Chapter 1

Immunohistochemistry Fundamentals

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Overview

Demonstrating changes at the cellular level via morphology is the current gold standard in diagnostic histopathology. Most pertinent to tumour pathology, cells expressing observable antigenic proteins provide valuable information as to their lineage, disease status and operational conditions. Detecting these 'expressions' and evaluating them within a clinical context gives confidence to the clinician considering relevant therapies. Likewise, interpreting disease pathways and simulating models helps researchers address relevant hypotheses (de Matos et al. 2010).

Immunohistochemistry (IHC) is a technology employed to 'tag' specific sites within tissue and observe the reaction. The ability to do so renders IHC, a powerful tool in diagnostics and research applications. The discipline is made up of three elements: *immunology*, *histology* and *chemistry*. Employing antibodies to identify antigenic sites within tissue utilizes concepts from immunology. Establishing the technique upon the study of cells and tissues explains the histology reference. Principles of chemistry are used to visualize the reaction detected from a 'label' bound directly or indirectly to the antibodies, an idea explored further in what follows.

Immunohistochemistry originated in 1890 with the discovery of serum antibodies and their use to combat disease (Childs 2014). This chapter begins with a homage to the pioneering work of early investigators and their valuable contributions throughout the years. A revolution of imagination, ingenuity and curiosity from scientists and clinicians worldwide, buoyed by their successes in research but also undeterred by their failures, led to discoveries still impacting disease diagnoses and treatments today. This chapter endeavours to highlight individual contributors to this progressing technology.

Although IHC is built from a solid theoretical basis centred upon the antigen–antibody attraction, setbacks and uncertainties often arise within IHC practice. A basic rundown of IHC principles is offered and includes the relationship of the antigen and antibody complex within IHC assays. Variations adopted from the 'standard' IHC protocol are then explored and compared to current standard practices. Common terms such as 'immunocytochemistry', 'immunolabelling', 'immunofluorescence' and 'immunoperoxidase staining' are delved into. All share the same principles with IHC but can be differentiated with subtle modifications to the technique. A discussion as to the differences is provided in this chapter whilst the highlighting of important phrases and explanations gives context to the technique. A brief mention of manual techniques and automation systems along with specific examples of using IHC in real practice should serve as a good introduction to the discipline as well as to the book. By this chapter's end, it should be evident how IHC has come to be regarded as the diagnostic tool of choice in anatomical pathology.

Laying the Foundations

From IHC's humble beginnings to the present day, the story of IHC is one to be celebrated and disseminated. It is an account of a collective effort from inquisitive minds whose discoveries contributed not only to the development of IHC, but also to the impact it has made on disease investigation and drug development. The effects have been so huge that possibilities from this technology continue to be redefined. Most will attest that IHC has been a 'game changer' in diagnostic histopathology.

Seeking treatment for diphtheria in the 1890s, researchers made a pivotal discovery in the form of serum therapy which can be viewed as one of the foundations of IHC. To combat the 50,000 German fatalities from the disease each year, three eminent scientists collaborated on researching how to effectively treat patients with what we now know is a toxin

originating from a bacterial infection. Dr Emil von Behring (Figure 1.1) at the Pharmacological Institute, University of Bonn, in association with Robert Koch and his colleague Shibasaburo Kitasato, found that rabbits, guinea pigs, rats and horses injected with attenuated forms of *Corynebacterium diphtheriae* produced 'antitoxins' (antibodies) in their sera that could then be used to treat patients. In 1891, the mortality rate from the disease reduced from more than 50% to 13%, a drop so important that in 1901, Dr von Behring received the first Nobel Prize in Physiology or Medicine for this important breakthrough and was instilled as the founder of serum therapy.

Although successful, this 'passive' serum treatment prevented diphtheria for only a short time. In 1901, while searching for a treatment that worked longer, Dr von Behring injected patients directly with attenuated bacteria or with mixtures of antiserum and attenuated bacteria so that patients produced their own antibodies. This discovery led to the first 'active' immunization strategy.

In 1891, a tetanus serum was introduced as a therapeutic agent against tetanus in animals valuable to agriculture. At the onset of the First World War, as many lives were lost not from the battlefield but from tetanus, the serum was injected into soldiers. For preventing the disease during battle and saving many lives, Dr von Behring was awarded the Prussian Iron Cross Medal as a 'Saviour of German Soldiers' (NobelPrize.org).

These two occasions directed investigators to the wonders of antibodies. Although antibodies were still mysterious in the early 1900s, researchers already knew their value in medicine. To define them and to establish a standard by which they could be measured, Dr von Behring worked with Professor Paul Ehrlich (Figure 1.2) at Robert Koch's Institute in collecting serum from large animals and working on the serum therapy protocol. By 1913, Dr von Behring had

Figure 1.1 Dr Emil von Behring

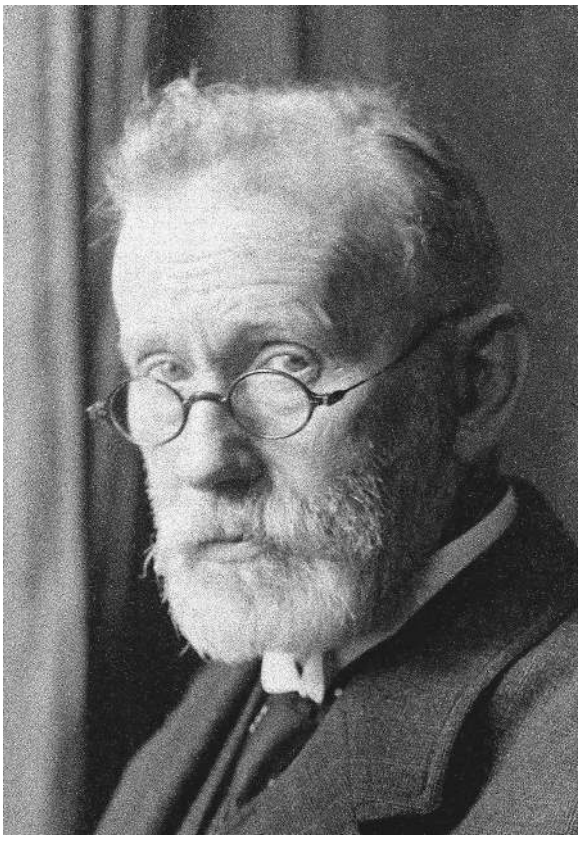

Figure 1.2 Professor Paul Ehrlich

produced his first active vaccine. Having attenuated both diphtheria antigen and antibody (mixing both active and passive therapy), the vaccine was aimed at both long-term protection against and short-term neutralization of the bacteria (Childs 2014).

Professor Paul Ehrlich was a pioneer in pathology renowned for his contributions in the fields of histochemistry, haematology and microbiology. Notable works include the theory and practice of staining animal tissues with aniline dyes, his research categorizing dyes into basic, acidic or neutral groups, and the classification of blood cell granules. In histology, Ehrlich is synonymous with a specific preparation of a working haematoxylin formulation bearing his name.

In 1896, Professor Ehrlich was appointed director of the Institute for the Control of Therapeutic Sera. Researching the characteristics and mechanisms of the antigen–antibody reaction, he found that the infective agent comprised two groups, the toxic region and the toxoid. The latter in his view was the group responsible for fuelling the production of antibodies. He noticed that heat enhanced the antigen–antibody reaction while cold restricted it, and that the binding was specific but could be broken with hydrochloric acid. For this groundbreaking work, Professor Ehrlich was awarded the Nobel Prize in Physiology or Medicine in 1908.

Basic Principles of Immunohistochemistry

The overall aim of IHC is to detect the presence, distribution and location of specific molecules (antigens) in cells and tissue. To make this possible, the three main concepts of *immunology*, *histology* and *chemistry* are required. Immunohistochemistry first employs antibodies to identify antigens and second affords the ability to visualize the reaction. Although less sensitive than Western blots or enzyme-linked immunosorbent assays (ELISA), IHC has the main advantage of enabling the investigator to observe and understand disease processes in a morphological context within an intact tissue environment.

Immunology Concepts

Antibodies, otherwise known as immunoglobulins, are serum proteins produced by B-cell lymphocytes that play an important role in an animal's defence system. They have the ability to bind with specific antigens that the body sees as foreign, thus initiating an immune response. The term 'antigen' is coined from two words: 'antibody' and 'generator' (Male and Roitt 1998). More precisely, it is the epitope on the antigen that the antibody recognizes. Multiple epitopes can be found in one antigen molecule.

Figure 1.3 represents an antibody with two light chains and two heavy chains arranged in a 'Y' shape. The light chain is connected to the heavy chain via a sulphide bond and the two heavy chains are joined together by a disulphide bridge. Note the distinct areas of the Fab and Fc regions. The binding site is at the Fab location and this is where the antibody latches onto the epitope. It is also called the variable region because this Fab site adapts to all of the variations an epitope can present to the animal. The Fc region belongs to a cascade of events that ultimately recruits phagocytes to destroy the pathogen. This body of knowledge came from the combined effort of astute workers, some of whom are now highlighted.

In 1897 at Vienna's Serotherapeutic Institute, Dr Rudolf Kraus demonstrated a method to detect antigen–antibody complexes. His precipitin reaction resulted from incubations of serum containing anti-cholera antibodies obtained from rabbits with soluble *Vibrio cholerae* bacterial infiltrates. He proved it again with serum containing anti-typhoid antibodies with *Salmonella enterica* serotype Typhi bacterial infiltrates. Each incubation resulted in the production of insoluble clumps of antigen–antibody complexes.

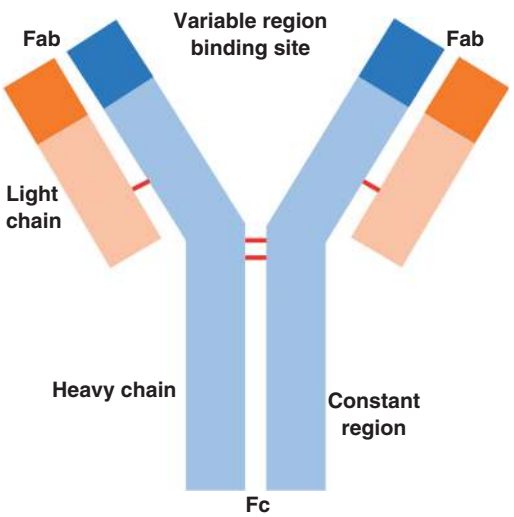

Figure 1.3 Schematic of an antibody

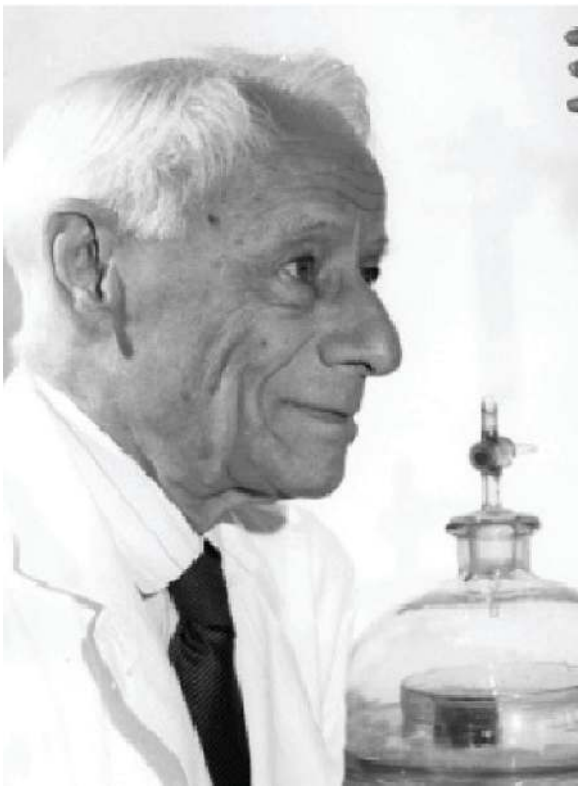

Figure 1.4 Dr Michael Heidelberger. Courtesy University archives, Rare Book & Manuscript Library, Columbia University Libraries

The precipitation reaction was of great interest to researchers, but it wasn't until 1920 that the next significant breakthrough was made. Figure 1.4 shows Dr Michael Heidelberger, a person highly regarded as one of the fathers of immunology and the founder of modern immunochemistry, a branch of biochemistry that examines the mammalian immune system on a molecular level. He established the first coloured antigen–antibody complex. Under the tutelage of Dr Oswald Avery, himself a notable figure in early DNA research, Dr Heidelberger identified the protein nature of antibodies and noted that the antigens he was working with were polysaccharides (Van Epps 2006). Further discoveries revealed antibody origin, function and specificity. These revelations assisted in understanding immune reaction chemistry and molecular mechanisms, which then led to more effective antisera for the treatment and prevention of bacterial infections.

Throughout his career, Dr Heidelberger received many awards in recognition for his work. He received 15 honorary degrees, 46 medals and 2 Albert Lasker awards in 1953 and 1978. For his work in developing quantitative analyses of antibodies, he was decorated with the Louis Pasteur Gold Medal in 1960. This prestigious honour is given by the Swedish Medical Society once every 10 years. Dr Heidelberger's other significant prizes include the Bronze Medal of the City of Paris (1964), the National Medal of Science (1967) and the Louisa Gross Horwitz Prize in 1977 (Stacey, 1994).

In the 1930s, Professor John Richardson Marrack was another researcher intrigued by the antigen–antibody complex and its crystal lattice-like formation. He believed the antibody as well as the antigen had more than one binding site and that the precipitating complex formed was the result of an antibody bridging with two multivalent antigen molecules. Working with diphtheria, he showed the toxin–antitoxin precipitate was made up of mostly antitoxin and this compound consisted of serum globulin. In 1934, Professor Marrack succeeded in attaching a dye to antibodies and through quantitative tests showed that for optimal precipitation, concentrations of antigen and antibody needed to be ideal. Researchers still adhere to this practice today when diluting out concentrates for IHC assays. In 1938, he wrote a book about antigens and antibodies, explaining the relationship between specificity and chemical structure and highlighting his thoughts on the nature of the antigen–antibody complex. In 1971, for pioneering work in the chemical interpretation of antigen–antibody interactions, Professor Marrack received the Distinguished Service Award of the First International Congress of Immunology (Complete Dictionary of Scientific Biography).

The works concerning antibodies and antigens from these early pioneers at the dawn of the twentieth century laid the groundwork for the continued evolution of IHC. The next leap was to bring the technology *in vivo* and observe the antigen–antibody reaction from a morphological viewpoint.

Histology Notions

When a tissue sample is analysed for disease, the first step is gross (macroscopic) examination noting structures and changes to the tissue that are large enough to be visible to the naked eye. Areas of particular interest are sampled and placed into histology cassettes for processing. Routinely, the submitted tissue pieces must be no larger than 25 mm long x 20 mm wide x 4 mm thick. Samples at this size will fix and

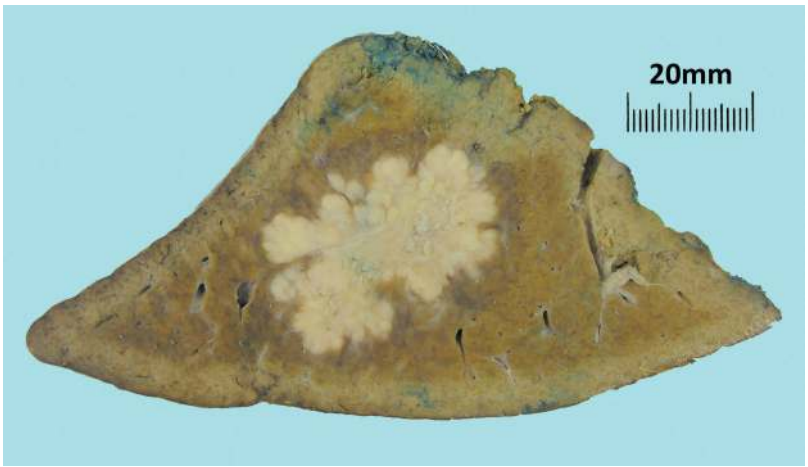

Figure 1.5 Gross macroscopic 'cut-up' is the foundation of anatomical pathology

process optimally on a routine overnight schedule. For formalin fixed paraffin embedded (FFPE) blocks, tissue fixation and processing are fundamentally the most important factors for IHC success. Figure 1.5 shows a section of liver sampled at the macroscopic 'cut-up' to visualize the lesion within the organ. A complete description and measurements are recorded before further dissection into cassettes. The main objectives in this clinical situation are finding answers to questions such as:

- What is the nature of this lesion?
- The description of the morphological features observed under the microscope
- Is this tumour a primary liver cancer or a metastatic deposit from somewhere else in the body?
- Are margins involved? Is there evidence of local spread or metastasis to distant sites?
- What are the prognostic implications for the patient?
- Are specific therapies suitable for this type of cancer?

A series of histological procedures ensues: tissue dehydration and clearing before paraffin wax infiltration, block embedding before microtomy and mounting onto glass slides. Sections are stained for haematoxylin and eosin (H&E), providing the first glimpse of morphology under microscopy. The H&E, using the basic haematoxylin dye to stain nuclear components blue, and eosin, an acidic dye, to stain cytoplasmic components pink, is the gold standard morphology stain in histopathology. A rather simple stain to perform, it highlights significant features of the tissue that if left unstained would be invisible. The subtle hues of the H&E stain allow the trained pathologist to microscopically assess tissue structure and morphology.

Not all structures, however, are demonstrated by the H&E stain. Some structures require the use of special stains to identify and reveal them. As shown in Figure 1.6A, reticulin fibres require silver impregnation to highlight the black type III collagen within organs of the reticuloendothelial system such as the liver. A trichrome stain developed by Dr Pierre Masson is seen in Figure 1.6B. This technique uses a selection of dyes to differentiate muscle from collagen fibres in connective tissue (blue). Other tissue entities such as fibrin and erythrocytes are also identifiable in trichrome stains. Note also the magnification given for Figure 1.6 is 'x40'. This indicates the objective lens on the microscope used to capture the image is rated at x40. The magnification in real terms is achieved by multiplying the objective lens magnification by the eyepiece magnification. For all images used in this chapter and throughout the book, the eyepiece objective is always x10. Therefore the 'real' magnification of images shown in Figure 1.6 is 400x (40 x 10).

The main objective of performing H&E and special stains on tissue sections mounted onto glass slides is to enable the pathologist to observe the morphological picture via microscopy and relate the findings back to the clinical history. A comprehensive illustration of the pathological processes occurring within the tissue specimen is the ultimate aim. Often, details are seen which are not easily recognized or understood. This is because many diseases mimic each other with similar cellular structures but actually differ in their behaviour and how they are treated. Where accurate diagnoses

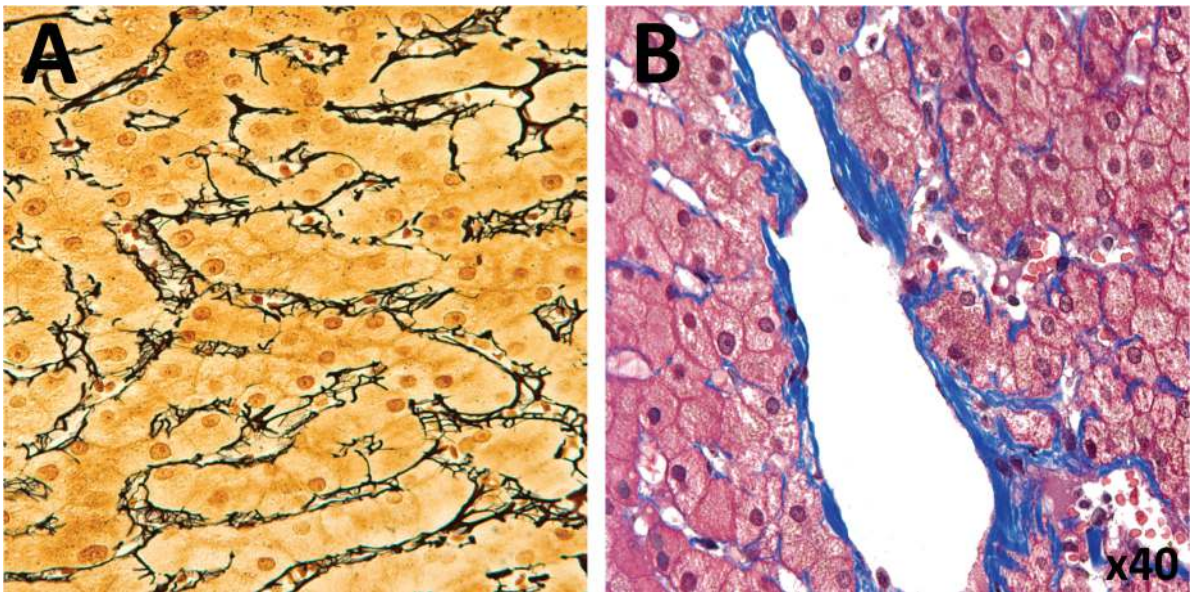

Figure 1.6 Reticulin techniques (A) and trichrome methods (B) are common histology special stains

are concerned, knowing the differences and interpreting them precisely is vital. Immunohistochemistry adds value to this endeavour. It is applied in both diagnostic anatomical pathology and research applications to assist with:

- diagnosis of disease and determining whether a tumour is benign or malignant
- identification of cell type and lineage to histologic origin and primary site of tumours
- identification of prognostic factors and therapeutic indications of some diseases
- identification of structures, organisms and materials secreted by cells
- diagnosis of morphologically non-differentiated neoplasms
- subtyping of neoplasia (such as lymphomas)

Combining immunology concepts to a morphological context is only possible if the investigator can see the reaction. To fulfil this criteria, IHC practice is grounded in the science of chemistry. Without it, microscopic examinations would not be possible.

Chemistry Influences

Performed on tissue sections that are either freshly frozen or FFPE, IHC detects various entities within tissue via a label attached directly or indirectly to an antibody, hence the often-used nomenclature of 'immunolabelling'. The nature of this label dictates the chemistry required to develop and observe the antigen–antibody reaction. The advantage is the spatial information offered, the specific locale within the tissue specimen that is tagged for evaluation via light, electron or fluorescence microscopy. The label can be a fluorophore, chromogenic dye, metal element or radioisotope with each requiring specific protocol development. The science behind two of the most commonly used labels in IHC standard practice is now explored further.

Visualization via Fluorescence

Fluorescence is a phenomenon whereby light at a certain energy (wavelength) has the ability to excite certain compounds to such a degree that when absorbed, it will emit light at a longer wavelength. Naturally occurring substances displaying this marvel are called fluorophores. Dyes and chemicals added to materials to confer fluorescence are known as fluorochromes (Bancroft and Floyd 2008). Immunofluorescence (IF) employs fluorochromes as the label conjugated directly or indirectly to the antibody. However, a specialized microscope different to a conventional optical light set-up is required. Before discussing why this is so, it is pertinent to explore how IF originated.

In 1939, Dr Albert Hewett Coons at Harvard University was investigating the components of Aschoff nodules in rheumatic fever. He studied the nature of antigens and antibodies involved with their formation and wondered if it would be easier to localize the antigen by labelling the antibody molecule with something visible.

Working together with organic chemists Dr Louis Fieser, Dr Hugh Creech and Dr Norman Jones, he coupled anthracene isocyanate to anti-pneumococcal serum and succeeded in agglutinating the pneumococci. The bacteria fluoresced brightly under ultraviolet (UV) light, but this feat was hindered by unwanted background fluorescence seen in many other structures at the blue or red wavelengths. Dr Coons then switched the label to fluorescein isothiocyanate (FITC) to observe the characteristic apple green fluorescence. Visualization of the label was possible via a fluorescence microscope apparatus newly developed by an assistant professor, Allan Graffin, at Harvard's Department of Anatomy. Dr Coon's success with the basic fluorescein-labelling procedure paved the way for diagnostic fluorescence microscopy techniques in bacteriology, immunology and anatomical pathology. For his pivotal work with IF and the discovery that plasma cells make antibodies, Dr Coons received the Albert Lasker Basic Medical Research Award in 1959. He was also the recipient of numerous international honorary academic degrees and awards. These include the Paul Ehrlich Award in 1961, the Passano Award in 1962, the Gairdner Foundation International Award in 1962 and the Emil von Behring Prize in 1966 (American Association of Immunologists).

Fluorescence microscopy is different to conventional light microscopy. The 'light source' must have enough energy to excite the fluorochromes. Commonly, wavelengths in the UV spectrum produced by mercury vapour or xenon gas lamps are utilized. Nowadays, metal halide and light-emitting diode (LED) technology may also be employed to create the required wavelengths. This light is called the excitation wavelength because it is used to excite the fluorochrome. After absorbing the light at the excitation wavelength, the fluorochrome undergoes a change at the atomic level, resulting in a variation to the electron distribution pattern. Energy in the form of emitted radiation is released from the fluorochrome at a longer wavelength and therefore the fluorochrome possesses less energy. This change in energy state is referred to as 'Stoke's shift'. To observe these changes, special filters fitted to the microscope are required. Often termed 'dichroic mirrors', they are designed to transmit light at a certain wavelength to the fluorochrome whilst reflecting away other unwanted wavelengths. These mirrors are often called filter sets and they must be matched to the fluorochrome characteristics. For example, FITC has maximum excitation at 494 nm and emission spectra at 519 nm (Horobin 2002). The dichroic mirror must allow light at wavelength 494 nm through to excite the fluorochrome and then permit light at 519 nm to reach the eyepieces. Notice the reason we see FITC as a green colour is because 519 nm is in the green zone of the visible light spectrum. The path of light after passing through the dichroic mirror to reach the fluorochrome is via the objective lens to excite the specimen and then back up the objective lens again to reach the eyepiece.

To summarize, let's follow the light path. From the lamp source, light in the UV band passes through a dichroic mirror (filter), down through the objective lens and then on to the specimen containing the fluorochrome. It then travels back up the objective lens, through the dichroic mirror again on its way to the eyepiece for visualization. Hence the term 'reflected light microscopy' is often used to mean fluorescence microscopy. To get the most out of fluorescence microscopy, a darkroom is required and since fluorescence fades over time, it is common practice to take digital images for record-keeping. Although it is quite a sensitive marker, fluorescence does have advantages and disadvantages. Fortunately, investigators have other options and technologies with which to 'label' their antibodies.

Visualization via Enzymes

For more than 40 years, IF was considered a powerful diagnostic aid. However, the method clearly had faults. The most frustrating were the need for an expensive fluorescence microscope, the instability of fluorescein over time, autofluorescence in some tissues – especially with formaldehyde fixation – and the failures at the electron microscope (EM) level. Many attempts were made to counteract these issues; some workers used radioactive antibodies while others relied on heavy metals to tag antibodies. In 1966, it was Dr Richard Graham and Dr Morris Karnovsky's studies of enzyme activity that paved the way for others to successfully attach enzymes and confer them as labels conjugated to antibodies.

The enzyme label is visualized by the enzyme's involvement in the chemical reaction with a suitable chromogen inducing a visible coloured or election-dense precipitate with its substrate. The enzyme commonly used for IHC is horseradish peroxidase (HRP). As shown in Figure 1.7, in the presence of the substrate hydrogen peroxide (H_2O_2), HRP drives the oxidation of 3,3'-diaminobenzidine (DAB), resulting in an insoluble, photostable, brown-coloured precipitate at the antigen–antibody site. Addition of nickel, cobalt or copper salt renders the DAB precipitate different shades of brown through to black. Use of enzymes as the label to observe reactions with a conventional optical microscope proved popular. In addition to HRP, different enzymes can be used for different situations, depending on the desired colour for the end product. Other enzymes deployed include alkaline phosphatase, β-galactosidase and glucose oxidase.

Horseradish Peroxidase

This enzyme has a molecular weight 40kDa glycoprotein that consists of 18% carbohydrate isolated from the root of the horseradish plant. Horseradish peroxidase has an iron haem group (haematin) as its active site which forms a complex with the substrate H_2O_2 and decomposing it to H_2O and O_2 when a H^+ donor is available. Chromogens such as DAB are employed as H^+ donors, resulting in an oxidization to form an insoluble brown product. Note the substrate for HRP is hydrogen peroxide (H_2O_2) and not to be confused with the chromogen DAB. Table 1.1 lists alternative chromogens suitable for HRP-based IHC.

Alkaline Phosphatase

This enzyme, derived from calf intestinal alkaline phosphatase (AP), has a molecular weight of 100kDa. The histochemical reaction can be described as a simultaneous coupling or azo coupling whereby the enzyme removes and transfers phosphate groups from organic esters (substrate) by breaking the P-O bond. The esters are hydrolysed to phenolic compounds. The phenols then couple to diazo salts to produce the coloured end product as highlighted in Figure 1.8 using naphthol AS-MX phosphate as the substrate and Fast red as the diazo salt. The reaction product is a red insoluble precipitate but labile to alcohols. Mounting is possible in solvents such as DPX, but the dehydrating steps with alcohol should be avoided and replaced with oven drying.

Other possible substrates include Naphthol AS-BI phosphate, Naphthol AS-TR phosphate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Table 1.2 lists alternative chromogens for AP.

Glucose Oxidase

This enzyme, derived from *Aspergillus niger*, has a molecular weight of 185kDA. Not commonly utilized when compared to HRP and AP, the enzyme facilitates a simultaneous redox reaction in which the substrate glucose is oxidized at the same time as a colourless tetrazolium salt is reduced to a coloured formazan. The colour of the formazan depends on the tetrazolium salt used. Three common salts include:

i. Nitro blue tetrazolium (NBT) = blue reaction product

Table 1.1 A selection of chromogens capable of development with horseradish peroxidase

Hydrogen donors (chromogens)	Colour	Solvent
3,3'-diaminobenzidine tetrahydrochloride (DAB)	brown	resistant
3 amino-9-ethylcarbazole (AEC)	red	labile
2-methyl-2 H-isothiazol-3-one	green	resistant
4-chloro-1-Napthol	blue	labile
p-phenylene diamine dihydrochloride & pyrocatechol in 1:2 ratio (Hanker-Yates)	blue	resistant

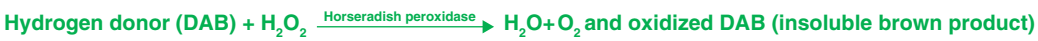

Figure 1.7 The chemical reaction for diaminobenzidine (DAB) using horseradish peroxidase (HRP)

Figure 1.8 The chemical reaction for Fast red using alkaline phosphatase

ii. Tetra nitro blue tetrazolium (TNBT) = brown reaction product
iii. Iodonitro tetrazolium (INT) = red/purple reaction product

β-galactosidase

This enzyme has molecular weight 500kDa and is isolated from *Escherichia coli*. Not commonly used for IHC, it employs the indigogenic histochemical method as highlighted in Figure 1.9.

For standard practice in diagnostic IHC, the two enzyme labels most commonly used are HRP and AP. Although other colours are available, the conventional pairing is DAB with HRP and Fast red with AP. Both pairings are counterstained with haematoxylin for routine optical light microscopy.

Conjugating Antibodies

When the early researchers first bound antibodies to antigens, the specific class of antibodies most often used were immunoglobulin G (IgG) molecules as depicted in Figure 1.3. These molecules have two variable regions specific for antigen binding as well as a constant region where labels can be attached. A quick and short single-step 'direct approach' as highlighted in Figure 1.10 was applied by Dr Coons when he conjugated the label FITC directly to antibodies for attachment to the antigens in his experiments. Using a fluorochrome in this instance, the label can vary with the most popular being an enzyme or metal particle such as colloidal gold. Radioisotopes have also been used.

Even though the direct approach is still in use today due to its simplicity, there are inherent practical shortcomings. The method is insensitive, producing low-signal yields, which necessitates the need for copious amounts of primary antibody to increase the signal-to-noise ratio. This workaround is not always possible, especially with epitopes that are naturally 'low expressors'.

Later research revealed that antibodies could effectively act as 'antigens'. This led to the discovery of secondary antibodies produced against the IgG of the species that made the primary antibody. Secondary antibodies are often produced in larger species offering plentiful sera. Using a two-step 'indirect approach' as highlighted in Figure 1.11, an unlabelled primary antibody reacting with the tissue antigen is applied first, followed by a labelled secondary antibody. The label conjugated to the secondary antibody indirectly locates the epitopes. A big advantage over the direct method is that multiple labelled secondary antibodies can bind with different antigenic sites on a single unlabelled primary antibody, which, in turn, increases the sensitivity of the assay. Another advantage is the ability to use the one labelled secondary antibody with many primary antibodies (clones) raised from the same animal species. Likewise, the secondary antibody can also be conjugated with fluorescent dyes (indirect fluorescence method) or enzymes (indirect immunoenzyme method).

When researching the enzyme–antibody conjugation at the EM level, Dr Stratis Avrameas of the Pasteur Institute in Paris used glutaraldehyde to conjugate peroxidase to IgG. Using these conjugates, in 1971, he developed the enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA), both of which are 'close relatives' of IHC and widely used for clinical and research purposes.

While Professor Avrameas worked on enzyme immunoassays, Dr Paul Nakane, an assistant professor at the University of Michigan, also researched immunoenzyme techniques and successfully conjugated peroxidase to IgG with the bifunctional reagent p,p'-difluoro-m,m'-dinitrodiphenyl sulphone. Another

Table 1.2 Chromogens capable of development with alkaline phosphatase

Ester compounds (chromogens)	Colour	Solvent
Fast blue BB	blue	labile
Fast red LB	red	labile
New Fuchsin	red	resistant
Fast Garnet GBC	green	labile
Nitro blue tetrazolium (NBT)	blue	resistant
Iodonitro tetrazolium violet (INT)	purple	resistant

5-bromo-4-chloro-3-indoxyl - β-D-galactoside —Enzyme→ indoxyl group —Azo salt→ Azo indoxyl dye (blue)

Figure 1.9 The chemical reaction for β-galactosidase

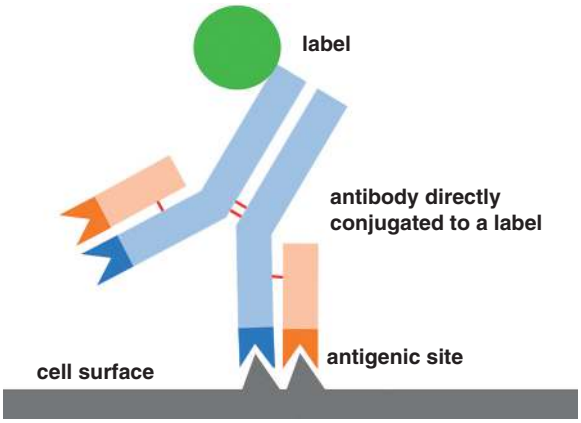

Figure 1.10 Single-step 'direct' immunolabelling

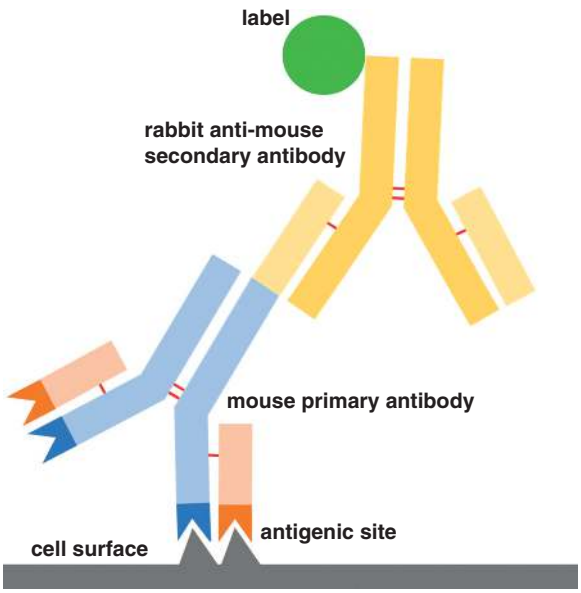

Figure 1.11 Two-step 'indirect' immunolabelling

challenge Dr Nakane undertook was exploring whether immunoenzyme techniques could be applied to the detection of multiple antigens in the same cells or tissues. We now call this endeavour 'double IHC staining' or 'multiplex IHC'. He experimented with a sequential-type multiplex technique using indirect peroxidase labelling on rat pituitary tissue localizing luteinizing hormone, growth hormone and thyroid-stimulating hormone on the one tissue section (Nakane 1968). The method can be summarized as employing the indirect peroxidase technique several times with different primary antibodies each time to locate discrete epitopes and developing them with separate colours. Although primitive compared to today's standards, using multiple sequences with various peroxidase substrates, he and his co-workers established that this could be done.

As a result of further developments in the late 1960s and 1970s to eliminate endogenous enzyme activity whilst maintaining tissue structure, the enzyme-based immunolabelling techniques proved superior to IF methodologies. Less (more diluted) primary antibodies could be used in detecting antigens. The labels did not fade and could be adopted for both the electron microscope and the standard optical brightfield microscopes.

Variations on the Theme

Even though enzyme-immunolabelling techniques were now preferred, there were practical problems still to be solved during the late 1960s to early 1970s. Most prominent was finding solutions to increase the efficiency of labelling and the ability to conjugate enzymes to all antibodies. Three prominent research groups emerged. They were headed by Dr Samuel Spicer from the Medical University of South Carolina, Dr Ludwig Sternberger, a professor at Johns Hopkins University, and Dr Stratis Avrameas of the Pasteur Institute in Paris.

Each team focused on finding suitable alternatives to the use of chemical conjugates for attaching enzymes. They found that anti-peroxidase antibodies can be used as a bridge to introduce the peroxidase enzyme to the assay. This new technology was coined the 'immunoglobulin-enzyme bridge method' by Dr Spicer, the 'unlabelled antibody method' by Dr Sternberger and the 'mixed-antibody method' by Dr Avrameas.

Each research group's technique used indirect immunolabelling in a series of layers, the first layer being the unconjugated primary antibody followed by the second layer of the secondary antibody. The third layer is the anti-peroxidase antibody produced in the same species as the primary antibody. The fourth layer constitutes peroxidase enzyme, which is added to the assay in order to bind to the tertiary antibodies already present in the system. Finally, the fifth layer is added to the system containing the peroxidase substrate H_2O_2. Emphasized schematically in Figure 1.12, this IHC assay was known as the 'sandwich approach' and despite possessing a low-affinity anti-peroxidase-to-peroxidase bridge layer, the method was more sensitive and effective than any before it.