Advances in Histopathology – Immunohistochemistry; the quiet Revolution continued

Whilst the Haematoxylin and Eosin (H&E) stain continues to be the universally accepted routine stain of choice, over the past couple of decades there have been significant advances in immunohistochemistry (IHC) which have revolutionised our diagnostic approach to tumour and to a less extent non tumour histopathology specimens. The immunohistochemistry field is rapidly changing with improving technology and an increasing array of commercially available antibodies are now available. Prior to the immunohistochemistry age, pathologists only had a limited number of histochemical stains (for instance mucin and silver stains) together with electron microscopy to assist in classification of tumours.

### Immunohistochemistry Definition

Immunohistochemistry combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. Immunohistochemistry makes it possible to visualise the distribution and localisation of specific cellular components within a cell or tissue on microscopic slides. The term immunohistochemistry is often used interchangeably with immunocytochemistry and immunostaining.

### Milestones in Immunohistochemistry

The concept of immunohistochemistry has existed since the 1930s and in 1942 Coons and his colleagues used fluorescent labeled antibodies to demonstrate pneumococcal antigens in infected tissues. Since this time there have been a series of progressive scientific discoveries and technological improvements which now make immunohistochemistry a routine and essential technique in laboratories. Key advances are summarised in Table 1.

### Development of Antibodies

Antigens have multiple determinants (epitopes) all inducing the formation of antibodies of differing specificity and affinity. Even a single epitope can induce the formation of several antibodies of differing specificity and affinity.

Polyclonal antibodies were initially available, derived from the serum of laboratory animals which had been exposed to antigens. A major advance was the ability to fuse single immunoglobulin producing plasma cells with non secreting myeloma cells resulting in hybrid cells which produced monoclonal antibodies. The hybridoma clones in cell culture medium produced monoclonal antibodies for research and commercial use. With the advent of molecular biology it is now possible to clone the segment of DNA responsible for a specific antibody into a vector, for instance phage, E.coli or yeast and commercially produce monoclonal antibodies.

### Development of Sensitive Detection and Amplification Systems

The site of localisation of antibodies (and their corresponding antigens) in tissues required the development of detection systems. Initially antibodies were tagged with fluorescent markers on frozen section slides, which added significant collection and procedural difficulties. Practically this was difficult and not applicable in routine histopathology laboratories with standard formalin fixed tissues. A number of detection systems have been developed which work on routine formalin fixed tissues (following antigen retrieval), often used in conjunction with an amplification step to highlight previously undetectable antibody-antigen binding sites. The commonest detection method is the peroxidase antiperoxidase system (PAP), see Figure 1. The PAP reagent comprises antibody against horseradish peroxidase and horseradish peroxidase antigen which gives a brown immunostaining reaction.

### Table 1

**Key Advances in Immunohistochemistry**

- Antigen retrieval systems enabling immunohistochemistry to be consistently and reliably performed on routine formalin fixed and paraffin processed tissue. (Previously much of this work could only be done on frozen section material)
- Development of a broad range of polyclonal and monoclonal antibodies
- Development of sensitive detection and amplification systems, enabling demonstration of previously undetectable antigens
- Availability of automated immunostainers

Figure 1 Peroxidase Antiperoxidase system

![Peroxidase Antiperoxidase system](image)
Antigen Retrieval Systems

Formaldehyde fixation continues to be the fixative of choice for routine laboratories. Aldehydes fix tissues by crosslinking amino acids with methylene bridges, causing denaturation of molecules and masking antigenic sites (epitopes). The longer the specimen remains in formalin, the greater the degree of antigen masking.

The antigenic sites (epitopes) were initially unmasked on formalin fixed tissue sections using proteases (PIER – protease induced epitope retrieval). Whilst this was useful, the technique had its limitations. A significant advance was made when it was discovered that heating formalin fixed paraffin sections in a fluid medium exposed previously masked antigens (HER – heat induced epitope retrieval). A range of heating systems are used including microwave ovens, autoclaves, pressure cookers, vegetable steamers, ovens and thermal cyclers. The duration of heating required depends upon the maximum temperature reached and the time that the tissue had been fixed in formalin.

Range of Antibodies Available

In general monoclonal antibodies can be developed against virtually any antigen and an extensive range of antibodies are available for routine diagnostic use. Examples include antibodies directed to cell adhesion and surface markers, cytoplasmic elements and structures (for instance intermediate filaments – keratins and vimentin), nuclear proteins, hormone receptors, markers of muscle, neuroendocrine, endothelial and melanocytic differentiation. Cell proliferation markers include proliferating cell nuclear antigen (PCNA) and Ki-67. A number of tumours have chromosomal translocations and these cells have expression of gene products which can be identified with immunohistochemistry (which serves as a surrogate marker for the chromosomal translocation). Tumours with known translocations which can be detected by immunohistochemistry include Ewing’s sarcoma/primitive neuroectodermal tumour, desmoplastic round cell tumour and alveolar soft part sarcoma.

Table 2
Application of Immunohistochemistry

| Diagnosis of primary malignant tumours – some tumours are so poorly differentiated that their histogenesis is not apparent on routine haematoxylin and eosin stained sections |
| Determining the likely site of origin of metastatic tumours |
| Categorisation of leukaemias and lymphomas e.g. T and B cell markers |
| Detection of molecules that have prognostic or therapeutic significance e.g. oestrogen and progesterone receptor protein. HER2 receptor |
| Detection of minimal disease – highlighting small numbers of tumour cells, which may be difficult to appreciate on routine sections, small volume residual tumour in resection specimens or sentinel lymph node biopsies |
| Use in conjunction with fine needle aspiration cytology – evaluation of cell block material to ascertain nature of primary tumour and or metastasis |
| Highlighting proximity of poorly differentiated single tumour cells to surgical margins |

Application of Immunohistochemistry in Routine Laboratories

Immunohistochemistry is valuable in the diagnosis and management of tumours and it has applications in a number of scenarios, some of which are listed in Table 2 (previous page).

Immunohistochemistry is an adjunct to the diagnosis of tumours and the findings must be interpreted in the light of the features seen on the routine haematoxylin and eosin sections and the clinical setting. Figure 2. demonstrates positive S100 protein staining, which in conjunction with a panel of other immunostains assisted in the diagnosis of invasive malignant melanoma. Also note that on this section the stain highlights greater numbers of tumour cells, than initially appreciated on the haematoxylin and eosin stained section.

Limitations of Immunohistochemistry and Approach to Diagnosis

There are a number of causes of false negative and positive staining. In addition some tumours may develop aberrant expression of various antigens, not detected in normal mature cells. Other tumours may lose or only partially express antigens which are present in normal mature cells.
Clinical Benefits of Immunoperoxidase Tests

- Immunoperoxidase testing can be performed on biopsy samples and cell block material, enabling rapid diagnosis, without the need for frozen section and or excisional biopsy prior to definitive therapy.
- Definitive diagnosis based upon biopsy material enables pre-operative chemotherapy and radiotherapy, decreasing tumour size prior to definitive surgery and improving prognosis for some advanced tumours.
- Diagnosis based upon at times relatively minimal tissue enables accurate prognostication and in some clinical circumstances therapy without definitive surgery e.g. hormone and chemotherapy for breast carcinoma metastases.
- In rare circumstances tumour diagnosis can be made on partially crushed biopsy material which in the past would have been not diagnostic of malignancy; enabling on going patient management, rather than open biopsy.
- Reduction in reports with indeterminate results.
- Advances in tumour molecular biology and immunoperoxidase techniques have improved classification of tumours and new entities have been discovered enabling more accurate prognostication and improved targeted therapy. An example of this is the significant advances which have been made in our understanding and classification of lymphomas.
- More specific diagnosis and prognostic information enabling targeted therapy. The advances in immunohistochemistry and tumour diagnosis coincide with the increasing array of tumour specific chemotherapy agents and therapy regimens that are currently available.
- Ascertaining the likely primary site of origin of metastatic tumour enables a targeted search for the primary, considerable saving of time and cost and allows specific therapy to be commenced with less delay.

In view of this and the differing specificity and sensitivity of antibodies a panel of immunohistochemical stains is usually performed in conjunction with control material on the slide. The control material may either be intrinsic to the specimen, for instance normal blood vessels in the specimen, or a section of known positive staining tissue is placed on the same slide as the test section (to ensure the same conditions for the control and test specimen).

The antibodies selected in the panel depend upon the clinical scenario. For instance if the tumour is a high grade breast carcinoma tests for oestrogen, progesterone and HER2 receptor proteins are used; if the latter stain is positive (and subject to other confirmatory tests) the patient is then eligible for Herceptin (Trastuzumab) therapy. A relatively common scenario in our practice is the differential diagnosis of spindle cell tumours of skin and a panel of stains is used, as shown in Table 3 and illustrated in Figure 3.

Table 3. Panel for the Workup of common Pleomorphic Cutaneous Spindle Cell Tumors, with Expected Immunophenotypes

<table>
<thead>
<tr>
<th></th>
<th>Cytokeratins</th>
<th>S100 Proteins</th>
<th>Melanocytic Markers (HMB-45, Melan-A)</th>
<th>Smooth Muscle Actin</th>
<th>Desmin</th>
<th>Endothelial Markers (CD31, CD34)</th>
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<tbody>
<tr>
<td><strong>Sarcomatoid SCC</strong></td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td><strong>Melanoma</strong></td>
<td>−/+</td>
<td>+</td>
<td>−/+</td>
<td>−</td>
<td>−/+</td>
<td>−</td>
</tr>
<tr>
<td><strong>AFX</strong></td>
<td>−</td>
<td>−</td>
<td>−/+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><strong>Leiomyosarcoma</strong></td>
<td>−/+</td>
<td>−/+</td>
<td>−</td>
<td>−</td>
<td>−/+</td>
<td>−/−</td>
</tr>
<tr>
<td><strong>Angiosarcoma</strong></td>
<td>−/+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<table>
<thead>
<tr>
<th><strong>SCC</strong></th>
<th>Squamous Cell Carcinoma</th>
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<tbody>
<tr>
<td>+</td>
<td>positive in more than 90% cases</td>
</tr>
<tr>
<td>+/−</td>
<td>positive in 50 to 70% of cases</td>
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<table>
<thead>
<tr>
<th><strong>AFX</strong></th>
<th>atypical fibroxanthoma</th>
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<tr>
<td>−</td>
<td>negative</td>
</tr>
<tr>
<td>−/+</td>
<td>usually negative but anomalous expression may be seen in up to 25% of cases</td>
</tr>
</tbody>
</table>
Figure 3. Atypical Fibroxanthoma (and overlying epidermis)

Haematoxylin and Eosin
Negative cytokeratin
Positive vimentin

References
3 Rosai and Ackerman’s Surgical Pathology. Juan Rosai. Ninth Edition
4 Seminars in Diagnostic Pathology. Immunohistochemistry in Tumour Diagnosis. Volume 17, No 3. August 2000
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