Module:
Techniques in Neuroscience

Week 3
Immunohistochemistry: Preserving and studying cells of the brain

Topic 1
An introduction to immunohistochemistry – Part 4 of 4

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In this section, we will discuss immunotechniques. Immunostaining, immunodetection, immunolabeling, are all terms used for a technique that visualises specific molecular targets, mostly proteins in tissues. We call these targets antigens and visualise them by the use of antibodies raised or made against the antigens. For example, antibodies can be raised against a protein, the antigen, in a rabbit. Let us assume that this protein is called NueN. This is a nuclear protein expressed by neurons. The serum from the rabbit will contain antibodies against NueN, what we call anti-NueN antibodies, and these can be harvested, and applied to a tissue section. If the NeuN protein is present in a section, then the anti-NueN antibodies in the serum would bind to it and generate a NueN/anti-NueN complex. I will explain this process in a little more detail shortly. Once a complex is formed, it may be visualised by subsequently cutting out an enzymatic reaction on a section. This is called immunohistochemistry. Alternatively, it may be visualised by using fluorescent dyes. This is called immunofluorescence. The term immunocytochemistry that you may come across, refers to the process when an enzymatic reaction is used to visualise antibody antigen complexes on cells grown in a laboratory in a tissue culture dish. You will hear more about cell culture later in this module. I should mention that the term immunoflorescence is also used to describe the immunolabeling of cultured cells by florescence dyes.

Immune detection methods take advantage of the interaction between antibodies and the molecular targeting tissues called antigens. Before we go any further, let's look at some terminology. So antigens are large macro molecules usually a protein or a molecule with a protein component, like a glycoprotein or a lipoprotein. An epitope is a small section of the antigen. Normally at eight to 15 amino acid sequence that is recognised by the antibody. So, each antigen will have many potential epitope sites. As I'm sure you know, different cell types express specific groups of proteins, as well as many proteins that are common to all cells. Also, many proteins are compartmentalised within a cell. For example, they exist within the nucleus, or the mitochondria, or the rough endoplasmic reticulum, or even in the membrane. With immunodetection techniques, it is possible to not only distinguish between cell types. For instance, neurons versus oligodendrocytes, because a specific cell type will express a specific protein or groups of proteins. But also to visualise a location of proteins within the cell, for

example, in the cell cytoplasm or on the cell surface. This is important because a normal location of a protein maybe altered in disease.

There are two main types of antibodies made for research: monoclonal antibodies and polyclonal antibodies. I will first introduce monoclonal antibodies. A monoclonal antibody preparation contains only a single antibody type that's specifically recognises a single epitope on the antigen molecule. Monoclonal antibodies are produced by immunising or injecting an animal, for example, a mouse, with an antigen. In this case, the protein that we wish to generate an antibody against. This can be a human protein. B cells are then extracted from the spleen of that animal. These B cells are then fused with myeloma cells. These are immortal cells and so can be grown indefinitely in a laboratory. By fusing the B cells with the myeloma cells, we generate a cell called a hybridoma cell and this cell can also be grown indefinitely in a laboratory and can secrete antibodies. So now, we have a population of hybridoma cells that can secrete antibodies. However, not all hybridoma cells in this mixture will generate the antibodies that we want, but some will. And each of these will generate an antibody that interacts with the specific epitope on our protein of interest. We are able to isolate individual hybridoma cells from this mixture and then expand them to generate a population of hybridoma cells. All of which generate a specific antibody that recognises a specific epitope on the antigen used for immunisation.

Polyclonal antibodies are also produced by injecting an antigen of interest into an animal, usually a rabbit. The animal's B cells will produce antibodies against the antigen, and these antibodies can be found in the serum of the animal. As mentioned earlier, a protein has many epitopes and different B cells will make antibodies against a different epitope of the protein. So the serum will contain a collection of antibodies that recognise different epitopes of the same protein. Hence the term, polyclonal. Polyclonal antibodies may vary between production batches. And they also cross react with other proteins. This is because the more epitopes that are recognised by an antibody mixture, the more opportunity there is that any one particular epitope, that is amino acid sequence, may be found on an entirely different protein.

Here is a very brief introduction to antibody structure. Antibodies belong to the immunoglobulin protein family. They are Y-shaped as you can see in the cartoon and are composed of four polypeptide chains. Two identical copies of a heavy chain and two identical copies of a light chain,so called because of their relative molecular weights. The arms of the Y contain the antigen binding site. Antibodies can be divided to five different types: IgG, IgM, IgA, IgD and IgE. However, most antibody reagents are IgG, or occasionally, IgM.

Having briefly discussed antibodies, let us now look at how we can actually use them to detect proteins in our sections. Let us think about how we can visualise a protein P, in the membrane of cell within the section. We can do this using a direct method or an indirect method. In both cases, antibodies are applied to the tissue section. In a direct method, the primary antibody, seen in green, so called because it binds directly to the antigen, is directly linked to a report molecule shown in red. These report molecules, and we will talk about these on the next slide, enable us to visualise the bound antibody. This direct technique is suitable only for highly expressed proteins. Because if there are very few epitopes available, the reporter signal may be too weak for us to see. The indirect method is a method that allows us to significantly amplify the reporter signal strength. See the right-hand side image. Again, the primary antibody binds to the antigen. But in this case, this antibody has no reporter linked to it. The section is then incubated with the second antibody, termed a secondary antibody that binds specifically to the primary antibody. It is a second antibody that has a reporter linked to it. The secondary antibodies are polyclonal antibodies, so will react with epitopes all over the primary antibody. The figure on the right illustrates this, but only shows two of the bound secondary antibodies. Thus, multiple secondary antibodies will bind to the primary antibody. and as all of the secondary antibodies carry a reporter, the signal is amplified. The reporter molecules may be an enzyme or a fluorochrome. We will look at these different types of reporters next.

In the fluorescence method, the antibody, primary or secondary, is linked to a fluorochrome. The fluorochrome is a reporter molecule that is not itself colored, but will emit colored light at a specific wavelength in the visible spectrum when illuminated by UV light. We have specialised microscopes, called fluorescence microscopes, that enable us to view such fluorochromes. This method can be used to detect the presence of more than one protein at the same time by using two or more different antibodies, each linked to different fluorochromes that emit light at different wavelengths, as shown in the cartoon on the left hand side. In the case of the enzymatic detection method, the antibody, again, primary or secondary, is linked to an enzyme, most often an enzyme called horseradish peroxidase, or HRP for short. This is found in the root of horseradish. A substrate is then added to the tissue. And the enzyme and substrate interact to generate an insoluble coloured product at the site of the antigen-antibody complex that can be visualised under a light microscope. Such a substrate is called a chromogen. The direct and indirect methods of automatic detection are shown on the right-hand side of this slide where the yellow depicts a coloured precipitate being formed.

However, there are some other factors that we need to consider when carrying out immunohistochemical experiments. These are: the incorporation of positive and negative controls, antigen retrieval, and blocking of non-specific binding. I will talk about each of these next.

It is very important to include positive controls to assess fidelity of the technique and specificity of primary antibody. I will explain with an example. BrdU, or bromodeoxyuridine, is a synthetic analogue of the nucleoside thymidine, one of the four bases in our DNA, and is used to identify dividing cells. When injected into the brain of a mouse, for example, BrdU can be taken up by cells and incorporated into their DNA in place of thymidine when they divide, as shown in the cartoon. The presence of BrdU in a cell can then be detected using an anti-BrdU antibody. Such a method is often used to assess changes in adult neural stem cell division in the hippocampus. Let me explain. In the adult brain, the dentate gyrus, a region within the hippocampus, is one site where new neurons are generated throughout our lives. This process of generating new neurons is called neurogenesis. Neurogenesis within the hippocampus is important for memory and also hippocampal neurogenesis has been associated with changes to our mood. For example, in animal models of depression, antipsychotics have been shown to increase adult hippocampal neurogenesis. An example of BrdU staining in the hippocampus can be seen on the slide. So, in our hypothetical experiment, let's assume that mice have been injected with BrdU to assess changes in stem cell proliferation in the hippocampus after drug treatment. So we are looking for BrdU staining in the hippocampus as a means to assess neural stem cell proliferation. What if we see no antibody staining within the hippocampus? Does this mean that there was no BrdU incorporation and hence no division? Or did the anti-BrdU antibody not work? We can confirm that our anti-BrdU antibody and staining protocol worked by including a positive control. For example, we could use a piece of small intestine isolated from the same animal, and look for BrdU incorporation in its tissue. This is because we know we can always find dividing cells here in the crypts of Lieberkühn at the base of the villi. If we see staining here, this would confirm that BrdU was incorporated and the staining procedure worked, even if was not seen in a treated animal hippocampus. So this is example of the benefits, the necessity, of using a positive control.

For a negative control, it is mostly sufficient to make the primary antibody. Any positivity seen is then assumed to be caused by non-specific binding of the visualisation reagents. One then compares results of the negative control and the positive control against the test results before drawing a conclusion.

Let's now think about antigen unmasking, or antigen retrieval. We heard in the first part of this lecture how fixation is important for preserving tissue morphology. But fixation techniques can also affect your ability to detect antigens using immunohistochemistry. In essence, fixation procedures can mask or alter epitopes, so that they can no longer bind to the primary antibody. Antigen unmasking or retrieval refers to any technique where the masking of an epitope is reversed so that the antibody can again bind to it. In this slide, two such techniques are

highlighted, heat-induced epitope retrieval, or HIER, and protease-induced epitope retrieval. The mechanism by which HIER is achieved is not really known. But HIER can be performed using microwave ovens, pressure cookers, steamers, or water baths. However, it is recommended to use commercial systems specifically designed for HIER. HIER is normally carried out with sections immersed in a buffer solution. Such a solution resists change to pH. That is, the acidity or alkalinity of a solution. A list of buffers can be seen on the slide. Each will be tested to see which works best with the antibody being used. In protease-induced epitope retrieval, sections are pre-incubated in enzymes. And Proteinase K, trypsin, and pepsin have all been shown to have some effect. Which enzyme to use and for how long is decided by trial and error. An over digestion will destroy both the antigen and the tissue section so this method has inherent difficulties. On top of that it has been shown to only work for a small proportion of antigens.

Unfortunately, the antibodies we use can sometimes bind to nonspecific components in cells and tissues with low affinity, by that I mean low strength. And can result in a false positive signal. This is more often true for polyclonal antibodies. To prevent this happening excess protein is added that will compete for and block binding to these components, as shown in the cartoon. Serum contains a range of proteins that can compete out the antibody from binding to sites other than its target epitope. Normally researches block with the serum of the animal species that was used to raise a secondary antibody. For example, if a secondary antibody is obtained by inoculating a goat with a mouse IgG antibody the serum used to block nonspecific binding would be goat serum. Alternatively, we can use an excess of proteins like bovine serum albumin to block nonspecific binding and this is very effective.

So when we carry out an immunostaining experiment we always need to consider the following:

- what positive and negative controls to include
- do we need to use antigen retrieval
- and what should we use to block non-specific binding.

Let me finish by showing you a couple of examples. On the slide is an image of a paraffin wax section from a mouse model of Alzheimer's disease showing astrocytes surrounding an amyloid plaque. These are protein aggregates that build up in the Alzheimer's diseased brain. The method used to stain this section was a double indirect immunofluorescence staining. To do this the section was incubated simultaneously with two primary antibodies that each recognise a different protein: glial fibrillary acidic protein, or GFAP, and beta-amyloid. GFAP is a protein expressed by astrocytes in the brain and beta-amyloid is a protein fragment that makes up the amyloid plaques. The anti-GFAP primary antibody was then visualised with a secondary antibody linked to a green fluorochrome. And the anti-beta-amyloid primary antibody was visualised with a secondary antibody linked to a red fluorochrome. The blue that you can see in the image are due to the nuclei of cells being stained with a fluorescent blue stain called DAPI.

For my final example I'm showing you a paraffin wax section from a normal mouse brain showing an area of the hippocampus. This section, again, stained with an anti-GFAP antibody to visualise astrocytes in the section. But this time the binding of this antibody is detected by indirect immunoperoxidase staining using DAB. The section is counter stained with hematoxylin and a blue dye to show all nuclei.

To summarise, from this presentation I hope you can see that histotechniques are invaluable tools for neuroscience and have contributed significantly in the elucidation of many normal and disease processes. Today we have talked about the importance of tissue preservation by fixation, or freezing and about the processing and embedding techniques used to support the tissue for sectioning. We have discussed some of the staining techniques that can be used to identify the cell types and proteins within a section and provided some examples

about such techniques can help us identify changes that can occur in the diseased or damaged tissue. In topic two of this week Carl Hobbs will demonstrate how we actually carry out these techniques in the laboratory.