Module:
Techniques in Neuroscience

Week 3
Immunohistochemistry: Preserving and studying cells of the brain

Topic 1
An introduction to immunohistochemistry – Part 1 of 4

Carl Hobbs
Histology and Imaging Manager, Wolfson Centre for Age-Related Diseases, King's College London

Voice over by **Dr Brenda Williams**

Hello, I am Dr Brenda Williams, senior lecturer in neuroscience and your module lead. Over this week, Carl Hobbs the histology and imaging manager at the Wolfson Centre for Age-Related Diseases and I will introduce you to some of the histological techniques that are used in the neurosciences.

Histological techniques are some of the oldest methods available to scientists to study structure and function of the nervous system, and are still essential tools in biology and medicine today.

In this lecture, we will outline methods of tissue and cell preparation, and the principle of histological stains, metal impregnation methods and immunohistochemistry techniques and give examples of how these methods are used in neuroscience research.

So, in this lecture we are going to start by talking about the sample preparation methods. This involves fixation of the tissue, followed by processing and embedding the tissue and mounting the sections on slides. We will then look at some basic histological staining techniques, that is, dye staining and metal impregnation techniques such as the Cajal method and finally we will consider immunostaining, which involves the use of antibodies to demonstrate the presence of specific proteins with the tissue

Why examine tissues and cells? Well, by doing this, we can study tissue anatomy, and cytoarchitecture. We can study the distribution of proteins within the tissues and, most importantly, we can study and understand pathological changes associated with disease.

So histological study of tissue is essential for clinical diagnostic neuropathology and also basic and translational neuroscience research.

Let us next think about tissue sources. For this, we can use animal models, post mortem donor tissue, pathology samples and or even surplus surgery material.

Let us first think about the use of animal models and their advantages and disadvantages. One advantage of animal models is that they enable us to examine tissue throughout the course of disease from early to end stages. Although some animal models occur naturally, most are generated based on specific genetic defects that have been identified in humans, so we can use animal models to assess the effect of these specific mutations. We can also use these models to analyse whether therapeutic strategies have any effect. However, we must also remember that there are limitations of using animal models for translational research. This is because animal models often do not always fully recapitulate the human disease. Therefore, we must always interpret the results of studies that use models organisms carefully. There is also the need to carefully consider the welfare of animals that are used in research, so there are always ethical concerns to be taken into account when undertaking any type of animal research.

So, what about using human tissue? Some of the most valuable research comes from the study of donated human tissues. Most donations are done post-mortem with full consent given by the donor or members of the donor's family. In some cases, if consent has been given, pathology samples or surplus surgery material can be used for research.

What are the advantage of post mortem donor tissue? Well, using human tissue can reduce the need for animal research. It is arguably a better material to study human diseases or diseases for which no animal model exists. However, the use of human tissue is carefully regulated. Also, as you may imagine, most post-mortem tissue that is available generally comes from the end stage of the disease and there is little availability of such tissue from early disease stages.

Now that we've obtained tissue for our research project we have to prepare our sample so that it can be used effectively. The first thing to remember is that once tissue is removed from a living organism or a deceased donor, the irreversible processes of autolysis and necrosis will begin, leading to cellular damage. Also, tissues from post-mortem donations are more likely to have signs of cellular damage than samples obtained from living donors.

So, when we prepare tissue for histology our aim is to preserve this tissue in as life-like a manner as possible and to prevent irreversible cell or tissue destruction, which can be caused by the presence of pathogens. There are two common methods for tissue preservation: chemical fixation and cryopreservation, and we shall look at these in turn.

There are many chemicals that act as fixatives. The choice depends upon what is required in experimental design, as no fixative is ideal for all applications. Fixatives that are best at preserving cellular ultrastructure, such as glutaraldehyde, can interfere with the subsequent staining processes, while other chemical fixatives, such as acetic acid and methanol, can produce some good staining results but give inferior cellular and tissue morphology. Most applications use formaldehyde, which gives best balance between optimal morphology and stain quality.

So what do chemical fixatives do? Well they stabilise proteins and other macromolecules which are enmeshed amongst these proteins, such as carbohydrates. Cross-linking fixatives - for example, formaldehyde and glutaraldehyde - create covalent bonds between proteins in the tissue. They lead to good preservation of morphology as the process anchors proteins relative to each other inside the cells and between the cells.

Protein tertiary structure is maintained, but the fixation process tends to be slow. Precipitating fixatives, such as ethanol and methanol, disrupt hydrophobic bonds between proteins causing them to irreversibly precipitate. While this can be useful for some techniques, this type of fixation is much less suitable for antibody based techniques –what we call immunostaining.

Now let's consider how to fix tissue. We can fix the first tissue by simply immersing it in a chemical fixative as a liquid. The fixative, in solution, will diffuse through the tissue over time. While this is more applicable for small, already dissected samples, larger samples can easily be placed into the same tissue but fixed with agitation – this means stirring.

Alternatively, we can inject fixative as a fluid directly into the circulatory system. This is called perfusion. Perfusion requires an intact circulatory system but can deliver the fixative very quickly throughout the organ or entire experimental animal via the blood vessels. This method is always going to be preferred, if possible, because it gives superior preservation.

Several factors will affect the quality of tissue fixation. Changes in pH, for example, will affect the reactivity of the fixative. The rate of the fixative diffusion will determine the length of incubation in the fixative. Formalin, for example, will penetrate tissue at approximately one millimetre per hour.

The size of the specimen is also very important to consider. So where possible, samples are dissected to no larger than five millimetre cubes as this facilitates optimal fixation.

Temperature also plays a role. Fixation at four degrees centigrade will retard degenerative changes but will also reduce the penetration rate to the fixative. In contrast, room temperature fixation will accelerate fixative penetration, but also potential degenerative changes.

We will now move on to consider cryopreservation. Cryopreservation is the preservation of tissue structure and components by freezing them rapidly without fixation, although, in many cases you can fix the tissue and then freeze it. Using cryopreservation, we can stop tissue degradation by rapidly cooling sample with dry ice or liquid nitrogen – what we call 'snap-freezing'. This method is very rapid, and unlike chemical fixatives, does not modify protein structure. However, the morphology is relatively poorly preserved, as ice crystals form during the process can damage cellular structure. This is particularly true if the snap-freezing is not carried out correctly.

However, if the sample is rapidly cooled to below -70 using liquid nitrogen, the liquid water is converted to vitreous water without going through the crystalline phase, and this will minimise damage to the tissue, or what we call the ice crystal artefact. Cryopreservation does not permanently fix the tissue, thus, over time degradation will occur and will progress rapidly if the sample is not stored at the appropriate temperature - most often, in -80 freezers.

The next step in tissue processing for histology is embedding. Chemical fixation will not harden tissues sufficiently to allow thin sections to be cut - for example, at six microns thin. So the sample is embedded in a solid medium that will give extra support for tissue structure and also give the sample sufficient rigidity to enable cutting of thin sections.

There are many embedding mediums available, and the choice depends upon experimental requirements. For example, if we wish to study neural architecture, we would use harder embedding media, such as plastic resins, since we can cut much thinner – for example, one to two micron sections – using this method.

However, paraffin wax is the most common embedding media in histology laboratories. Essentially, the sample is impregnated with liquid paraffin wax which hardens and forms a matrix in and around the sample, preventing tissue distortion. It is a very versatile embedding medium and allows for sections of different thicknesses to be cut. Softer embedding media, such as agarose and gelatine, are suitable for production of much thicker sections.

Cryopreserved tissue is already hard enough to allow sectioning, but small or irregularly shaped samples can be further supported with media that freezes at the same density as most soft tissues.

Commercial, water soluble, embedding media – such as OCT - are viscous at room temperature, but upon freezing, solidify and act as a support medium for the tissue, and also allows for the tissue to be handled for a short time without risk of thawing.

We are now going to discuss the processing of paraffin wax embedding and the stages involved. Paraffin wax, with a 56 degrees to 60 degrees melting point, is used. It's a widely used embedding medium. It is sufficiently hard to support tissues, yet soft enough to allow sections to be cut using a microtome blade. However, it is not soluble with water, thus tissues need to be processed before it can be embedded in paraffin wax.

There are many variations in times and chemicals used, but the sequence of four events and the rationale are the same. Initially, you have to dehydrate your tissue – that is, remove all of the water from it – because water is not miscible with molten paraffin wax. So it must be completely removed by sequential immersions in increasing concentrations of alcohol, commonly 70 per cent, 90 per cent and 100 per cent alcohol. The number of changes in each of those is up to each laboratory.

Once the tissue has been dehydrated, it needs to be cleared of alcohol because the alcohol is not miscible with molten paraffin wax either. We have to remove it completely by a solvent that is miscible in both alcohol and paraffin wax: for example, xylene or Histo-Clear. Once the tissue has been cleared, we need to replace the xylene with molten wax. This is called infiltration.

Finally, once the tissue has been infiltrated with molten wax, we have to embed the tissue in a block of paraffin wax. The tissues are oriented in metal moulds containing fresh molten paraffin wax, and then they are allowed to cool. Upon cooling to four degrees C, the wax blocks are easily removed from the metal mould, ready for sectioning or storage.

In the next part of this lecture, Carl Hobbs, the Histology and Imaging Manager at the Wolfson Centre for Age-Related Diseases at KCL, will tell you about the machinery we use in the laboratory to process and cut tissue sections.