The Basics of Immunohistochemistry in the Kidney

We use Immunohistochemistry (IHC) in both a qualitative and a semi-quantitative manner, i.e. to determine if/where an antigen is expressed and how much. The first thing to consider is the choice of primary antibody. Monoclonal antibodies offer many advantages; high homogeneity, no limitations on supply, absence of contaminating antibodies and minimum batch-to-batch variation. There are however some drawbacks; the antibody used must be tailored to the intended use e.g. fixed or not, cross-reactivity can not be removed by pre-absorption and they are more selective in terms of protocol requirements. You also have to consider other practicalities when choosing your tools such as the fact that long term storage of polyclonal antibodies can lead to polymerisation and aggregation which increases non-specific binding. Storage conditions are variable between antibodies but there are some commonalities such as, always adhere to the manufacturers recommendations for temperature, use containers that have minimal protein adhesive properties such as polypropylene and use a carrier protein (such as 0.1-1% BSA) if you are storing at a concentration of less than 10-100microg/ml. The expiry date on the batch of antibody is not necessarily a hard rule about discarding them as this will just be the amount of time that the manufacturer has tested them for. So as long are you are running good quality controls in your experiments then it is fine to carry on using them. Also the differing affinities of the types of antibodies lead to differences in the concentrations they can be used at. As a general rule of thumb polyclonals are used between 1:100-2000, monoclonals from tissue culture supernatants 1:10-1000 and monoclonals from ascites up to 1:1000000.

There are a number of ways in which you can collect and preserve your tissue of interest but they fall into two categories; frozen or fixed. For frozen tissue the method of choice for morphological preservation is to embed the tissue in OCT (optimum cutting temperature) in a mould then immerse in isopentane cooled in liquid nitrogen. In some cases this can be preceded by a brief fixation in acetone or alcohol. Snap freezing of unfixed tissue avoids the problem of antigens being masked by fixation and allows semi-quantitiative immunofluorescent microscopy. In the renal field this is the method of choice for detection of immunoglobulin and complement in glomeruli. The drawbacks of using this process is that long-term storage is not perfect as the tissue tends to dry out, if care is not taken then the morphology is not well preserved and cutting these types of sections requires considerable expertise and patience. Fixed tissue on the other hand provides a much better morphology and prevents elution, degradation and changes in position of the target antigen. Additionally if there is an infection risk this is the recommended solution. However, as intimated above, some antigens may become masked and therefore need to be retrieved. If the tissue is further paraffin-embedded then additional masking can be observed. Traditional fixatives include Paraformaldehdye (which can include periodate), formalin and Carnoy's. Formalin varies greatly and will change during storage; it should be made fresh and its pH controlled to ensure consistency. This fixative is good for small molecules but not good for immunofluorescence. Epitopes are either classed as formalin sensitive or resistant.

Antigen retrieval is often a process of trial and error and techniques are evolving. Proteolytic digestion etches the tissue and its effectiveness varies with antigen and tissues, in fact it can destroy some antigens. It works best on glycoprotein-rich targets. Conditions are optimal at 37°C and it is best to optimise the technique and then adhere religiously to this regime. The other standard methodology is heat treatment in buffer. The buffer is often citrate at pH 6 but 0.01M Tris HCl pH 1 or 10 may be a better solution. The duration and temperature of this process is crucial and typically 10-60min at near boiling is used with a slow cool. Other techniques include steam and a protease in EDTA pH8.

Blocking is performed to decrease non-specific binding and staining. An irrelevant protein is applied to the tissue in question and forms links with a variety of molecules. As the primary

antibody has a much higher affinity for its target antigen it easily displaces the block. The kidney has a particularly high level of biotin which should be blocked or avidin systems avoided. If tissues/cells are likely to have Fc receptors present then these can also be specifically blocked.

Detection systems are either direct or indirect. Directly conjugated primary antibodies are most commonly used in immunofluorescence (IF). This is the preferred method for quantifying staining and double labelling but is subject to photobleaching or quenching. To avoid bleaching use the lowest illumination possible when viewing samples. For quenching avoid compounds such as certain salts and use anti-fading mounting media. Some tissue exhibits more autofluorescence than others, i.e. ones that contain higher levels of collagen, keratin or fats but detergents such as Tween 20 also contribute as well as fixing and embedding. Incubation times are often crucial and anything up to 48h is used with dilute concentrations. In general if a higher incubation temperature is used then the incubation time needs to be more stringent. The use of a secondary antibody system to visualise the binding of the primary is more commonly used and this provides an amplification of the binding and therefore an increase in sensitivity. Further amplification can be achieved by using techniques which include avidin-biotin steps or polymer-based methods, but the later may be subject to steric hindrance. The choice of enzyme conjugate in the secondary system is also influenced by tissue and the two options are horse radish peroxidase and alkaline phosphatase. The frozen kidney (proximal tubule) has a high content of endogenous alkaline phosphatase and this can be blocked with 1-5mM levamisole. There are a reasonable variety of substrates available for both of these enzymes some of which are suitable for use with alcohol and organic solutes and some which aren't. With all substrates an excess can inhibit enzyme activity. In addition cyanide and azide can inhibit HRP activity. Whatever system is selected thought must be given to the use of controls such as an irrelevant isotype matched to the primary antibody, preabsorption of the antiserum with the antigen of interest and the use of antibodies to the same target but different epitopes to confirm results.

Other tips and tricks for IHC - If you use a buffer system of pH6 you can often get better staining with less antibody. Avoiding NaCl and azide will give you better staining. PBS can suppress monoclonal staining but is often used in the washing step for renal biopsies. Antibodies are positively charged in general but this will vary with the diluent due to changes in isoelectric points. Slides can be coated (albumin or lysine) or bought charged to aid with adhesion of tissue.

Some references and websites that may be helpful are;

http://www.immunohistochemistryprotocols.com/
http://icg.cpmc.columbia.edu/cattoretti/Protocol/Immunohistochemistry/FAQ.html
http://pri.dako.com/08002_25may06_ihc_guide_book.pdf
http://www.protocol-online.org/prot/Immunology/Immunohistochemistry/
http://www.histochem.net/
http://www.ihcworld.com/introduction.htm
http://biowww.net/browse-125.html
Double immunofluorescence labelling of routinely processed paraffin sections David Y. Mason et al. J Pathol 191:452 (2000).
Immunoassays with rolling circle DNA amplification: A versatile platform for ultrasensitive antigen detection. Barry Schweitzer et al. PNAS 97(18):10113-9 (2000).

As always you can contact a member of the Renal Scientists group via our webpage. Happy staining!
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