Immunostaining and in situ hybridization essay

The study of gene expression provides invaluable insight into an organism’s structure and function; how it is that from a mere embryo genes can control which cells, tissues, organs and limbs will develop, providing the organism with the inherited traits that specifically adapt it to its forthcoming environment. Mutations of particular genes are sometimes associated with certain birth defects. Should an organism with a mutation in just one gene be born with a specific birth defect, it gives a clue as to the gene’s function.

The question of its intended function may be better understood by discovering whether it became inactive during development to cause the defect. Of the collective amount of genes inherited by an organism not all are actively expressed (“ turned on” and functioning). If expressed, it will be transcribed into RNA, specifically mRNA, providing the coding blueprint for a particular protein. When inactive and not expressed, no RNA is transcribed and thus no protein produced, perhaps because the protein is no longer needed or not required in a particular area of the body.

To prevent the unnecessary expenditure of energy a core feature of many organisms was similarly evolved to inactivate genes that serve no current purpose. Therefore a technique that enables the visualization of the internal embryonic environment will confirm whether RNA is present; its presence in an area is a good indicator that the gene coding for the RNA is performing a task required by the organism. In Situ Hybridization is just one biological technique that enables researchers to study such prenatal development.

Specific probes are utilised to detect RNA. Due to its single-stranded nature, a complimentary length of single-stranded nucleic acids (the probe) is

synthesized to hybridize through specific base pairing with the RNA of interest (see Figure One*). An embryo is placed in a solution containing this specific probe. The probe then locates the places in the organism where the RNA is present and hybridizes to it, hence the term “ in situ hybridization” (‘ In situ’ being Latin for ‘ in its original place’).

The probe must be chemically labelled, whether by a radioactive label, a label that fluoresces under Ultraviolet light or one that can be detected by an antibody. This label, coupled with the probe, becomes the ‘ reporter’ molecule, its visualization of which, enables localisation of RNA (or in fact DNA) sequences in heterogeneous cell populations. This includes tissue samples as well as environmental samples. Riboprobes even allow the researcher to localise and assess the degree of gene expression, particularly useful in neuroscience.

Figure One: Diagram to illustrate nucleic hybridization during In Situ Hybridization techniques. Other applications of In Situ Hybridization include its use in microbiology, studying the morphology and population structure of microorganisms; the method is extremely sensitive and can detect the amount of mRNA contained in a single cell. Its application in profiling embryonic tissue (developmental biology) and establishing abnormal gene expression is reflected in other areas such as the profiling of pathogens in pathology.

Furthermore, the technique is used in karyotyping and phylogenetic analysis due to the revolutionary ability Fluorescence in Situ Hybridization (FISH) had upon viewing chromosomal aberrations. Unique FISH patterns can be

displayed even on individual chromosomes, showing defects. This is integral to a great deal of prenatal analysis, as it allows diagnosis of potentially fatal abnormalities before premature mortality occurs i. e. miscarriage. A suitable treatment can then be administered, or in some cases, defective development terminated.

Physical mapping of clones on chromosomes is made possible because of this. We can directly assign previously-mapped clones to chromosomal regions associated with heterochromatin or euchromatin. In Situ Hybridization is distinct from immunohistochemistry (IHC), which usually localizes proteins in tissue sections, rather than nucleic acid sequences. ‘ Immunostaining’ was later developed as a term to encompass a wider variety of antigen-antibody staining methods used in histology and cell biology.

The principle of this staining is that, by taking advantage of antigen-antibody (Ag-Ab) specific interactions, the presence of a specific protein in a tissue can be established, by using its known antibody to identify it. A specific monoclonal or polyclonal antibody is bound to the target immunogen before the Ag-Ab link is amplified (more true for ‘ indirect’ immunostaining, see below) and visualized by an indicator system. Labelling with colouring (fluorescent) agents or electron-opaque substance (ultrastructural tags) beforehand allows such visualization.

When using colouring agents, for instance, the emission of fluorescence when exposed to light of a particular wavelength (immunofluorescence) detects the Ag-Ab reaction. There exists two means for utilizing

immunostaining practices: a direct method and an indirect method. Following the evolution of IHC it has become typical to employ the indirect approach, which is not only cheaper but has the distinct advantage of producing a stronger signal. In the direct method there is no such amplification and therefore the Ag-Ab reaction appears very weak.

It is a one-step staining process involving the labelling of one antibody which reacts directly with the antigen contained in the tissue section; it is simple and relatively fast. This method has since been improved, techniques adapted to progressively increase the sensitivity without the detriment of increasing the non-specific staining (background matter) as the lower levels of antigen are visualized. What is now commonly used as the successor to this method is the indirect technique for detection, which involves a primary unlabelled antibody, detected by a secondary antibody.

The secondary antibody can be visualized with the aid of an enzyme conjugated to it (such as peroxidase, alkaline phosphatase or glucose oxidase; this is the indirect immunoenzyme method). With further secondary antibodies attaching to the primary (see Figure Two*), as well as even incorporating a third tertiary layer, the Ag-Ab signal is amplified even more with greater sensitivity. Concerning the three-stage methods, their early use did not label the second antibody and added an anti-enzyme antibody to which the enzyme had formed a complex. Figure Two: Direct and Indirect Immunostaining

Today, more complex arrangements of antibodies and molecules detect antigenicity. Biotin and (strept)avidin in these complexes provide the best

sensitivity and is used in most laboratories either as a conjugate or complex. As well as correct antibody choice for the target antigens, full preparation of the samples is crucial to maintain cell morphology and the antigenicity of target epitopes (antigen component recognised by the paratope of an antibody). Often the sample tissue is perfused or blood-rinsed to prevent interference of hematologic antigens with those target antigens needing to be detected.

Tissues are typically fixed in crosslinking reagents, (i. e. paraformaldehyde) before being embedded in paraffin to keep its natural architecture for storage. Tissue fixation immobilizes antigens while retaining subcellular structure, however because it chemically reduces protein solubility it may also mask target antigens if not done correctly, thus proper fixation is vital to ensure optimized results. Antigen retrieval techniques may be necessary should this occur, particularly if there is long fixation incubation or a high percentage of crosslinking fixative used.

The solvent Xylene is commonly used then, to remove paraffin from IHC slides. Cells are permeabilized after formaldehyde fixation by covering the cells of the tissue with a permeabilization buffer temporarily (e. g. Diaminobenzidine solution, “ DAB”). It should only be required for intracellular epitopes when the antibody required access to the intracellular matrix to detect the protein. However it is also required for detection of transmembrance proteins if the epitope is in the cytoplasm. Acetone, ethanol and methanol all permeabilize, though the latter is not always suitable.

Permeabilization of tissues is also an important stage regarding In Situ Hybridization, enabling the probe access to the target. Embedding is a prerequisite for sectioning, should the decision to view the sample in sections be positive; whole-mount IHC allows visualization of even a whole organism, i. e. an embryo. Finally, blocking is a technique used when a staining approach depends on biotin, peroxidases or phosphatases for amplification (or enzymatic antigen detection), whose quenching or masking of their endogenous forms prevents false positive and high background detection.

Physical blocking of all endogenous biotin or chemical inhibition of all enzymatic activity will combat this. In reducing background staining blocking solutions are particularly effective, since they bind to any open protein binding sites that do not have attached any antibodies or antigens. Though they preferentially bind to specific epitopes, antibodies will also partially bind to a weaker degree on non-specific proteins at reactive sites, if they are similar to those of their target antigen’s own binding sites.

This non-specific binding significantly raises background staining and masks detection of the target antigen. Common buffers then, to incubate the samples with that blocks the reactive sites of proteins that the primary or secondary antibody may otherwise bind to weakly, include BSA, gelatin, non-fat dry milk or normal serum. Immunostaining applications are as varied as they are numerous. Similar to In Situ Hybridization, it is most typically operated as an important diagnostic, predictive and investigative tool in histopathology for the diagnosis of abnormal cells. That is, in certain types of cancers, i. . breast cancer.

Specific molecular markers are characteristic of cellular events at one moment in the cycle, such as cell death (apoptosis) or proliferation. Particularly useful then, is IHC in developmental biology, when stages of development can be effectively frozen in time for examination. The exploratory potential concerning the cellular distribution of proteins, their absence or presence in combination with their intracellular location, as well as changes in protein expression or degradation, gives studies using immunostaining techniques a steep advantage over others.

The staining can be carried out on a variety of different cells and tissues preparations, such as (as mentioned) formalin-fixed, paraffin wax-embedded material, frozen sections and cytological preparations. This allows antigens and their expression to be visualized in material that is under long-term storage. The results are permanent and so slides can always be reviewed at later dates, particularly useful for relating levels of protein expression in cancers to clinicopathological data of patient outcomes; comparisons can be drawn with further clarification of archived visual evidence.

Similarly to ICH, In Situ Hybridization (ISH) sample cells are treated to fix the target transcripts as well as increase the probe’s available access. The probe hybridizes to the target complementary sequence at raised temperatures before all excess is washed clear. The temperature and other such controls as salt and/or detergent concentration may be altered to optimize the removal of non-identical interactions (only truly complementary sequences that match will remain hybridized).

Then the labelled probe can be localized and its quantity measured in the target tissue using the most suitable method (e. g. fluorescence microscopy). ISH can use multiple probes to rapidly detect multiple transcripts simultaneously. Both ISH and IHC are methods that complement one another. For many genes, the sites where protein exists and can be visualized by IHC are not the same as the actual sites of synthesis, as revealed by In Situ Hybridization; often the protein is taken up elsewhere to target cells once it has been secreted.

Both methods shed combined understanding on the activity and function of a gene and its different aspects of expression. ISH is a successful means for tracking down sites of potential synthesis the moment cloning is complete, far faster than the production of an antibody can be accomplished. Used in combination a comparison of the patterns of expression of mRNA and protein can be formed of the same histology section, widening understanding of gene expression in relation to its subsequent protein purpose.