

Dyes, stains, and special probes in histology

WOLF D. KUHLMANN

Laboratory Diagnostics & Cell Science, D-56112 Lahnstein

Histological stains are traditionally important in order to study cell structures and intracellular or extracellular substances at the microscopical level; for historical views, stains and detailed descriptions of histological staining methods see WEIGERT C (1871), EHRLICH P (1877, 1878), GIERKE H (1884), GIEMSA G (1904), LEWIS FT (1942), GOMORI G (1952), PEARSE AGE (1953), GRAUMANN W and NEUMANN K (1958), ROMEIS B (1968), GURR E (1971), LILLIE RD and FULLMER HM (1976), HOROBIN RW and WALTER KJ (1987), BURCK HC (1988), TITFORD M (1993), KIERNAN JA (1999), TITFORD M (2001), HOROBIN RW and KIERNAN JA (2002), TITFORD M (2005), BANCROFT JD and GAMBLE M (2007), EISNER A (2007), KIERNAN JA (2008), LLEWELLYN BD (2009), TITFORD M (2009), LLEWELLYN BD (2019).

The staining of tissue sections was much influenced by the aniline dye chemistry in the second half of the 19th century (TITFORD M [1993] for review). P EHRLICH was among the first to study systematically synthetic dyes and to introduce scientific methods into the field of histological dye staining (EHRLICH P, 1877, 1878).

Aniline is an aromatic amine consisting of a benzene ring and an amino group. It was chemically prepared for the first time by distillation of indigo by O UNVERDORBEN (1826) who named it “Crystallin”. In 1834, it was also obtained from coal tar by FF RUNGE (1834) (“Kyanol”), a substance giving a blue color on treatment with chlorinated lime (calcium hypochlorite). The term “aniline” was given later by J FRITSCH (1839, 1840, 1841) by experimentation with indigo. The name “aniline” of the obtained compound ought to reflect its relation to the indigo-yielding plants (*Indigofera anil*). This compound had been isolated quite earlier in much the same way (UNVERDORBEN O, 1826), then named “Crystallin”. The various “aniline” preparations from coal tar and the coloring matters derived from were subsequently further investigated (HOFMANN AW, 1843; HOFMANN AW, 1845; HOFMANN AW, 1854; HOFMANN AW, 1859; HOFMANN AW, 1863; WITT OH, 1876), and all of them proved finally to be identical: phenylamine, aminobenzene. Aniline is still an important precursor substance in dye and organic chemistry (NIETZKI, R, 1881; KAHL T et al., 2002; JOHNSTON WT, 2008).

A crucial point in the further development of dye chemistry was making indole from indigo (BAEYER A, 1868; BAEYER A and EMMERLING A, 1869) and synthetic indigo by chlorination and subsequent reduction of isatin (BAEYER A and EMMERLING A, 1870). Yet, the first true synthesis of indigo did not come until isatin¹ was produced from phenylacetic acid (BAEYER A, 1878). With the introduction of cinnamic acid, a new synthetic way proved even more useful in the production of indigo; cinnamic acid was equally suitable for the synthesis of isatin and indole (BAEYER A, 1880). Finally, decoding the indigo formula was achieved when indigo was synthesized by 2- nitrobenzaldehyde and, thus, the structure of indigo was

¹ Isatin is an indole derivative. The compound was first obtained by OL ERDMANN (1840, 1841) and A LAURENT (1841) as a product from the oxidation of indigo by nitric and chromic acids.

obtained definitely (BAEYER A and DREWSEN V, 1882). Later on, other synthetic pathways were studied (HEUMANN K, 1890).

The aniline dye (synthetic dye) industry grew rapidly. The first industrial-scale use was in the manufacture of mauveine, a dye of the phenazine group being patented in 1856 by WH PERKIN (Producing a new coloring matter for dyeing with lilac or purple color stuffs of silk, cotton, wool, or other materials, British patent No. 1984, 26th August 1856); PERKIN WH, 1879). Many other dyes created from coal tar (and with ingredients other than aniline) followed.

The first artificial production of a natural dye was alizarin, produced by C GRAEBE and C LIEBERMANN (1868, 1869). They realized that alizarin was a derivative of anthracene occurring in coal tar. Industrially, anthraquinone (or alizarin) dyes are derived from anthracene which is a compound of anthracene oil, a coal tar fraction. The synthesis of *indanthrene* dyes (anthraquinone dyes, the name is derived from *indigo* and *anthracene*) is based on the fusion of 2-aminoanthraquinone molecules in alkali (BOHN R, 1901). BOHN's invention of the first anthraquinone vat dye "Indanthrene Blue" was patented in 1901 (Verfahren zur Darstellung eines blauen Farbstoffs der Anthracenreihe, DRP Nr. 129845, 6th February 1901). Then, other dyes became synthesized and recorded in a number of patent specifications.

From the many different classes of dyes, azo dyes form the largest and most important group of synthetic organic dyes. The structure of azo dyes is based on azobenzene and includes the diazo functional group (R-N=N-R') where the N=N group is the azo group between two aromatic rings. Dye synthesis follows reactions studied by P GRIESS (1858) which are diazotation and coupling. The reaction mechanism consists of two steps. In the first step, diazotation of primary/aromatic amines is performed to form diazonium salts (diazonium kations) and called *diazo component*. Because aliphatic diazonium salts are very unstable, aromatic diazonium compounds are preferred. In a second step, azo coupling is done with the *coupling component*: the coupler carries an electron-rich carbon atom to which the diazo component will become attached (principle of electrophilic substitution) bridging the gap between the two molecules with a new azo group (-N=N-).

Starting substances are aniline or appropriately substituted derivatives of benzene. The nature of the aromatic substituents on both sides of the azo group as well as the water-solubility of the dyes determine the colors of the azo compounds (BRUNNER; 1929; HUNGER K et al., 2002). Most azo dyes are red, orange or yellow. The development of azo and anthraquinone dyes completed the spectrum of synthetic dyes (HUNGER K and HERBST W, 2002; HUNGER K et al., 2002).

Histological specimens are prepared as is usual in routine histopathology which includes preservation and embedding (i.e. fixation, dehydration and embedding in paraffin or resin). Prior to staining, sections are cut with a microtome. In certain cases, cryostat sections or celloidin preparations are needed. Furthermore, cell smears from suspensions may be used.

Stains are applied to make cell structures more readily visualized than in unstained specimens, and, under certain conditions, they can reveal molecular compounds and differences associated with evolution or pathological conditions. Thus, stains in general are aimed as special probes which possess variable specificity depending on the selectivity with which they react. Comparable to molecular biology, the term *probe* may be used to define a reagent which is suitable to detect or to measure the presence of a particular molecule. The term *probe* can be applied to all stainings, yet more specifically in cases where reactions with highly

specific reagents are performed such as antibodies, nucleotide sequences or other high affinity binding molecules.

Old and classical histological stains will still maintain their importance in histology; some of them are even indispensable, even if the majority of them lack strictly defined specificity. Together with the below described high specific molecular probes, they constitute a valuable repertoire of the histologist for his microscopic studies. There exist hundreds of histological stainings from which some common methods are described in the following. For a comprehensive list of histological dyes with their respective Color Indices we refer to the homepages of BD LLEWELLYN (<https://www.stainsfile.info/stain/stainindex.htm>), CHROMA Gesellschaft “Ausgewählte Färbemethoden für Botanik, Parasitologie und Zoologie” (<http://www.chroma.de>) and A EISNER (<http://www.aeisner.de/daten/farbinh.html>).

Table: Characteristics of common histological stainings

Stain type	Reagent	Color	Cell structure
Acid dyes <i>synthetic</i>	Aniline blue Eosin Fast green	Blue Pink-red Green	Staining of acidophilic cell structures (anionic dye), f.e. cytoplasm
Basic dyes <i>synthetic</i>	Azures Methylene blue Toluidine blue	Blue	Staining of basophilic cell structures (cationic dye), f.e. nuclei and RER (RNA); demonstration of metachromasia: color shift from orthochromatic to metachromatic color in the presence of polyanionic substances, f.e. granules in mast cells
Natural dyes <i>basic dyes</i>	Hematoxylin (must be oxidized and used together with a mordant)	Blue/black	Staining of basophilic cell structures such as nuclei and some cytoplasmic substances (cationic)
Chemical reaction <i>Feulgen stain</i>	Schiff reagent (basic fuchsin)	Magenta	Schiff reaction of aldehydes from previously hydrolyzed DNA with basic fuchsin for the specific demonstration of DNA
Chemical reaction <i>PAS stain</i>	Schiff reagent (basic fuchsin)	Magenta	Pretreatment with periodic acid for the conversion of 1-2 glycol linkages into aldehyde groups followed by the Schiff reaction for the demonstration of 1-2 glycol moieties
Lipid stains	Sudan Oil red (lipid soluble)	Black Red	Lipid droplets, unsaturated lipids, phospholipids: staining principle due to differences in solubility of the dye within two media, i.e. diffusion from low concentrated alcoholic solution into the specimen
Metal stains <i>impregnation</i>	Silver (Bodian, Gomori) Gold	Brown Black	Silver impregnation of cell structures, f.e. for the demonstration of the Golgi apparatus, reticular fibres and neurofibrils Silver impregnation followed by gold chloride for stable and enhanced contrast

<i>oxidation</i>	Osmium tetroxide	Black	Several distinct application, f.e. demonstration of lipids (unsaturated lipids, phospholipids) which reduce osmium tetroxide to give a black compound; fixation and contrasting of membranes; impregnation according to Golgi followed by AgNO ₃ solution
Special stains: <i>acid and basic dyes</i> (Romanowsky type)	Giemsa Wright	Blue Purple Pink-red	Used for blood and bone marrow smears to demonstrate orthochromatic, polychromatic and metachromatic properties
Special stains: <i>acid and basic dyes</i> (defined pH)	Methylene blue and eosin	Blue Pink (light)	Buffered solution of acid and basic dye mixture to demonstrate cytoplasmic basophilia on tissue sections
Special stains: <i>acid and basic dyes</i> (polychrome stains)	Masson trichrome Mallory triple Movat pentachrome	Blue Green Blue-black	Selective staining of connective tissue compounds, muscle, fibrin
Special stains: <i>elastica stains</i>	Taenzer-Unna Weigert Orcein Resorcin fuchsin Verhoeff method	Brown (dark) Blue-black Purple Black	Elastic tissue, elastic fibrils
Special stains: <i>mucin stains</i>	Alcian blue at different pH values Combination with other cytochemical reactions (PAS etc.)	Blue Blue-green Magenta	Mucins (glycoconjugates), acid and neutral mucins in gastrointestinal epithelium
Special stains: <i>neurohistology</i>	Weigert hematoxylin Methylene blue Cresyl fast violet Classical Nissl or fast Nissl methods Luxol fast blue (Klüver-Barrera)	Violet (nuclei, Nissl bodies) Dark blue (myelin sheaths) Blue-black (myelin sheaths) Deep-blue (nuclei, Nissl bodies) Bright blue (myelin sheaths)	Distinct applications for neurohistology, staining of basophilic structures by basic dyes, f.e. perikaryon, nuclei, Nissl bodies, glial cells, fibers, myelin sheaths
Special stains: <i>colloidal susp.</i>	Trypan blue (vital staining)	Blue	Nontoxic colloidal particles which do not label living cells (vitality marker of cells in vivo and in vitro, cell suspensions, cultured cells); useful marker of phagocytic cells (cleared by phagocytic system)
Histochemistry <i>enzymes</i>	Specific enzyme substrates for endogenous enzymes	Chromogen dependent	Selective cell structure (site of endogenous enzyme)
Histochemistry <i>enzymes</i>	Specific probe and defined labels (immunohistology)	Chromogen dependent	Cell structure defined by the applied molecular probe (antigens, antibodies)
Fluorochromes: <i>vital staining or</i>	Xanthenes Acridines	Different colors (fluorescence)	Fluorescence: in vivo labeling of cells and tissue structures

<i>staining of sections</i>	Tetracycline (cationic, anionic and electroneutral fluorochromes)		Fluorescence: tissue sections stained with fluorochromes
Fluorochromes: <i>immunohistology</i>	Specific probe and selected labels (fluorochromes)	Different colors (fluorescence)	Cell structure defined by the applied molecular probe (antigens, antibodies etc.)
Fluorochromes: <i>in situ hybridization</i>	Specific probe and selected labels (fluorochromes)	Different colors (fluorescence)	Cell structure defined by the applied molecular probe (DNA, RNA)
Other labels e.g. enzymes: <i>immunohistology, hybridization and other studies</i>	Specific probe and selected labels (multiple detection principles)	Different colors (chromogenic or fluorescent)	Cell structure defined by the applied molecular probe (antigens, nucleotides etc.)

The interaction of dyes with tissues

Dyes are obtained either from natural sources or from synthetic production. With the introduction of the aniline dyes, starting from the mid-19th century, natural dyes have almost lost their role in histology with the exception of hematoxylin from Logwood trees which still remains its great importance in routine histopathology. P EHRlich was one of the first to study systematically aniline dyes and to introduce scientific methods into the field of histological dye staining (EHRlich P, 1878). Then, GRÜBLER'S *Verzeichnis der Farbstoffe* (1880) and the *Farbstofftabellen* (SCHULTZ G and JULIUS P, 1914) gave the first overviews of the existing natural and synthetic dyes. Several years later, *Colour Index* was published as a reference guide for manufacturers and consumers by the Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists (1925). *Colour Index* is now published as *Colour Index International* on the web (<http://www.colour-index.org/>). Colorants are listed according to *Colour Index Generic Names* and *Colour Index Constitution Numbers*. For each product name, *Colour Index International* lists the manufacturer, the physical form, and the principal uses.

Histological staining are based on chemical and physical principles that involve the following reactions:

- electrostatic reactions: dying substances are composed of chromophores (f.e azo groups) and auxochrome groups (f.e. hydroxyl, carboxyl or nitro groups), and the latter properties define the dye as an acid dye or a basic dye;
- metal impregnation: the inherent ability of certain cell structures (argyrophilic elements) to precipitate submicroscopic amounts metal ions (silver ions) which are further developed by a reduction process, or the metal impregnation of cell components in a so-called argentaffine reaction following oxidative pretreatments;
- selective stainings due to the solubility of the dyes: fat staining with Sudan, Oil red and other fat dyes which relies on differences in solubility of the dye within two media, i.e. diffusion from alcoholic dye solution into the fat of specimens;
- cytochemical reactions: the application of defined chemical reactions, f.e. chemical complex formation for Fe³⁺ staining, enzyme substrate reactions for peroxidases, phosphatases etc., or selective staining of tissue molecules after their chemical modification (f.e. introducing reactive groups such as aldehydes for the Feulgen and PAS reaction);

- ligand specific probes: the selectivity of cellular staining is defined by the applied molecular probe and their specificity for cellular molecules with which they react, f.e. antibodies, lectins, nucleotides.

Proteins behave as amphoteric molecules, i.e. they possess both acidic and basic groups which contribute to acidophilia or basophilia. The dye-protein interaction is mainly due to the net charge. At the isoelectric point (pI) the net charge is negligible and binding of charged dyes will be minimal. Then, below pI , proteins bind acidic dyes (anionic dyes) and above pI , proteins bind basic dyes (cationic dyes). At physiological pH, proteins may exhibit either net (+) or net (-) charges. Thus, their behaviour is either acidophilic with affinity for acid dyes or basophilic with affinity for basic dyes.

Many dye stainings are chemically due to the formation of salt linkages between dye and cellular substance. The whole mechanism is certainly more complex and is often not fully understood. The formation of salt linkage means that an acid dye (anionic dye) such as eosin will bind a basic group (positively charged) of the protein (= acidophilic substance). On the other hand, a basic dye (cationic dye) such as methylene blue will bind an acidic group (negatively charged) of the protein (= basophilic substance).

Special histological stains

On the basis of basic and acid dyes, a number of stains with orthochromatic, polychromatic and metachromatic colors have been experienced with the purpose to distinguish certain elements in histological preparations. For example, one of the first successfully applied dye combination was a mixture of acid and basic dyes by D ROMANOWSKY (1891) to stain parasites in blood smears. He used methylene blue and eosin simultaneously. The most interesting aspect of this mixture was the observation that the combined stain had properties which were different from the stains used alone. Such dye mixtures have been further refined and are now known as WRIGHT, GIEMSA or MAY-GRÜNWARD stains. They may be characterized as eosinates of methylene blue or azure derivatives of methylene blue. Today, the simultaneous application of such acid and basic dye mixtures are referred to as modified ROMANOWSKY stains and yield very nice differential staining of white blood cells.

The true staining mechanism of ROMANOWSKY stains is still unknown. At least three fundamental processes have been elucidated (HOROBYN RW and WALTER KJ, 1987), briefly

- orthochromasia: pink staining by eosin (acid dye component) and blue staining by methylene blue (basic dye component),
- polychromasia: layered staining with both dyes,
- metachromasia: color shift of basic dye from blue to violet/purple due to high concentration of polyanions (highly acidic substances) in the stained specimen.

Other special stain formulations include polychrome staining with mixtures of several various acid and basic dyes for the differential staining of connective tissue compounds (MALLORY FB, 1900; MASSON P, 1929; MALLORY FB, 1936; MALLORY FB, 1938; GOMORI G, 1950; MOVAT HZ, 1955; JONES ML, 2002). The staining affinity of tissue components is affected by several factors such as the molecular size of the used dyes, the density of the tissue, pH of the solution and the use of colorless “dyes”. The latter are phosphotungstic acid or phosphomolybdic acid and have a complex role in the staining process. Even if the chemistry of these techniques cannot be explained in detail, they are excellent stains and commonly used to distinguish collagen fibers, muscle and extracellular matrix from cellular cytoplasm.

Under special conditions, orcein, resorcin, aldehyde-fuchsin methods or Verhoeff's hematoxylin differential stain are highly specific for elastic fibers (UNNA PG, 1890; WEIGERT C, 1898; VERHOEFF FH, 1908; GOMORI G, 1950; FULLMER HM and LILLIE RD, 1956) from which orcein can be also applied in electron microscopy (NAKAMURA H et al., 1977). The staining principle is not clear, but it is thought that aldehyde residues are responsible. When orcein is used as stain, then dye-amine reactions may be involved.

Alcian blue (a group of polyvalent, basic copper phthalocyanine dyes with many positive charges on their molecules) is a useful dye for the localization of ionisable moieties of mucins (STEEDMAN HF, 1950) and, together with other chemical reactions, excellent to distinguish acid and neutral mucosubstances (glycoconjugates). To this aim, histochemical procedures using periodic acid-Schiff (PAS) and Alcian blue at pH 2.5, Alcian blue at pH 1.0 and high iron-diamine/Alcian blue were introduced. In further developments, defined chemical or enzymatic alterations were induced in tissue sections to stain selectively reactive groups in epithelial mucins (MOWRY RW, 1963; LEV R and SPICER SS, 1964; SPICER SS, 1965; FILIPE MI, 1979; KUHLMANN WD, 1984).

Long before the introduction as fixative in electron microscopy, osmic acid (osmium tetroxide) has been used as stain for lipid substances (STARKE J, 1895; HOERR N, 1936). Osmium is chemically bound to fat and reduced to a black compound, and as such acts as a fixative. The black substance is easily observed in the light microscope. Today, lipid staining is preferentially done with so-called lipid soluble reagents such as Oil red and Sudan dyes. The principle relies on differences in solubility of the dye within two media whereby diffusion occurs from a low concentrated alcoholic solution into the specimen. Some important work on the understanding of lipid staining have been performed by L MICHAELIS (1901) and H H ESCHER (1919).

There exist a number of impregnation and metal methods which are applied to stain structural details with reasonable preference (GOLGI C, 1873). The staining of reticular fibres, neurofibrils and nerve cells or other structures is readily achieved by several silver impregnation methods; the results depend on the particular method applied.

Silver or silver/gold impregnation methods do not fall into the category of classical dye staining (BIELSCHOWSKY M, 1902; BIELSCHOWSKY M, 1903; BODIAN D, 1936; GOMORI G, 1946; ROMANES GJ, 1950; FERREIRA-MARQUES J, 1951; BREATHNACH AS, 1965). For reasons not yet completely understood, certain tissue elements react preferentially with silver salts. The metal (silver) becomes precipitated onto the structural components from the respective salt solution under defined reducing conditions (f.e. hydroquinone); the silver salts are made visible by conversion into metallic silver by processes which are comparable to black and white photography. Preferably, sections are subsequently toned with gold chloride. The whole impregnation procedure is a complex technique and needs careful handling.

There exist a number of special stainings which have been preferentially developed for neurohistology to reveal the essential parts of the nerve system: the nerve cells (perikaryon), the Nervenfortsätze with their myelin sheaths and the glial substance. Generally, methods applied to localize nuclei and tigroid bodies in nerve cells are called *Nissl stains* (NISSL F, 1894). Excellent results are obtained with the basic dyes such as methylene blue, toluidine blue and cresyl fast violet. For the detection of myelin sheaths, C WEIGERT (1882) introduced a special procedure involving a mordant step and an alcoholic hematoxylin solution. This method became subsequently refined. Instead of an alcoholic hematoxylin solution, a combined cell and myelin staining technique was developed by KLÜVER and BARRERA (1953)

using Luxol Fast Blue (sulfonated copper phthalocyanine) and cresyl fast violet with high color contrast of the cyto- and myeloarchitecture in neural tissue sections.

Histochemistry, defined microchemical reactions

The Feulgen reaction and the PAS (periodic-acid-Schiff) technique are typical example for chemically well defined stainings to reveal certain cell structures. Both methods use the SCHIFF reagent (SCHIFF H, 1866; basic fuchsin) for the detection of aldehyde groups which have been selectively produced in tissue specimens by defined chemical pretreatments. This reaction has found numerous applications in cytochemistry (BAUER H, 1933; FEULGEN R, 1914; FEULGEN R and ROSSENBECK H, 1924; MCMANUS JFA, 1946; LILLIE RD, 1947; HOTCHKISS RD, 1948; MCMANUS JFA, 1948). Due to its high specificity, the Feulgen *nuclear reaction* has become one of the most prominent stains for nuclear DNA. In a first step, DNA is hydrolyzed with HCl to yield aldehydes which in turn react in the second step with Schiff reagent to form a stable magenta reaction product. The Feulgen reaction is very specific and widely used for staining and densitometric quantification of nuclear DNA (HARDIE DC et al., 2002).

An important progress in specific microscopical stainings was the introduction of enzyme histochemistry. The first histochemical procedures were reported for the detection of the peroxidase reaction in leukocyte granules (FISCHEL R, 1910) and for hemoglobin in red blood cells (LEPEHNE G, 1919) by the use of benzidine. Several systematic studies followed later (GOMORI G, 1952; GOMORI G, 1953). In the meantime, many peroxidase substrates have been proposed for a great spectrum of applications including colorimetric, fluorescent and chemiluminescent measurements as well as histochemical stainings (KRIEG R and HALHUBER KJ, 2004; KRIEG R et al., 2007; KRIEG R et al., 2008; PETERSEN KH, 2009).

The early descriptions of cytochemical peroxidase reactions were followed by studies on alkaline phosphatases in various tissues (GOMORI G, 1939; TAKAMATSU H, 1939). Then, new cytochemical approaches for many other enzymes (including methods for electron microscopic cytochemistry) were published: oxidases, dehydrogenases, oxidoreductases, lyases, ligases (f.e. GOMORI G, 1952; PEARSE AGE, 1953; SHELDON H et al., 1955; BRANDES D et al., 1956; BARNETT RJ and PALADE GE, 1958; ESSNER E et al., 1958; GRAUMANN W and NEUMANN K, 1958; NACHLAS MM et al., 1958; MITSUI T, 1960; KUHLMANN WD, 1970; KUHLMANN WD and AVRAMEAS S, 1971; PEARSE AGE, 1980; KUHLMANN WD and PESCHKE P, 1986; KIERNAN JA, 2007). Even if a large number of specific cytochemical substrates exist now, appropriate cytochemical substrates for many other enzymes are still lacking. The common names of all enzymes, enzyme subclasses, along with their EC numbers, and further details on enzymes including pathways in which they act are given by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB <http://www.chem.qmul.ac.uk/iubmb/enzyme/>); for common names of enzymes, see

- subclass EC 1 - Oxidoreductases
- subclass EC 2 - Transferases
- subclass EC 3 - Hydrolases
- subclass EC 4 - Lyases
- subclass EC 5 - Isomerases
- subclass EC 6 - Ligases

Enzyme histochemistry has several limitations which are primarily related to tissue fixation and tissue preparation in general. With these inherent problems in mind and due to the

growing demand for more and other specific detection methods, the development of alternative techniques was pushed.

Labeling by use of special probes

With the demand for specific histological stainings (apart from enzyme histochemistry) in cell research, histopathology and molecular biology, a great number of specific methods have been developed during the last 20 years enabling selective detection of dedicated molecules. For example, antibodies, lectins and nucleotides can be applied as special probes with important significance in studies of phylogeny, embryogenesis and organ disease.

Immunohistological techniques were invented by AH COONS and co-workers (COONS AH et al. 1941; COONS AH, 1958). These techniques makes use of the principles of immunology to identify and (in the sense of histology) to locate cellular antigens by antibodies. To this aim, so-called *direct* or *indirect* methods and a great variety of marker molecules as labels are applied (see chapter *Cell staining with direct and indirect assay formats*). Fluorochromes and enzymes are the most widely employed labels in light microscopy. In the case of electron microscopy, ferritin, enzymes and colloidal gold particles are very popular.

There exist many variants of the immunohistological principle. Basically, the following steps are involved:

- preparation of specific antibodies;
- use of these antibodies to localize the corresponding antigen in cells and tissues;
- selection of appropriate marker for direct or indirect labeling technique;
- detection system: coupling the antigen-antibody complexes with chromogen (marker molecules);
- visualizing and detecting the chromogen (enzyme substrate, UV light, others) in order to locate the antigen.

The sensitivity of immunohistology can be greatly enhanced by a variety of amplification methods, f.e. antibody bridge techniques (with secondary and tertiary antibodies) or other high affinity bridge techniques such as avidin-biotin are in use. Then, in the case of enzymes as labels, the cytochemical reaction product may be further intensified by chemical procedures.

Radioautography is of considerable interest in tracing radio-elements in the body and in studying dynamic events such as secretion pathways and nucleic acid synthesis. First developed for light microscopy, this techniques proved also very useful in electron microscopy (BELANGER LF and LEBLOND CP, 1946; PELC SR, 1947; TAYLOR JH et al., 1957; BACHMANN L and SALPETER MM, 1967; SALPETER MM, 1967, LEBLOND CP, 1981). Several radioactive tracers may be used for labeling purposes: (a) radiolabeled precursor molecules such as sugars, amino acids or nucleotides in synthesizing studies; and (b) certain finished “products” such as hormones in receptor binding studies. It must be kept in mind, however, that one of the main problems of autoradiography is its resolution in microscopy. Tritium as label is a good choice. It could be shown that tritium provided the highest resolution available since the beta particles have a maximum energy of only 18 keV which corresponds to a range of about one micron in photographic emulsions.

Radioautography has significantly contributed to the understanding of many fundamental processes in cell research such as protein or carbohydrate synthesis, secretory processes,

exocytosis, endocytosis, receptor bindings and cell division. Also, the combination of radioautography and immunohistology (double labeling experiments) enabled substantial studies on cell division/differentiation and antigen/antibody synthesis in the course of immune response or in the course of carcinogenesis (KUHLMANN WD et al., 1975; KUHLMANN WD, 1978); KUHLMANN WD and PESCHKE P, 2006).

Cell proliferations have been performed for a long time following [³H] thymidine incorporation into DNA. The use of [³H] thymidine labeling has declined meanwhile due to the development of an alternative method for the detection of DNA replication using the thymidine analog 5-bromo-deoxyuridine (BrdU) in an immunohistochemical procedure (GRATZNER HG, 1982). The incorporation of BrdU into nuclear DNA during S-phase is localized in tissue preparations using monoclonal antibodies against BrdU and a chromogen detection method. Comparable to immunohistology, a variety of detection principles and marker molecules (e.g. fluorochromes, enzymes) can be applied.

The technique of *in situ hybridization* is another example of “special probe” in histological staining. Double stranded DNA in solution can be “melted” into single strands (by heat or elevation of pH), and this manipulation is reversible so that the single strands can be recombined (“annealed”). This hybridization process is specific and only complementary strands will combine: hybrids between DNA-DNA, DNA-RNA and RNA-RNA are possible.

The formation and detection of DNA-DNA and RNA-DNA hybrid molecules in cytological preparations were first described in 1969 (GALL JG and PARDUE ML, 1969; JOHN HA et al., 1969; PARDUE ML and GALL JG, 1969). Although the annealing of DNA or RNA molecules to their complementary sequences has been applied earlier for several purposes and by several techniques (e.g. in solution for scintillation counting or on nitrocellulose membranes for radioautography), *in situ* hybridisation needed special histological preparation. Since then, the technique has become established for the identification of DNA and RNA within cells and tissues (GUITTENY AF et al., 1988; WARFORD A, 1988; HANKIN RC and LLOYD RV, 1989; PRINGLE JH et al., 1989; LARSSON LI and HOUGAARD DM, 1990; SHORROCK K et al., 1991).

Especially the availability of synthetic oligonucleotide probes and the introduction of non-radioactive detection methods have made *in situ* hybridization techniques accessible for many laboratories. Marker molecules including detection principles known from immunohistology play a major role in cellular hybridization techniques in a variety of fields such as gene expression, genetic diseases, location of genes on chromosomes and chromosome abnormalities.

Selected publications for further readings

Unverdorben O (1826)
Runge FF (1834a, 1834b, 1834c)
Fritsche J (1839)
Erdmann OL (1840)
Fritsche J (1840)
Erdmann OL (1841)
Fritsche J (1841)
Laurent A (1841)
Hofmann AW (1843a, 1843b, 1843c)
Hofmann AW (1845)

Hofmann AW (1854)
Perkin WH (1856)
Griess P (1858)
Hofmann AW (1859)
Hofmann AW (1863a, 1863b, 1863c, 1863d)
Schiff H (1866)
Baeyer A (1868)
Graebe C and Liebermann C (1868)
Baeyer A and Emmerling A (1869)
Baeyer A and Emmerling A (1870)
Weigert C (1871)
Golgi C (1873)
Witt ON (1876)
Ehrlich P (1877)
Baeyer A (1878)
Ehrlich P (1878)
Perkin WH (1879)
Baeyer A (1880)
Grübler G (1880)
Baeyer A and Drewsen V (1882)
Weigert C (1882a, 1882b)
Gierke H (1884a, 1884b, 1884c)
Nietzki R (1886)
Heumann K (1890)
Unna PG (1890)
Romanowsky DL (1891)
Nissl F (1894)
Starke J (1895)
Weigert C (1898)
Mallory FB (1900)
Bohn R (1901)
Michaelis M (1901)
Bielschowsky M (1902)
Bielschowsky M (1903)
Giemsa G (1904)
Verhoeff FH (1908)
Fischel R (1910)
Feulgen R (1914)
Schultz G and Julius P (1914)
Escher HH (1919)
Lepehne G (1919)
Feulgen R and Rossenbeck H (1924)
Colour Index (1925-2009)
Brunner A (1929)
Masson P (1929)
Bauer H (1933)
Hoerr NL (1936)
Bodian D (1936)
Mallory FB (1936)
Mallory FB (1938)
Gomori G (1939)
Takamatsu H (1939)

Coons AH et al. (1941)
Lewis FT (1942)
Belanger LF and Leblond CP (1946)
Gomori G (1946)
McManus JFA (1946)
Lillie RD (1947)
Pelc SR (1947)
Hotchkiss RD (1948)
McManus JFA (1948)
Gomori G (1950a, 1950b)
Romanes GJ (1950)
Steedman HF (1950)
Ferreira-Marques J (1951)
Gomori G (1952)
Gomori G (1953)
Klüver H and Barrera E (1953)
Pearse AGE (1953)
Movat HZ (1955)
Sheldon H *et al.* (1955)
Brandes D *et al.* (1956)
Fullmer HM and Lillie RD (1956a, 1956b)
Taylor JH et al. (1957)
Barnett RJ and Palade GE (1958)
Coons AH (1958)
Essner E *et al.* (1958)
Graumann W and Neumann K (1958)
Nachlas MM *et al.* (1958)
Mitsui T (1960)
Mowry RW (1963)
Lev R and Spicer SS (1964)
Breathnach AS (1965)
Spicer SS (1965)
Bachmann L and Salpeter MM (1967)
Salpeter MM (1967)
Romeis B (1968)
Gall JG and Pardue ML (1969)
John HA *et al.* (1969)
Pardue ML and Gall JG (1969)
Kuhlmann WD (1970)
Gurr E (1971)
Kuhlmann WD and Avrameas S (1971)
Kuhlmann WD *et al.* (1975)
Lillie RD and Fullmer HM (1976)
Nakamura H *et al.* (1977)
Kuhlmann WD (1978)
Filipe MI (1979)
Pearse AGE (1980)
Leblond CP (1981)
Gratzner HG (1982)
Kuhlmann WD (1984)
Kuhlmann WD and Peschke P (1986)
Horobin RW and Walter KJ (1987)

Burck HC (1988)
Guitteny AF *et al.*, (1988)
Warford A (1988)
Hankin RC and Lloyd RV (1989)
Pringle JH *et al.* (1989)
Larsson LI and Hougaard DM (1990)
Shorrock K *et al.* (1991)
Titford M (1993)
NC-IUBMB (1992-2009)
Kiernan JA (1999)
Titford M (2001)
Hardie DC *et al.* (2002)
Horobin RW and Kiernan JA (2002)
Hunger K and Herbst W (2002)
Hunger K *et al.* (2002)
Jones ML (2002)
Kahl T *et al.* (2002)
Krieg R and Halhuber KJ (2004)
Titford M (2005)
Kuhlmann WD and Peschke P (2006)
Bancroft JD and Gamble M (2007)
Eisner A (2007)
Kiernan JA (2007)
Krieg R *et al.* (2007)
Johnston WT (2008)
Kiernan JA (2008)
Krieg R *et al.* (2008)
Llewellyn BD (2009)
Petersen KH (2009)
Titford M (2009)
Llewellyn BD (2019)

Full citation of publications is given in chapter in chapter *References*
link: https://www.immunologie-labor.com/cellmarker_files/References.pdf