Tissue Who? — A Foray into Histology and Tissue Identification

Mysterious Figure #180165120

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1 Introduction

Histology leverages techniques like microscopy and tissue staining to glean insight into the structure and function of tissues. It serves as a tool for both scientific discovery and clinical diagnosis [1, 2].

Depending on the techniques used, histology can reveal a lot more than just the overall structure of a tissue. Specialized staining techniques can reveal things like localized enzyme activity, the presence of certain classes of chemicals, and even the localization of particular antigens and proteins. This sort of information is critical to not only understanding the organisation of tissues, but also in diagnosing the pathologies of real patients.

Even traditional techniques can reveal the internals of individual cells and capture processes like mitosis. The level of detail that can be obtained in histology skyrockets even farther with the introduction of electron microscopy techniques which allow for imaging detail smaller than the wavelength of visible light.

In most applications of histology, there are three steps taken between the operating table and the microscope: *Fixation, Sectioning,* and *Staining.*

1.1 Fixation

During fixation, the tissue sample is protected from degradation via autolysis and / or the embedding process [3, 4]. While the aim of fixation is to preserve the tissue and change it as little as possible, in practice, every fixation method does some sort of physical or chemical damage to the tissue.

Alcohol-based fixatives, like methanol, might be great for immunohistochemistry, (because precipitation preserves many of the antigen epitopes that cross-linking doesn't) but they have the side effect of denaturing many proteins and washing away small molecules and lipids that could have been valuable in traditional histochemical or empirical methods [1, 3, 5]. Similarly, an acid-based fixative might be good for simultaneously demineralising and fixing bone samples (via coagulation), but leads to the hydrolysis of nucleic acids and lysis of red blood cells [6].

While specific applications may benefit from these alternative fixing methods, in most applications, formaldehyde / formalin (which forms cross-links without denaturing the proteins) has become the "gold standard" and preserves tissue in the most life-like form possible [4]. Do note that formaldehyde and formalin are not *entirely* synonymous — formalin is buffered 40% formaldehyde solution that has been preserved with 10% methanol [5, 6].

As an alternative to chemical fixation, it is possible to flash-freeze a sample using either liquid nitrogen or a dry-ice slurry. This has the advantage of preserving nearly all of the molecules within a cell (enzymes, antigen epitopes, nucleic acids, etc.) but if the sample is too large or frozen too slowly, poor morphology can result from the formation of ice crystals [7]. This damage can be mitigated through the use of cryoprotectants like sucrose and glycerol which disrupt the polar interactions between water molecules [8, 9].

1.2 Embedding & Sectioning

After fixation, the tissue sample must be sliced thinly enough to be examined under a microscope (conventionally 5–8 µm thick) — this is done using a specialized cutting tool called a *microtome.* As a general rule, the thinner the sample, the better (as it leads to greater resolution and light transmission). However, before the sample can be sectioned, it must be made solid enough to be cut by the microtome (otherwise it would just be sheared and damaged by the cutting process). This usually involves *embedding* the sample in another solid substance or simply freezing it [1].

Paraffin wax embedding is a cheap and simple method for hardening a tissue sample. To allow the hydrophobic wax to impregnate the sample, it must first be completely dehydrated via an alcohol series and cleared with an organic solvent like xylene [1]. The tissue is then submerged in molten paraffin and, once the wax has full permeated the tissue, left to cool. The embedded sample can then be loaded into a standard microtome and cut into sections between 2–7 µm thick [1]. While this method is easy to automate and can produce relatively thin sections, it involves the use of many harsh solvents and can damage the tissue and wash away lipids.

Alternatively, after dehydrating the tissue, the tissue can be impregnated with an acrylic or epoxy resin (as opposed to paraffin). This resin embedding sets much harder than paraffin and allows much thinner sections to be cut (1–2 µm thick for acrylic and 0.5–1 µm thick for epoxy resins) [1]. While these thinner sections can greatly increase resolution and epoxy can even enable *Transmission Electron Microscopy* (TEM), special glass or diamond bladed microtomes are required to cut them. Additionally, epoxy embedded samples are impenetrable to most common stains and the will either need to be dissolved using something like an alcohol-hydroxide solution or stained with a special stain like Toluidine Blue (which is capable of penetrating the epoxy) [1, 10].

In lieu of embedding the sample in resin or wax, it's also possible to make frozen sections using the same flash-

freezing techniques (or a more gradual freezing if the cells have been cryoprotected) as before in a refrigerated microtome called a *cryostat*. Tissue samples are placed on "chucks" in the cryostat and covered in the glycerol-based OCT (optimal cutting temperature) compound. The chucks are then cooled by the cryostat and once the OCT has frozen, the sample is sectioned in the cryostat in the same way a paraffin embedded sample would be. While this method often leads to the thickest sections (5–10 µm thick) it preserves the most chemical detail (great for the various histochemistries) and if paired with frozen fixation methods, it's possible to go from biopsy to analysis under the microscope within 5 minutes. [1, 8]

1.3 Staining

The final and most important step of preparing a histological sample for examination is staining. Once a fixed sample has been sectioned and made only a few micrometers thin, its almost entirely transparent. In order to actually see any of the structures that have been isolated, it's necessary to stain the tissue with either a dye, fluorescent molecule, or some other visible marker. Different dyes have different chemical properties which lead them to stain different cellular components in different ways — ultimately revealing some sort of overarching structure.

See the Histological Staining section for a continued discussion of histological dyes.

1.4 Experimental Aim

The aim of this experiment was to take three unstained tissue sections of an unknown origin, stain them using both the H&E and PAS protocols, and finally examine the slides under a microscope to determine which tissue classification each sample belonged to.

2 Materials & Methods

The samples were fixed in 4% formaldehyde and subsequently cryoprotected in a 15% sucrose solution before being frozen in OCT and sectioned on a cryostat. The frozen sections had already been mounted onto 6 slides (two of each tissue type) when our team received them.

2.1 H&E Staining

In preparation for staining, 3 of the slides (one of each tissue type) were loaded onto a rack and washed for 5 minutes in a bath of distilled water (dH_2O) to dissolve the OCT freezing medium. Once the freezing medium was removed, the samples were transferred into a second bath to be stained in Harris modified Haematoxylin for 1 minute. The slide-rack was then transferred to a basin and gently rinsed with tap water for 4 minutes (until the stained samples appeared blue). The samples were then counterstained in a bath of 1% Eosin Y solution for 5 minutes. Once counterstained, the samples were quickly immersed (for 2 seconds) in a bath of distilled water before being dehydrated in the alcohol (methylated spirit) series — 2 seconds in 70%, another 2 seconds in 90%, and finally 1 minute in the 100% alcohol solution (all percentages are weight to volume). Finally, the samples were transferred to a fume cupboard and cleared in a bath of filtered xylene for 1 minute. After clearing, coverslips were mounted on the slides using the xylene-based DPX mountant.

2.2 PAS Staining

The remaining 3 unstained slides were loaded onto a rack and washed for 5 minutes in (dH_2O) before being transferred to a bath of 0.5% periodic acid for 10 minutes. Once oxidised by the acid, the samples were washed for 1 minute in (dH_2O) and stained for 15 minutes in Stiff reagent. Once stained, the samples were transferred to a basin and rinsed with running tap water until the magenta colouration could be clearly seen (5 minutes). The samples were then counterstained in Haematoxylin for 30 seconds and then rinsed, as before, under running tap water for 5 minutes. Finally the slides were swiftly immersed in (dH_2O) for 2 seconds before being dehydrated, cleared, and mounted as described previously.

2.3 Microscopy & Imaging

Two weeks after staining, the slides were collected and examined under the Motic BA310 microscope. For imaging, the microscope was outfitted with the Moticam 3.0 and the software Motic Images Plus 2.0 was used. Prior to imaging, the image scale was calibrated using a set of known-size dots and their respective magnifications. When switching between objectives on the microscope, the scale factor in the software was updated appropriately. In total, 12 images were captured — there were 3 tissue types, an H&E and PAS stained version of each, and every slide was imaged at both a high and low magnification. From these 12 images, 4 were selected (all of the same tissue type) to undergo further analysis.

3 Results

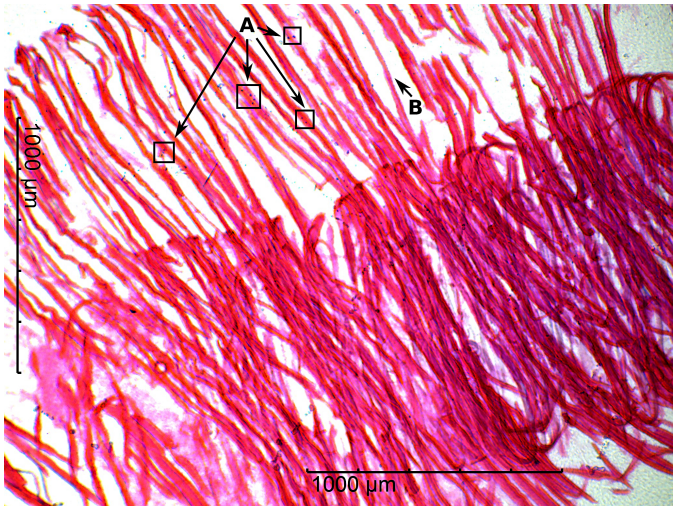
The tissue section shown in figure 1a clearly shows fibres running longitudinally across the slide (their cytoplasm stained red by the Eosin). The fibres appear to be comprised of cells that have fused together and left no visible division between them.

Also visible in figure 1a are the nuclei of these cellular fibres (stained blue / purple by the Haematoxylin). While they are quite small at the 5X objective, several nuclei can be seen in any given fibre indicating the multinucleate nature of the cells.

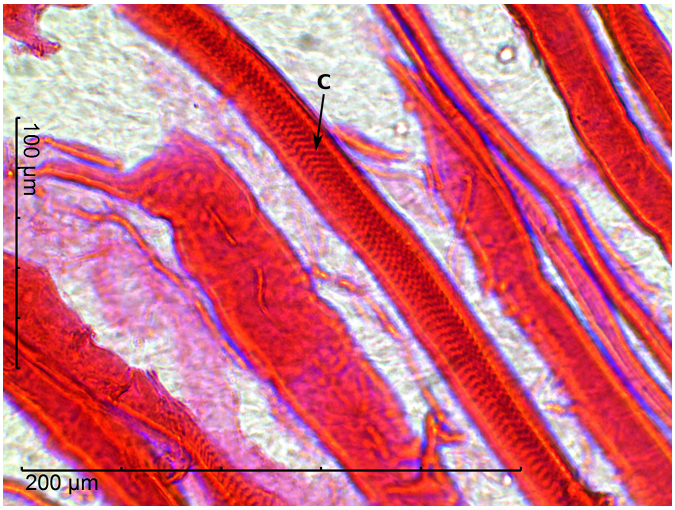
When the same slide is viewed under the 50X objective, as in figure 1b, striations can be clearly seen within each of the fibres as alternating bands of light and dark red (the colouration, again, a result of the Eosin staining).

Moving on to the PAS stained samples, figure 1c shows more fibre-shaped cells, but with much less definition than the H&E stain. Many of the fibres appear to be broken or torn, seemingly leaking the PAS stain, but others remain continuous throughout the whole frame.

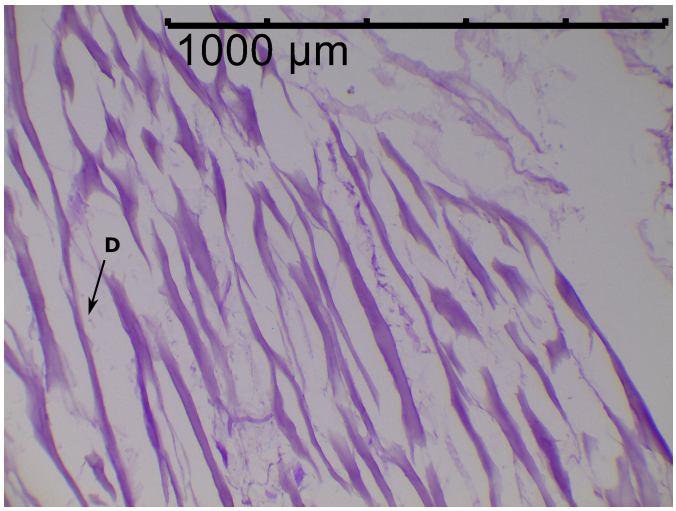
Finally, taking a closer look at figure 1d, striations can again be seen, though they are of much lower contrast than the ones produced by the H&E stain in figure 1b.



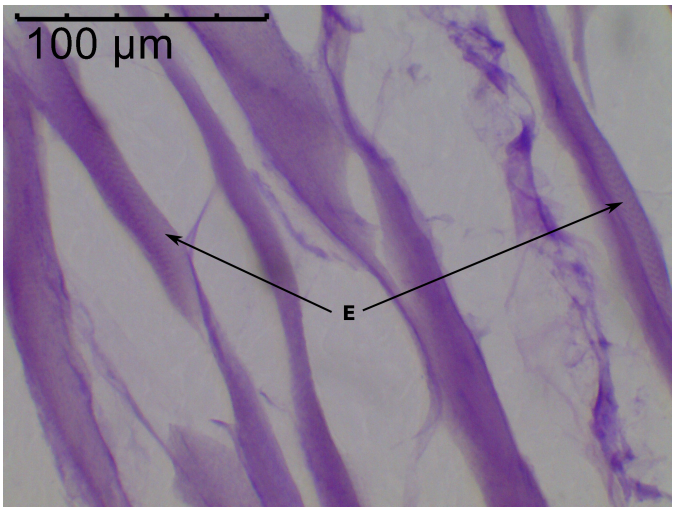
(a) H&E Stain at 5X magnification



(b) H&E Stain at 50X magnification



(c) PAS Stain at 10X magnification



(d) PAS Stain at 50X magnification

Figure 1: **(A)** Highlighted are clusters of nuclei (blue / purple) that appear in close proximity to each other as an example of multinucleation. **(B)** An example of the fibrous nature of the tissue sample. **(C)** Indicated is a rather strong striation pattern. **(D)** In the PAS stain, sugars in the cell fibres are being stained purple. **(E)** Faintly shown is another striation pattern present on the fibres, this time in the PAS stained sample.

4 Discussion

4.1 Interpretation of Results

After piecing together the histological clues, it seems safe to conclude that the tissue examined within the Results section is *skeletal muscle*.

The first clue is the fibrous nature of the myocytes. This was made easy to see by the longitudinal nature of the section and long cylindrical cells. These incredibly long cells are the result of many smaller myoblasts (muscle forming cells) fusing during myogenesis [11].

The second clue is the multinucleate nature of the tissue observed. Another consequence of myogenesis is that each myoblast's nucleus is retained within the final muscle fibre. This means that skeletal muscle cells end up having many nuclei located near the outside of the fibre. [11, 12].

As to why skeletal muscle would light up on the PAS stain, the answer is likely to be glycogen. Muscles store much of their energy as the highly-branched carbohydrate glycogen and play an important part in its regulation [13]. The histochemical nature of the PAS stain means that the presence of complex carbohydrates (like glycogen) in the cells is shown as a purple colour [1]. It's therefore more than likely that the purple staining of the muscle fibres is a result of their glycogen content.

Finally, the giveaway was the striation visible at higher magnifications. Skeletal muscle appears striated as a result of its repeated contractile units (called sarcomeres) which contain a dark A-band and a lighter I-band [12]. It's the alternation of I-bands and A-bands that produces the signature stripes of skeletal muscle.

4.2 Histological Staining

While there are countless histological stains in use today, for everything from lighting up a particular molecule to colouring in the entire structure of the tissue, they can all be placed into one of four categories: *Empirical, Histochemical, Enzyme Histochemical, and Immunohistochemical* [1].

4.2.1 Empirical

Empirical stains have been around for the longest time and were discovered mostly via trial and error. These dyes stain quite generally (a wide range of structures) and what gets stained is believed to be dependant on the size of the dye molecules and non-specific chemical interactions (like electrostatic attraction) [1]. The ubiquitous Haematoxylin & Eosin (H&E) stain used in this lab belongs to this category.

4.2.2 Histochemical

While empirical stains stain a wide variety of structures, histochemical stains are designed to stain specific classes of molecules. Once again in contrast to empirical stains, histochemical stains have a well understood chemistry. The Periodic Acid Schiff (PAS) stain, for example, first oxidises the carbon bond between two hydroxyl groups in a hexose sugar, then reacts with the formerly colourless Schiff reagent to tag carbohydrates with a magenta colouration. [1, 14]

4.2.3 Enzyme Histochemical

Unlike the previous two classes of stains, this staining method isn't designed to simply visualise the structures already present in the sample, this method can be used to test enzymatic activity. This stain directly connects biochemistry and morphology by incubating the sample with a substrate and then adding a visualizing agent to stain the *product* that is produced [1, 2]. Because the enzymes are responsible for forming the product (which is what shows up on the stain) this reveals not only where a particular enzyme is located, but also the degree to which it is functional. This technique has been used to diagnose conditions like Hirschsprung disease from live patient biopsies [2].

4.2.4 Immunohistochemical

Immunohistochemical staining is one of the most modern and powerful approaches to histological staining and allows for the detection of anything that has an antibody designed for it (a rapidly growing proportion of molecules) [1]. This method relies on antibodies being tagged with either chromatic of fluorescent markers (enabling advanced techniques like immunofluorescence) and makes it possible to locate very specific molecules throughout the tissue even when they don't exhibit enzymatic activity [15].

4.3 Applications & Limitations

As mentioned previously, histology is an important tool in both research and clinical settings. In research, histology is used to piece together the structure of different tissues and connect that to their chemistry and function. Here, lengthy fixations and thin, perhaps acrylic embedded samples are worthy trade-offs for increased resolution. In a clinical setting, however, when the patient is on the operating table, time is of the essence and cryogenic techniques enable a rapid response time. From anatomy to pathology, histology comes in handy.

With that said, there are a number of limitations that are difficult to overcome. For example, histology relies on the preparation and examination of extraordinarily thin tissue samples. This introduces the problem of dimension reduction (going from 3D tissue to a 2D section) and means that it can often be very difficult to piece together a complete, three dimensional picture from only histological sections. Additionally, histology is still a messy and empirical process. Different tissue samples will doubtlessly need to be prepared in slightly different ways (fixed for X amount of time, stained for Y minutes, etc) and many of the required steps damage the tissue in different ways. This means changing the staining step often necessitates the rewriting of the entire procedure so that the structures of interest are preserved. Finally, histology will always be somewhat limited by microscope technology. There is an upper limit to the power of optical microscopes (the wavelength of visible light) and even electron microscopes, while capable of higher magnifications, lose often-enlightening colour information.

5 Conclusions

While one can be reasonably confident that the tissue sample examined in the Results section was skeletal muscle, those are cherry-picked images from the 12 that were originally taken. While the staining procedure seemed to go over rather well, the sectioning, mounting, and imaging steps of the process could have used some improvement. Many of the tissue samples appeared sheared or torn and one of the tissue samples was nearly unrecognisable (possibly as a result of the sectioning). Additionally, the mountant on the slides was far from clear and riddled with artefacts. A massive delay between software and microscope in addition to an eye-piece focus that didn't quite match the camera meant many of the images were blurry. Future experiments should focus on ironing out these flaws and possibly explore some alternative stains in order gather additional information that could be used to classify the samples.

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