Module:
Techniques in Neuroscience

Week 5
Molecular biology: Going inside the cell

Topic 1
An introduction to molecular biology methods – Part 3 of 3

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Western blotting is a laboratory method we use to detect specific proteins from a mixture of proteins. In Western blotting, rather than staining and imaging the polyacrylamide gel, we process it further. In the same way that we used an electrical charge to separate the proteins in the gel during electrophoresis, we can also transfer the proteins in an electric field from the gel onto something else: a membrane which blots the proteins from the gel.

This is the principle of western blotting. Essentially, we put the gel and the membrane together in a sandwich between two electrodes. The sandwich is filled out with filter papers and sponges, as you can see here. The membrane that we want to transfer the proteins onto is made from nitrocellulose or sometimes PVDF. We run an electronic current through this sandwich, and as the proteins are still negatively charged, they're forced towards the positive electrode - this means they move out of the gel and onto the membrane. Nitrocellulose and PVDF, either of which are used at the membrane, have good protein retaining properties i.e. they're very sticky to proteins.

So once the proteins move out of the gel, they become immobilised on the membrane. When it has come out of the transfer, only the parts of the membrane which were adjacent to proteins on the gel will have protein on them. Before we can process it further, we need to block the membrane: this means we cover up any parts of the membrane which do not already have protein on. We do this by incubating the membrane with a solution containing a protein that we'd not expect to find in our samples. We usually use milk because it's cheap, contains a lot of proteins, and is easily available. After the membrane has been blocked, we add primary antibodies - these are antibodies that will bind to the protein we're interested in.

Antibodies are very specific and will only bind the protein we are interested in. The rest of this protocol is actually very similar in concept to histochemistry, which you studied last week. Following the primary antibody, we then incubate the membrane with secondary antibodies - these are antibodies that will bind to the primary antibody we used. They either have a fluorescent tag, which gives off light of a certain wavelength, or they have the enzyme horseradish peroxidase, HRP, attached. When you add the substrate for HRP, the enzyme catalyses a chemical reaction that produces light. We can then detect this light using photographic film, or an imager, designed to pick up the signal.

This is a picture of a gel and the corresponding western blot. You can see how in the gel, there are so many proteins that we have no idea which one is ours, whereas in the blot, we only see the protein we interested in. Even though we transferred all of the proteins from the gel onto the membrane, the use of specific antibodies means we've only detected our protein of interest. We can then go on to analyse the blot, and there are various free and easy-to-use computer programs available to measure the intensity of the band on the blot. Within a certain range, the intensity corresponds to the amount of protein.

To summarise, the samples are transferred from the gel onto a membrane, again by electric current. The membrane is blocked with an excess of nonspecific protein, then probed with a protein of interest using primary antibodies that bind to it, followed by horseradish peroxidase-tagged secondary antibodies, which bind to the primary antibody. Substrate for the reaction carried out by horseradish peroxidase is added to the membrane and the enzyme carries out the reaction, which generates light. The light signal is detected, which shows up as specific bands on the membrane. The size and intensity of the bands detected is proportional to the amount of protein in the sample, so western blots can be used to analyse the amount of specific protein in different samples.

Western blotting is a very commonly-used technique in molecular biology and cell biology labs. Let's take a few minutes to look at why it's so popular and also at some of its limitations.

Starting with advantages: western blotting is relatively quick and takes only about one and a half days, though it can be a lot faster as companies come up with better equipment. Western blotting doesn't require a lot of expensive, delicate, specialist equipment: all you need is some glass plates, the running and blotting tanks and a power supply. The gels and buffers are cheap and easy to make. It will work for a wide range of proteins, all you need is a good antibody. If you have a good antibody, they can be quite sensitive and very specific: you see only your protein on the gel. It will work with many procedures. You can use pretty much any cell type or tissue. It's also semi-quantitative: if you see a bigger signal, it means you have more protein, so you can use it to look at changes in protein levels.

Okay, but what about its drawbacks? The main drawback comes from fact that western blotting is only as good as the antibody you use, and antibodies can be very expensive. Many antibodies do not bind very specifically- they pick up proteins other than the one you're interested in, making the results confusing to interpret. In addition, an antibody may not work once a protein has been denatured - if it recognises a 3D epitope for example (an epitope being the part of an antigen molecule to which an antibody attaches itself). This is why we might want to tag a protein with a short sequence which antibodies bind very well to.

A further disadvantage is that sometimes proteins become modified by the cell once they've been synthesised. Part of the amino acid sequence can be chopped off or different chemical groups can be added with consequences for how the protein functions in the cell or is controlled. These modifications will affect how the protein runs on the western blot. While this can be an advantage, as it means that the technique can be used to determine where the modifications have happened, it can also make the blots harder to interpret. Western blotting can be more difficult if the proteins are very large or very small: large proteins will move slowly through the jar and doesn't transfer well through the membrane, so need very low-percentage gels which are hard to handle and don't transfer well; small proteins run very fast and will need high-percentage gels and can easily run off the end of the gel off through the membrane in the transfer.

While the technique is reasonably sensitive, if you are looking at changes in levels of a protein, then it may not robustly detect them. You need a change of at least 10 per cent in your protein levels to be able to see it on a Western blot. Other techniques, such as QRT-PCR or ELISA are much more sensitive for detecting low levels and small changes in protein.

And finally, Western blots are only semi-quantitative. Using ELISA and QRT-PCR, you can measure exactly how much protein you have, whereas you can only see approximate changes within a blot. For example, you can't compare between membranes as things like efficiency of the transfer, the antibody addition steps and the blocking all vary between membranes.

Let's now look at two examples of western blots of the kind that you might come across in a paper. The first one is a very simple example and shows the levels of a protein called BIN1 in the brains of people with Alzheimer's disease versus age-matched controls. Genetic studies have shown that BIN1 has a role in the development of Alzheimer's disease; however, it's currently not clear what that role is. To start to understand how BIN1 may contribute to Alzheimer's pathology, the author has determined how it's altered in Alzheimer's disease. From the blocks, you can see a reduction in the signal in the samples from people with Alzheimer's compared to control. So, we know there's a reduction in this protein.

This second example is a bit more complicated and involves the use of DNA technology. Stoicca et al., (2014) used western blotting to investigate how two proteins involved in motor neuron disease, PTPIP51 and VAPB, interact. Cells were transfected with an empty vector or the vector which codes for protein PTPIP51 (shown by the arrows here) and then incubated with different fragments of another protein called VAPB. The different VAPB fragments - 1 to 124, 89 to 207, 142 to 207 and the full length, 1 to 220 - were immobilised on beads. This is why it says 'GST', it means they're fused to a protein that will bind to the beads - an example of using a tag to help purify and investigate a protein.

The cell lysate was incubated with the beads, and everything that had bound to the beads could then be determined by western blotting. From this blot, you can see that PTPIP51 only bound to the full-length of VAPB protein, it did not interact with the fragments. There are no bands on the gel indicating it did not bind to the beads. This suggests that PTPIP51 binds to a 3D epitope that's formed when the entire VAPB protein folds, rather than a linear sequence of amino acids on just one part of the protein. As VAPB and PTPIP51 are potentially involved in disease, uncovering as much information as we can about their interaction could be important in the future for drug design.

To finish this section, let's highlight the main points and make some concluding remarks. Using these techniques in conjunction with each other, we can manipulate our cell systems in a wide variety of ways. Using plasmids, we can overexpress our protein of interest in some cells we're investigating. We can then use western blotting to see the impact this has on a variety of things, such as the expression levels of other proteins, the processing or modification of other proteins, the subcellular location of the protein or of other proteins, which we can do by fractionating the cell up into different cellular compartments- nucleus, mitochondria, etc. - and then western blotting the different fractions.

It's important to remember when assessing the results from these experiments though, that we're dealing with an artificial system. The cell is making much more of the protein than it usually does and sometimes the tag on the protein might affect the way it behaves. All mutations we've introduced may not reflect the real processing of the protein, therefore it is always important to use a variety of different techniques to get the best understanding of molecular events inside the cell that we can.