Histological staining techniques

WOLF D. KUHLMANN

Laboratory Diagnostics & Cell Science, 56112 Lahnstein

The first stainings were tried by A VAN LEEUWENHOEK (apparently in the years 1714-1719) and by R VIEUSSENS (about 1684) studying their objects with alcoholic saffron dyes (LEWIS FT, 1942). At last, genuine histological work began much later when T HARTIG, C WEIGERT, J GERLACH, P EHRLICH and H GIERKE started in the second half of the 19th century with their systematic studies of natural and synthetic dyes (aniline derivatives). Among the numerous famous names in histology, very outstanding persons and particularly responsible for pioneering the use of the microscope for the study of tissues were J MÜLLER, F HENLE and W WALDEYER, because their work influenced anatomy, improved the knowledge of diseases and opened the way of *pathology* as a medical discipline.

The principle of histological staining relies on the treatment of tissue sections with dyes in solution which react more or less specific with defined cell and tissue structures (see chapter *Dyes, stains and special probes in histology*). In its classical way, a uniform theory of tissue staining does not exist because the mechanisms of dye binding with the various cell components are quite heterogenous. Thus, conventional histological stainings must be seen in connection with the chemical and morphological behaviour of cell structures, and, histological stainings are very complex. In a particular case, both natural and synthetic dye staings may be due to (a) chemical reactions; (b) physical adsorptions or absorption; and (c) physico-chemical processes. Usually, chemical and physical processes act in parallel (BURCK HC, 1988).

Indigo: Indigo is a blue, crystalline organic chemical compound. Indigo can be obtained from the Indian indigo plant (*Indigofera tinctoria*) or the European woad (*Isatis tinctoria*). Since the end of the 19th century, indigo has mainly been produced by chemical synthesis.

Madder: Madder is a natural plant dye, also known as dyer's red or madder, a plant species from the genus *Rubia tinctorum* in the Rubiaceae family. Since the colouring agent alizarin could be produced synthetically from coal tar, the much more expensive madder cultivation has declined drastically.

Campeche: The bluewood tree or campeche tree (*Haematoxylum campechianum*) is a plant species found in Central America and the northern part of South America that belongs to the carob subfamily (*Caesalpinioideae*). Bluewood can be used to dye wool, cotton, linen and silk. The substance of bluewood used in histological techniques is haematoxylin. The combined use of haematoxylin and a metal salt goes back to F BÖHMER (1865). There are over 100 different recipes for the production of colour solutions containing haematoxylin.

The application of defined chemical and biochemical reactions in histology represented a breakthrough in specific histological staining. One of the earliest microchemical reactions was the discovery by H F LINK (1807) in which he used iron sulfate for determining tannic acid in leaves. Some years later, M RASPAIL (1825) used iodine for the microchemical detection of starch. Pioneering work in histochemistry was done by R FEULGEN, and the *Feulgen reaction* (FEULGEN R and ROSSENBECK H, 1924) is such an example. In its initial formulation, this stain was developed for the detection of DNA in the nucleus. In this context it is important to note that the *Feulgen reaction* is an ordered series of chemical reactions and not simply a stain. Since the demonstration that this technique is both specific and stoichiometric for DNA, this reaction has become an important means for staining nuclear DNA for densitometric quantification. In his treatise, K ZEIGER (1938) has given an excellent overview of the physicochemical basics of histological and histochemical staining methods.

During further developments of microscopical stainings, a significant step forward was obtained with the introduction of *enzyme histochemistry* when G GOMORI (1939) and H TAKAMATSU (1939) described independently methods for the demonstration of phosphatases in tissue sections. A grest number of histochemical reactions have appeared since then for the specific localization of enzymes at both light and electron microscopic levels which have been cumulated in well known treatises. The first have been published already in the years 1952 and 1953 (G GOMORI, *Microscopic Histochemistry - Principles and Practice, 1952*; AGE PEARSE, *Histochemistry Theoretical and Applied*, 1968, 1980). All these developments proved useful in the analysis of organ systems and cell compounds.

In the meantime, however, enzyme histochemistry in its strict sense has become a limited role. With the use of antibody labelings, originally introduced by AH COONS in 1941 as immunofluorescent technique, immunohistological methods are now regarded as tools with greater potential.

Immunohistology for specific cell analysis

Immunohistology was invented for the histologist as a specific histological tool for the identification of biomarkers which are relevant for the understanding of structure-function-relationships in cells and organs. Biomarkers are molecules with particular features that make them useful for histological tracing, measuring and monitoring under certain conditions such as phylogenetic, ontogenetic and disease development.

Methods which use antibodies are of special importance due to the inherent specificity of the antigen antibody reaction. Since their discovery by E VON BEHRING and S KITASATO (1890) more than a century ago, antibodies are used as highly selective ligand binding proteins and are widely employed for the detection and separation of antigenic molecules.

Immunohistology will give complementary diagnostic information to that obtained by the traditional morphological observations. The concept of cellular antigen staining in tissue

sections by labeled antibodies dates back to the early work of A H COONS and co-workers in 1941 describing an immunofluorescence technique for the microscope. In the beginning, simple methods were used which lacked sensitivity and specificity. Since then, improvements have been made in conjugation techniques, tissue fixation, selection of labels and microscopes.Now, immunohistochemistry has become part of molecular tissue pathology. The technique is a powerful but very complex tool, especially the use of high sensitive procedures and their application to detect one or multiple antigens in the same cell or tissue preparation.

Antibodies have applications in research, in diagnostics and in many specialized purposes which include pathology, forensic medicine, biochemistry and biology. Together with the modern concepts of molecular biology (notably nucleic acid probing by specific interaction of nucleic acid sequences with complementary probes and the polymerase chain reaction), antibody techniques are part of the most important tools in the study of the molecular and structural composition of cells. The extraordinary potential of immunocytochemistry can bridge molecular, cellular, organismal, and clinical investigations. Embedded in current proteomic technologies including cancer proteomics, immunocytochemistry contributes to the identification and understanding of complex changes in protein profiles.

Ligand-binding molecules for selective cell labeling

Antibodies possess a high degree of specificity towards antigenic determinants. Because of the narrow range of specificity of an antibody molecule to bind with its antigenic determinant, immunochemical methods are very sensitive techniques. With respect to the definition of antigenic molecules (substances which initiate the formation of and react with antibodies are called antigens), immunological methods are widely employed in qualitative and quantitative approaches since the very early years of the last century e.g. by EHRLICH, LANDSTEINER, WITEBSKY, HEIDELBERGER, MARRACK, KABAT, OUDIN, GRABAR and schools derived from these pioneers in immunochemistry.

Several types of antibody preparations exist for immunocytochemistry. These include antibodies of polyclonal or monoclonal origin; a pool of monoclonal antibodies is also useful. Polyclonal antibodies are readily obtained from animals (e.g. rabbit, goat, sheep, mouse or rat) upon immunization with appropriate antigen. Also, autoantibodies from patients with certain diseases may be used. These immune sera, however, will contain a mixture of numerous antibody populations directed against the different epitopes of the immunizing antigen. Hence, hyperimmune sera will suffer from the drawback of limited reproducibility. In order to overcome this problem, monoclonal antibodies will be a good alternative; they can be obtained by hybridoma techniques. Their great reproducibility and inherent antigen specificity for defined antigenic epitopes make monoclonal antibodies useful tools in immunological techniques.

Furthermore, genetic engineering such as phage display enables the production of recombinant antibodies. Usually, the sophisticated protein folding and modification machinery of mammalian cells is needed to obtain biologically active antibodies. Some of these limitations can be overcome by using smaller antigen-binding fragments of antibodies like Fab fragments which can be produced in *E. coli*. Then, alternative protein architectures which are easier to handle may become attractive. A novel class of engineered ligand-binding proteins such as the anticalins provide suitable properties as laboratory and diagnostic tools.

A number of phenomena which resemble antibody reaction are shared by lectins. These occur in a variety of plants, invertebrates and vertebrates and are used for the study of carbohydrate moieties. With their ubiquitous role in virtually all biological systems, glycans are vitally important molecules. Glycans are a large group of compounds consisting of sugars with diverse structures that are present inside and on the surface of cells. More than 50% of all proteins carry various glycan chains. They fulfill many different roles by interacting with proteins in a variety of biological events which underlie the development and function in multicellular organisms. Because lectins possess a high affinity and a narrow range of specificity for defined sugar residues, they are a powerful tool in carbohydrate studies. Lectins are today the most specific molecular probes for the histological localisation of glycoconjugate glycosylation. The principles described for immunohistology hold also true for lectin histology. In a similar way to immunohistological techniques, lectins which are either conjugated with markers such as fluorescein, enzymes etc. or not can be employed in "direct" and "indirect" staining procedures. In the latter techniques, a defined labeled antibody system is needed to localize the reaction site of the lectin itself.

In situ staining of molecules in cells and tissue preparations

With respect to immunocytochemistry, the purpose of most described procedures is the identification and characterization of cellular structure and function *in situ* rather than immuno-staining of physicochemically isolated constituents. Because the principles of immunohistology hold also true for nucleic acid staining by DNA and RNA hybridization techniques, both techniques are candidates to be joined in the study of life processes which depend so much on multiple external and intrinsic conditions. Usually, only parts of the genome become expressed as RNAs which concentrations, however, are not strongly correlated with the expression of proteins. Thus, studies of gene expression together with analysis of the proteome can provide useful information about the presence of characteristic proteins in defined conditions.

To this aim, the respective ligands are usually "labeled" in a way so that the formed complexes become "visible". Suitable substances for labeling purposes are those which lead to distinct fluorescent or colored reactions (light microscopic level) or which give significant deflection of electrons at the electron microscopic level. A milestone for investigations at the electron microscopic levels was the conjugation of the metalloprotein ferritin with antibodies by S J SINGER (1959) which enabled a new era of ultrastructure research.

As mentioned above, immunohistological techniques date back to the early works of A H COONS and co-workers in the early forties of the 20th century. Their immunofluorescent approach opened specific investigations on cellular structure and function at the light microscopic levels. Apart from the popular fluorophore labelings, enzymes are very interesting alternatives as marker molecules because they can be utilized for both light and electron microscopic studies. The first successful enzyme conjugation procedure for cellular antigen staining was developped by P K NAKANE and G B PIERCE (1966). Immunocytochemical developments are still in progress with respect to both antibody labeling methods and microscopes including image analysers and cameras which make cell staining more sensitive. It can be expected that these techniques will become more quantitative.

When labeled antibodies are used, cell staining can demonstrate the presence and the subcellular localization of an antigen. Furthermore, double or multiple labeling methods allow the simultaneous detection of different antigens. Immunohistological methods can be also

used in conjunction with many conventional histological stains and with autoradiography in order to compare the localization of the antigen with other cell-type markers. Moreover, immuno-staining techniques can be performed in connection with other selective staining procedures as for example in the research of glycans by the use of lectins or in nucleic acid labeling by use of selective nucleic acid probes.

The use of nucleic acid probes for the detection of DNA/RNA at the cellular levels follows staining principles which are quite similar to immunohistology. As described above, similarities concern the choice of marker molecules as well as the staining protocols (direct and indirect stainings) coupled with various antibody based detection systems. Popular nucleic acid stainings include the detection of genomic aberrations by single chromosome specific probes (FISH, fluorescence in situ hybridization), genetic mutations and gene expressions as well as studies on foreign genetic material as in the case of infectious agents by specific nucleotide probes with the techniques of *in situ* hybridization and *in situ* PCR. Technical developments are certainly just at the beginning.

In any case, generalized standard procedures for the detection of cellular ligands are not available. Usually, immuno-stainings must be established to each cellular model, f.e. by adaptation from existing protocols. Staining schedules have to reconcile at least three main steps:

- Specific antibody production or selective choice of another ligand specificity (f.e. artificial receptor proteins, lectins etc.) or appropriate nucleic acid sequences when probing for chromosomes and genomic DNA/RNA by *in situ* hybridization/*in situ* PCR,
- Selection of a detection system suitable for histological studies,
- Cell and tissue preparation including an appropriate fixation procedure.

Even if a wide range of ready-to-use reagents can be purchased, now, one has always to consider two aspects in immunohistology: (a) type and characteristics of the necessary reagents, and (b) type and morphological features of the cells/tissues to be stained. It appears necessary to work out primary and secondary antibodies and the best labeling conditions. Then, different tissue sampling protocols including different fixation schedules must be studied. For immunohistological stainings, different antibody concentrations, different incubation times and temperatures as well as different methods of blocking non-specific cell labeling must be examined. Because of all the numerous technical details in the preparation of antibodies, the selection of markers and their conjugation with antibodies, the preparation of tissues and the performance of cellular stainings, these aspects will be described in separate sections.

Evaluation of stained tissue sections

Immunostained sections with or without contrast enhancement (e.g. by postfixation with OsO_4) and either counterstained (e.g. with hematoxylin) or not are observed in the light microscope with typical Köhler's bright field illumination; for fluorescent preparations see chapter *Fluorescence and fluorescence microscopy*. Photographs are taken with an automatic microscope camera equipped with an electronic switch unit. Photos can be recorded on 35 mm black and white negative film (e.g. 18 DIN/50 ASA), on 35 mm tungsten color reversal film (e.g. 18 DIN/50 ASA; 3200º K) or on daylight color reversal film (e.g. 18 DIN/50 ASA). In the latter case, the lamp temperature is corrected from 3100º K to 5500º K by a conversion filter. The exposure time is modulated by gray filter. Alternatively, viewed tissue sections are

recorded by an integrated CCD color video camera. Documentation and archiving is done on disk by appropriate software.

In the case of immuno-electron microscopy, ultrathin sections are viewed by conventional transmission electron microscopesoperating at 80 kV with bright field illumination. For contrast enhancement, sections may be counterstained with lead citrate (e.g. for 30 sec) or other means. Areas of interst are either photographed with commercial film material (e.g. 6.5 x 9 cm) or recorded by electronic video camera. The viewed areas are documented and archived on disk by appropriate software.

Electronic documentation is today state-of-the-art in light, fluorescent and electron microscopies which allow to take measures in signal gain, contrast enhancement, noise elimination etc. Especially in modern fluorescence microcopy, imaging and high resolution of fluorescent signals depend largely on sophisticated electronic means.

Selected publications for further readings

Leeuwenhoek A van (1673)
Link HF (1807)
Raspail M (1825)
Müller J (1838)
Gerlach J (1848)
Hartig T (1854a, 1854b)
Gerlach J (1858)
Henle F (1840)
Henle F (1855)
Waldeyer W (1863)
Weigert C (1871)
Ehrlich P (1877)
Ehrlich P (1878)
Weigert C (1878)
Gierke H (1884)
Ehrlich P (1886a, 1886b, 1886c, 1886d)
Behring E von and Kitasato S (1890)
Weigert C (1904)
Feulgen R and Rossenbeck H (1924)
Zeiger K (1938)
Gomori G (1939)
Takamatsu H (1939)
Coons AH *et al*. (1941)
Lewis FT (1942)
Gomori G (1952)
Coons AH (1954)
Weller TH and Coons AH (1954)
Coons AH et al. (1955)
Coons AH (1958)
Riggs JL *et al*. (1958)
Singer SJ (1959)
Beutner EH (1961)
Nakane PK and Pierce GB (1966)
Avrameas S and Lespinats G (1967)

Pearse AGE (1968)
Avrameas (1970)
Nairn RC (1976)
Kuhlmann WD (1977)
Sternberger LA (1979)
Pearse AGE (1980)
Kuhlmann WD (1984)
Burck HC (1988)
Masters BR (2006)

Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

 12.07.2023