cross-section stained with Lead ATPase. Vasculature can be seen in abundance around the small darker fibres and scarcely around the large pale fibres. This implies that the smaller fibres are more dependent on capillaries for proper functioning than the larger fibres.

A major part of the learning process is practice and trial-and-error. Everybody has heard of the phrase practice makes perfect, and as Peter mentioned earlier, it is just as true in the world of science as it is in life. When practicing a multi-step protocol, like Lead ATPase, there are many places where steps can be modified, whether intentionally or by mistake. These alterations can lead to unexpected discoveries that either aid the scientist in fine-tuning their techniques or, conversely, set them back.

When practicing Lead ATPase, Dr Hawke, suggested trying two different fixations on our muscle sections. One slide was fixed with acetone and the other in ethanol for two minutes before incubation. The rest of the experiment was carried out identically; therefore if differences did arise, we would know that they were caused by the difference in fixation.



A - Ethanol



B - Acetone

Figure 3. Lead ATPase with an (A) ethanol fixation and (B) acetone fixation.

Different fixatives can have a big impact on the outcome of an experiment. Researchers must always be aware of how various steps in their protocol are affecting their experiments.

Figure 3A and 3B clearly shows a different appearance of the same muscle. We can see that the ethanol fixation shows no colour change in different fibre types. The colour is less robust and the vasculature is clearly visible because of the contrast in colour. The acetone fixation, on the other hand, is very good at differentiating between different fibres, however, because of the darker colours, capillaries are somewhat harder to spot.

What Do You Think? Now that you know the pro's and con's of different fixatives, which would you pick for your experiments when looking for vasculature?

Oil-Red-O Intramuscular Fat

After this interesting discovery, Dr Hawke and the Lab Team decided to try a similar experiment with Oil-Red-O (for more information about Oil-Red-O refer to [Update #5](#)). One slide was fixed using acetone and the other was fixed using paraformaldehyde (PFA) for two minutes before submerging it into absolute propylene. The rest of the protocol was performed exactly the same

to control for other variables.

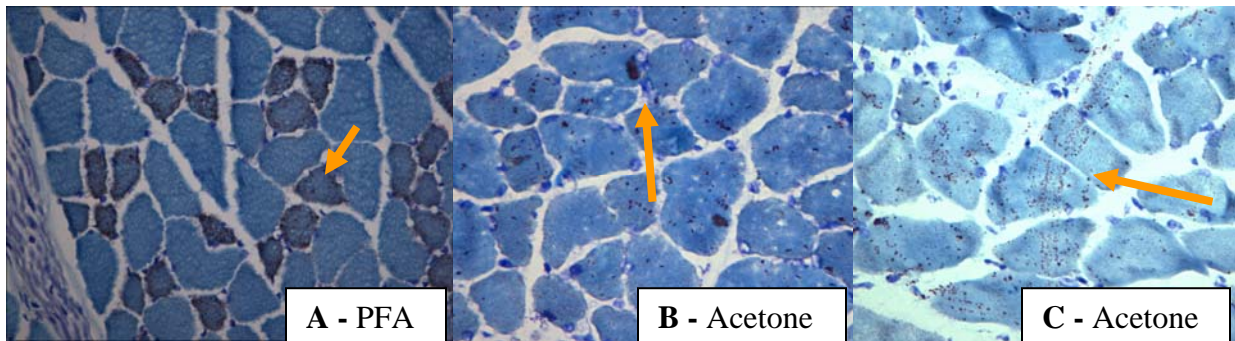


Figure 4. Oil-Red-O stain with (A) a paraformaldehyde fixation and (B,C) an acetone fixation. Fat droplets should appear as red dots confined in the muscle fibre, which can be seen with PFA fixation. Acetone fixation appears to show patchy red areas with some streaking, making it impossible to draw conclusions about the patterns of fat distribution.

Figure 4 is showing that both fixations were able to give a distinct red colour to the fat droplets. The PFA fixation in Figure 4A shows the red droplets are confined within the fibre and are consistently higher in number in the smaller fibres. This may signify that the smaller fibres are Type I, since they are more dependent on fat for ATP and energy production. This idea would also confirm our previous hypothesis taken from the Lead ATPase stain. However, as with the previous hypothesis, fibre type would have to be confirmed by Jesse with Metachromatic staining. Figure 4B and Figure 4C are Oil-Red-O stains performed with an acetone fixation. Figure 4B shows red droplets of fat within the muscle fibres as well as between the fibres. Figure 4C also shows staining between the fibres as well as staining in streaks. Both are showing a sporadic distribution of red droplets, and oversized droplets in certain locations while absolutely none in others.

It seems that fixing slides in acetone enhances Lead ATPase vasculature staining and produces inconclusive results in Oil-Red-O intramuscular fat staining. It is possible that

acetone fixation is making the muscle section 'stickier' helping colours bind easier, which is useful in some situations and not others.

Immunohistochemistry

Myoglobin

I have also been working with immunohistochemistry to look at myoglobin distribution within the muscle fibres. Immunohistochemistry is somewhat more complicated than Lead ATPase and Oil-Red-O,

with more factors to keep in mind. Thus far, I have had limited success. In *Figure 5* we can see that the myoglobin antibody has bound not only to muscle fibres, but also to other molecules within the cross-section. Ideally, the stain should be localized within the fibres. The red colour should predominantly be within the smaller fibres, since they rely on oxygen for energy production, while less abundant in the larger fibres. There should be no visible colour in surrounding and supporting tissue since myoglobin is only found within the muscle fibres.

To help narrow down the problem with myoglobin staining, I am going to perform a *positive control*. A positive control is an experiment that you are certain will give you a consistent perfect response. In the case of myoglobin, the heart would make a great positive control. The heart consists of muscle cells that are in constant need of oxygen to function. Even though heart muscle is very different from skeletal muscle (because it has the ability to contract spontaneously), it has a plethora of myoglobin.

Over the next few weeks I will make cryosections of the heart and test it for myoglobin. I will also practice my immunohistochemistry on dystrophin, because from Peter's experiments I know exactly what to look for. After getting the results of both stains Dr Hawke and I will make a decision on the next logical step for myoglobin. I will also continue practicing Oil-Red-O and Lead ATPase stains. When I feel confident that I can run my experiments without any unexpected surprises, I will try the stains on Weddell seal muscle sections. In the mean time Jesse, Peter and I will begin cryosectioning the pup, juvenile and adult seal tissue in preparation for the challenges to come.

If you have any questions comments or concerns don't hesitate to ask myself or the Lab Team. I looks forward to your answers to the challenge questions and your opinions about our experiments and progress.

Challenge Question:

It is clear that the Weddell seal undergoes physiological changes as it matures from a pup to a juvenile to an adult. Many of these changes have been discussed throughout the past few weeks.

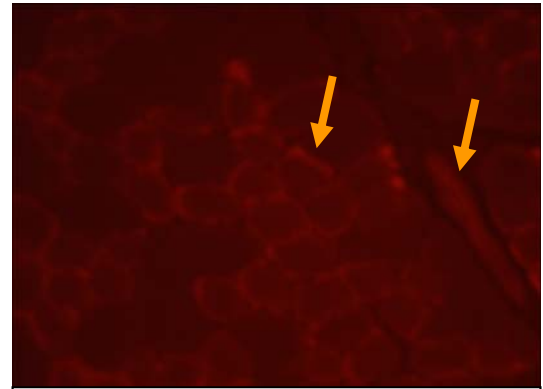


Figure 5. Immunohistochemistry for myoglobin.

Red fluorescence should be seen only within muscle fibres and predominately in Type I fibres with some colour seen in the Type II fibres. In this slide, the stain is seen in most fibres, localizing around the outside, as well as in the surrounding cells.

Difficulty level 1: Do you think these changes are caused by the ageing of the seal or by their change in swimming ability?

Difficulty level 2: What do you think myoglobin immunohistochemistry stain would look like on the heart tissue?

Difficulty level 3: How do you think the Weddell seal's daily diet effects these physiological adaptations?