

Update #5 from the Lab Team

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Greetings Polar Scientists!

It has been a busy week in the Hawke Lab at York University, and I am very excited to tell you about my experiences. Peter, Sophia, and I have been learning the staining and immunohistochemistry procedures that we will be using to study Weddell seal muscle. In this update, I will describe the two procedures that I will be using: *Oil-Red-O* and *Metachromatic* staining. As you might recall from previous updates, Oil-Red-O is used to identify intramuscular fat and Metachromatic staining is used to determine muscle fibre type.

When performing any experiment, it is important to make sure you have all the required equipment and solutions ready before starting. The first step in the



Figure 1

Hot Plate/Stir Plate

This device can be found in most laboratories. It is used to heat solutions to desired temperatures and mix solutions using a magnetic stir bar.

Oil-Red-O staining protocol involved making the Oil-Red-O solution. Sophia and I added the Oil-Red-O powder to propylene glycol, an oily liquid that is also known as propane-1,2-diol. After heating the solution on a hot plate (*Figure 1*) to dissolve all of the Oil-Red-O, we filtered the solution through a frittered glass filter. Frittered glass is a cotton-like material composed of small glass threads that can be used to filter out un-dissolved clumps of powder from the solution to ensure even staining of the muscle section. We placed the frittered glass in a plastic

funnel and poured our crude Oil-Red-O solution over the frittered glass. After the first filtration, we used a Nalgene Filter Unit (*Figure 2*) to further refine our solution. The Nalgene Filter Unit can be hooked up to an air pump, which pulls the solution in the upper compartment through a paper filter and into the container on the bottom. The top portion can then be discarded and the filtered Oil-Red-O solution can be stored in the bottom container. This way we don't have to remake the Oil-Red-O solution each time we want to perform the stain.

After filtering the solution twice, we were ready to prepare our slides for staining. Before any stain can be applied to a muscle section for analysis, the slides have to be taken out of the freezer and warmed to room temperature. We placed the slides in a coplin jar with paraformaldehyde (PFA).



Figure 2

Nalgene Filter Unit

Once it is hooked up to an air pump, the Nalgene Filter Unit can be used to filter out the impurities in a solution.

PFA preserves the integrity of muscle tissue by forming a bond between the



Figure 3

Coplin Jar

Slides are placed into slits in the coplin jar which hold them in place. When rinsing, the jar is put under a stream of distilled water. To reduce the direct impact of falling water, we placed a finger over the slides and allowed water to trickle in, thereby protecting the slides.

proteins in the muscle.(1) This prevents the tissue from decaying and ensures that the physiology of the muscle tissue is maintained. After fixing the muscle tissue with PFA, the slides were rinsed with distilled water. It is important to rinse the slides thoroughly to get rid of any excess fixative. To rinse the slides, we held the coplin jar under a stream of distilled water, ensuring that the stream hit our index finger first and then trickled into the jar, rather than the stream contacting the slides directly (Figure 3). If we allowed the stream of water to strike the slides directly, we would run the risk of the muscle sections being pulled off the slide.

After the slides were rinsed, we submerged them in absolute propylene glycol. Propylene glycol is a *hydrophobic* liquid, which means it will not mix with water. Placing our rinsed slides into this oily substance would help to repel any water molecules that were left over from the rinsing. This is important because our Oil-Red-O solution is hydrophobic and will be unable to bind to the muscle section if water is present.

Try It Yourself: Get a clear glass of water and add a tablespoon of vegetable oil. What happens when you stir your solution?

After leaving the slides in propylene glycol for at least 5 minutes, we removed the slides and wiped off the excess solution around the muscle section. We added the Oil-Red-O solution directly on top of the muscle cross-section. The slides were then placed in the humidifier and were left in an incubator overnight at 37°C. Incubating the slides ensures that the muscle tissue is stained thoroughly.

Some protocols recommend incubating the slides for shorter



Figure 4

Incubation chamber

This chamber serves a dual purpose of (1) maintaining desired temperatures and (2) shaking solutions over long periods of time.

periods of time. A big part of science is trial and error and seeing what works best for *your* experiment. If a certain incubation time gives inadequate results, then the logical step would be to increase or decrease the incubation time to see if the results can be improved. Thus, it is extremely important to record all the steps that you performed during an experiment so your results can later be replicated. As well, it is important for troubleshooting to know exactly what you have done so you know what to adjust to get a better outcome.

When Sophia and I fixed the muscle sections using PFA, we also attempted to fix the cells using acetone. However, when we looked at the slides that were fixed with acetone, we found that the Oil-Red-O did not bind specifically to the intramuscular fat and left streaks of Oil-Red-O stain throughout the section. Thus, from now on we will use PFA as a fixative rather than acetone.

After the 21 hour incubation period, we removed the slides from the humidifier and removed any excess stain that was still on the slides. We then thoroughly rinsed the slides in distilled water and applied a drop of Toluidine Blue. Toluidine Blue is used to define the muscle fibres and to give a background for the Oil-Red-O stain. After another thorough rinse, we air-dried the slides to remove any water droplets before mounting them with permount. If the slides were not completely dried before we applied the cover slip, humidity from the left-over water would cause our image to look cloudy under the microscope.

As you know, Oil-Red-O is used to identify intramuscular fat. The fat is stained with a red colour and appears as tiny droplets scattered throughout the muscle fibre. As you can see in *Figure 5*, the smaller muscle fibres, which are Type I fibres, have more red droplets due to the higher amount of intramuscular fat. The Type II fibres have less intramuscular fat, so they don't have as many red droplets throughout the fibre.

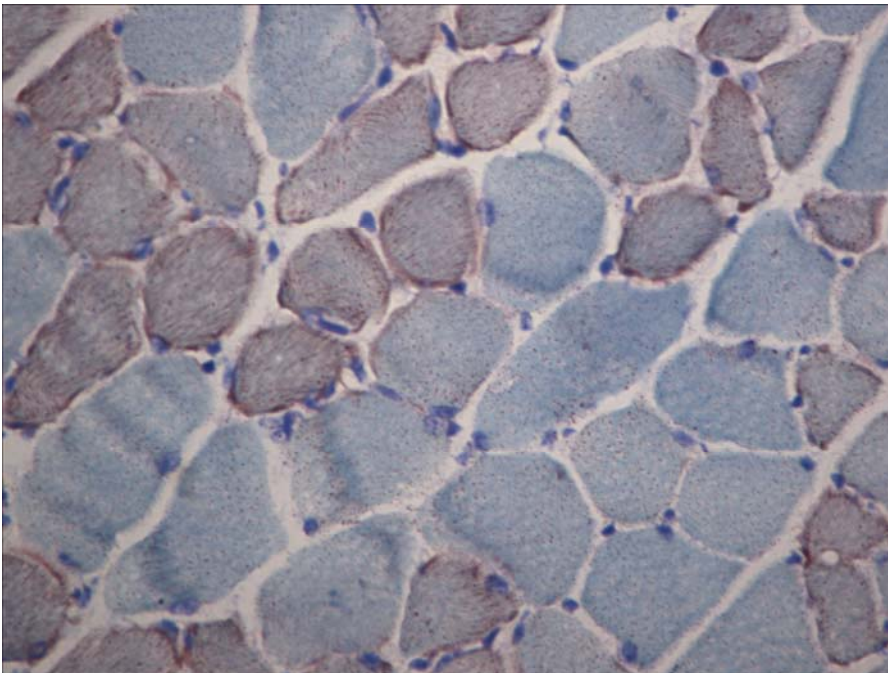


Figure 5
Mouse gastrocnemius muscle stained with Oil-Red-O as seen at 20x Magnification. Fat droplets are seen as red against the blue background of the fibres. There is a clear difference in the amount of fat stored in various fibres. However, additional staining would need to be done to confirm fibre type classification.

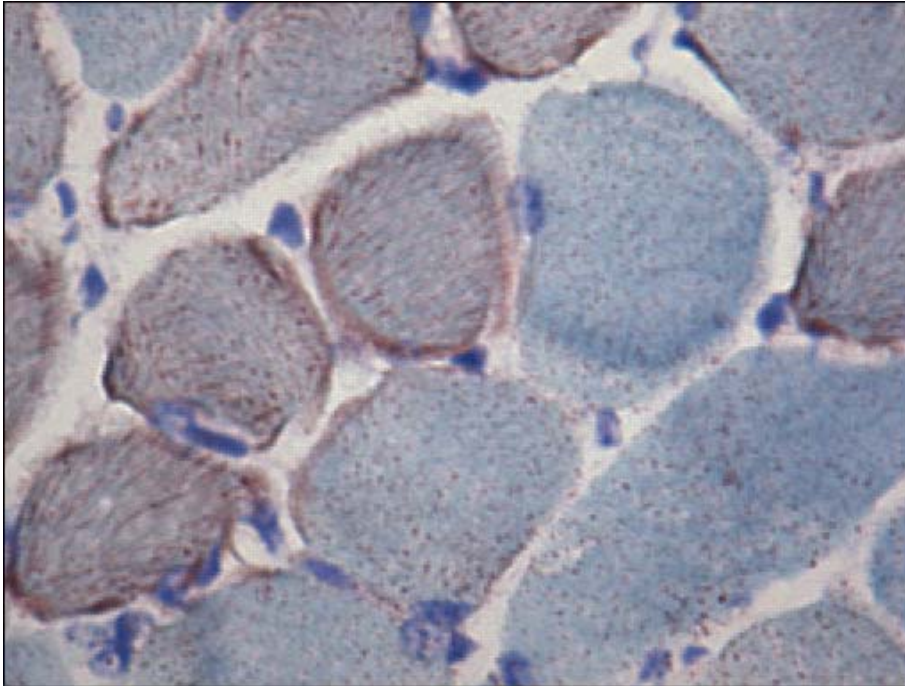


Figure 6
Mouse gastrocnemius muscle stained with Oil-Red-O as seen at 40x Magnification. Note the lipid droplets (little red drops) and how they are different between muscle fibres within the same muscle.

Figure 6 shows the same mouse gastrocnemius muscle under a higher magnification. Notice the difference in the amount of fat stored in the different muscle fibres.

Once I graduate to work with seal muscle, I will be studying the change in the distribution of intramuscular fat that occurs as Weddell seals develop from pups to juveniles to adults. As Sophia explained in her earlier update, there is a change in intramuscular fat as the Weddell seal grows to adulthood. The increase seen in the adult provides a larger reserve of energy and may be contributing to the adult seals' greater ability to dive under water for longer periods of time than the younger seals.

Challenge Question: Based on the physiology of muscle fibre types discussed in [my last update](#), why would slow twitch muscle fibres have more intramuscular fat than fast twitch fibres?

In addition to learning the Oil-Red-O staining procedure, I also learned Metachromatic staining. Before I could begin doing Metachromatic staining, I made five solutions from scratch. The five solutions were 1) Pre-Incubation Medium, 2) Tris Rinse, 3) Incubation Medium, 4) Calcium Chloride Rinse, and 5) Toluidine Blue Stain. The first three solutions required very specific pH levels in order for the staining protocol to work properly. pH is a measure of the acidity of a solution. Many physiological processes can only function within a small range of pH values. The pH scale is a numerical scale from 1 to 14, with 1 being most acidic and 14 being most basic.

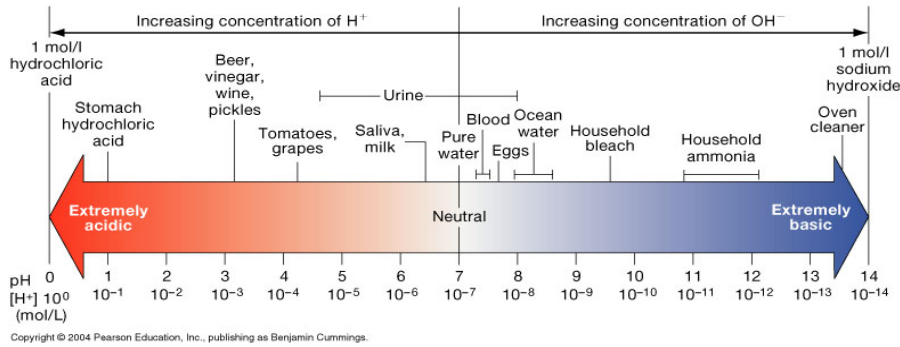


Figure 7
The pH Scale

The first step in Metachromatic staining procedure involved allowing the slides to air dry and then immersing the microscope slides in a series of incubation mediums and rinses. The purpose of the incubation medium was to maintain the physiology of the muscle section and promote the staining of the tissue. Once the incubation medium was thoroughly rinsed from the tissue, I stained the muscle sections with Toluidine Blue. After cleaning off the excess Toluidine Blue, I dehydrated the muscle sections with ethanol. Since the five solutions were aqueous solutions, it was necessary to dehydrate the sections to remove any excess moisture before mounting the cover slip with permount. Any excess moisture can make it difficult to see our muscle sections clearly using the microscope. Directly before the slides were mounted, I cleaned the slides in a cleaning agent called CitriSolv. CitriSolv is a natural, non-toxic compound that cleans the microscope slide and removes anything that may interfere with our image of the muscle section. It was extremely important to rinse the slides numerous times throughout this procedure to ensure that any excess solutions were removed from the slide. If we did not rinse well enough, the staining of the muscle sections would not be specific and we would see random blue areas throughout the tissue.

Metachromatic staining is used to identify Type I, Type IIa, and Type IIx muscle fibres. Type I fibres will stain with a dark blue colour, Type IIx fibres will stain a light blue colour, and Type IIa fibres will appear as an intermediate blue color. This staining procedure is based on the different ATPase enzymes found in the three muscle fibre types. ATPases are enzymes which break down ATP molecules, releasing energy that can be used to power chemical reactions that occur in our body. When I begin working with the Weddell seal tissue, I will be investigating the change in fibre type that occurs as the seal matures. I hypothesize that as the seal grows from a pup to a juvenile and then to an adult, there will be an increase in Type I fibres. Since Type I fibres are more resistant to fatigue, a greater amount of these fibres should contribute to the adult seals improved ability to dive for long periods of time. *Figure 8* is a mouse gastrocnemius muscle section stained with Metachromatic staining procedure. *Figure 9* is a more magnified image of a mouse gastrocnemius muscle section. The smaller fibres are stained with a darker blue colour, which confirms that these are Type I muscle fibres. The Type IIx fibres are larger, and thus can produce more force than Type I fibres. Most of the tiny blue shapes in between

the muscle fibres are the nuclei of the muscle fibres, while others may be satellite cells or other types of cells. This could be confirmed with immunohistochemistry.

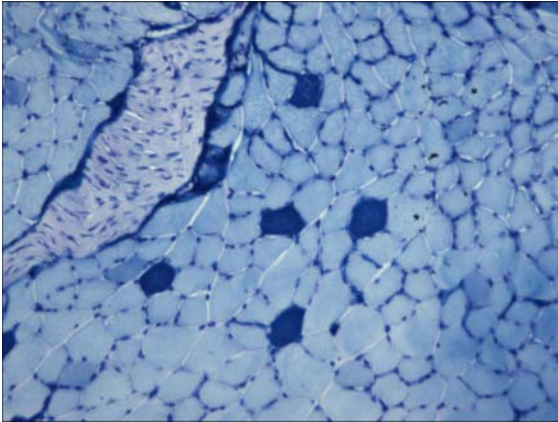


Figure 8

Metachromatic Stain at 10x Magnification
The dark blue fibres are slow twitch fibres and the light blue areas are fast twitch fibres.

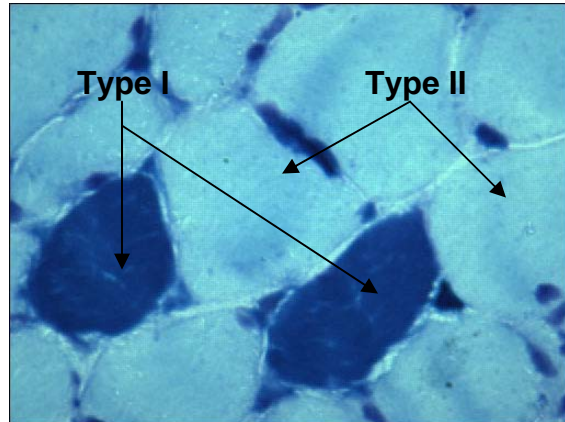


Figure 9

Metachromatic Stain at 40x Magnification
Notice the difference in size between the smaller Type I fibres and the larger Type II fibres. The little blue dots throughout the muscle are primarily muscle nuclei.

Over the next few weeks I will continue to refine my skills with Oil-Red-O and Metachromatic staining using mouse muscle tissue. As well, I will hopefully be graduating on to working with the Weddell seal muscle. The amount of muscle we have to work with from the Weddell seals is a lot smaller than the mouse tissue, so it will be a nice challenge to take 8 μm sections. My next update should include some more pictures for you, including Weddell seal tissue that has been stained with Oil-Red-O and Metachromatic. Until then, I look forward to reading about *your* work in your team blogs. Keep up the good work Polar Scientists and keep the questions coming!

Figure References

Figure 2: Lab Safety Supply. *Nalgene Filter Unit*. Retrieved Friday November 3, 2006

from https://www.labsafety.com/store/Lab_Supplies_and_Equipment/Lab_Supplies/Filtration/9907/

Figure 7: Pima Community College. *pH Scale*. Retrieved Friday November 3, 2006 from <http://drc.pima.edu/~biology/160alpha/lesson2/lesson2d.htm>

References

- 1) Lanier LL, Warner NL. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. *J Immunol Methods*. 1981;47(1):25-30.