While conventional histological staining methods have been established for decades, some for more than a century, immunohistochemical techniques are not yet routinely used in forensic diagnostics. They are used, however, when specific problems occur. In such cases, depending on the problem, routine diagnostics may be supplemented with specific microscopic techniques, including electron microscopy, laser scanner microscopy, and laser microdissection techniques, in order to isolate single cells or cell groups. For important routine diagnostics, established standard histological staining methods are discussed here. Basic information on immunohistochemical techniques and on the best-practice use of immunohistochemical and other methods are mentioned only briefly and therefore do not substitute reference to the specialist literature.

Immunohistochemical staining techniques, in particular the ABC method, the APAAP method, and the TUNEL technique, are used to label defined antigens with monoclonal and polyclonal antibodies. Commercially produced antibodies mostly originate from mice, less frequently from rabbits.

In these cases, a number of methodological and technical nuances must be considered in order to gain usable results. The degree of autolysis or putrefaction, the selection of fixation medium, fixation duration, incubation period, and concentration of the selected antibodies can be crucial. Different methods of antigen unmasking are significant in a number of immunohistochemical stainings.

The following chapter gives a general overview of staining and microscopy, highlighting the most important aspects, including potential sources of error and the recognition of typical mistakes and artifacts. For more detailed information, please refer to the relevant works on histological and immunohistochemical techniques.

### 2.1 Conventional Histological Staining

Conventional histological staining methods, including stain selection for specific situations, have long been established. Descriptions of the most frequently used staining methods should be sufficient for day-to-day practice (Table 2.1). Longer fixation in formaldehyde or in higher concentrations of formaldehyde can lead to sediments of formalin pigment. If the assessment of tissue sections will be affected by such sediments, pretreatment should be considered (Kardasewitsch reaction; Kardasewitsch 1952). Depending on which tissue is to be investigated, the fixation technique can influence the microscopic image. Thus, for example, the influence of fixation on the development of pulmonary alveoli has been investigated (Hausmann et al. 2004).

In some cases, alternative fixing solutions are used: Bouin’s solution, Zamboni solution, “NoTox” (Meyer et al. 1996), pure alcohol, etc. In cases where an electron microscopic investigation is needed, glutaraldehyde is typically chosen as a fixative (3% solution for 24 h at 4°C, followed by phosphate buffer solution; additional fixation in 1% osmium acid, embedded in Epon).

It should be noted that fixative selection and duration can have a direct bearing on potential molecular genetic investigations (Kuhn and Krugmann 1995). Such investigations can be difficult or even impossible and special pretreatment methods are sometimes
<table>
<thead>
<tr>
<th>Staining</th>
<th>Presented structures</th>
<th>Examples from forensic practice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcian blue</td>
<td>Detection of acid mucopolysaccharides</td>
<td>Mucoid lakes, for example, in cases of idiopathic cystic Erdheim–Gsell medial necrosis and dissected aortic aneurysm</td>
</tr>
<tr>
<td>Azan staining (azo carmine and aniline blue)</td>
<td>Connective tissue staining (red): azo carmine stains cell nuclei, erythrocytes, fibrin, fibrinoid, acidophilic cytoplasm, epithelial hyalin; Aniline blue (blue): collagen fibers, fibrous hyalin, basalophil cytoplasm, mucus</td>
<td>Differentiates basophilic and chromophobe cells in the hypophysis; loss of detectability, for example, in the case of Sheehan syndrome</td>
</tr>
<tr>
<td>Best’s carmine stain</td>
<td>Classified as a glycogen stain, but is not specific; also stains mucus, fibrin, gastric glands, and mast cell granules</td>
<td>Glycogen detection in kidney distal tubular cells in the case of hyperglycemia (Armanni–Ebstein cells)</td>
</tr>
<tr>
<td>Elastin staining according to Weigert</td>
<td>Stains elastic fibers violet-black</td>
<td>For example, elastic fibers in the aortic media</td>
</tr>
<tr>
<td>Elastika van Gieson (EvG)</td>
<td>Combined staining of collagen fibers (red) and elastic fibers according to Weigert (black and brown); cytoplasm, musculature, amyloid, fibrin, and fibrinoid (yellow)</td>
<td>Fibrotic zones in the myocardium, fibrosis in other organs, liver cirrhosis, cystial medial necrosis</td>
</tr>
<tr>
<td>Iron stain (Prussian blue reaction)</td>
<td>Stains trivalent iron, in particular hemosiderin; detection of iron deposits</td>
<td>Siderosis of the lung, posttraumatically deposited siderophages, e.g., for wound age determination</td>
</tr>
<tr>
<td>Fibrin staining according to Weigert</td>
<td>Blue: fibrin and bacteria Red: cell nuclei; is not considered a specific fibrin stain</td>
<td>Detection of microfibrin in the placenta, hyaline membrane in the lung post shock event</td>
</tr>
<tr>
<td>Gomori’s stain</td>
<td>Argyrophilic reticular fibers (silver)</td>
<td>Glomerular basal membranes in the case of a membrane-proliferative glomerulonephritis type I (MPGN) – so-called tram tracks; reticular fiber network in the case of hepatic peliosis</td>
</tr>
<tr>
<td>Grocott stain</td>
<td>Ideal fungal stain: fungal conidia, fungal fibers stain black</td>
<td>Fungal infection</td>
</tr>
<tr>
<td>Haematoxylin–eosin (H&amp;E) staining</td>
<td>Acidophilic cytoplasm is red, basophil nuclei are blue, erythrocytes are red</td>
<td>Routine staining</td>
</tr>
<tr>
<td>Congo red stain</td>
<td>Amyloid stain</td>
<td>Amyloidooses of any type, in particular cardiovascular</td>
</tr>
<tr>
<td>Kossa stain</td>
<td>Calcified bone tissue stains black in a non-calcified specimen</td>
<td>Sediments in renal tubules and vascular walls following ethylene glycol intoxication</td>
</tr>
<tr>
<td>Luxol fast blue (LFB)</td>
<td>Evidence of myelin and phospholipids</td>
<td>Myelin sheath staining</td>
</tr>
<tr>
<td>Mallory’s stain</td>
<td>Trichrome stain; collagen and reticular connective tissue is light-blue, nuclei are red, smooth musculature is violet, striated musculature orange-red, mucus is blue</td>
<td>Connective tissue stain, for example, in the case of liver cirrhosis</td>
</tr>
<tr>
<td>May–Grünwald–Giemsa stain (MGG)</td>
<td>Nuclei are purple-red, nucleoli are blue, cytoplasm is light blue-gray to red-violet, erythrocytes are pink to orange (except in the case of alkaline pH where they are green-blue)</td>
<td>Hematopoietic marrow, differentiation of cells of the myeloid and lymphatic line; eosinophil granula is red</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Nuclei are sharp blue, plasma cells are deep blue, erythrocytes are greenish</td>
<td>Suitable to detect agents, e.g., Helicobacter pylori</td>
</tr>
<tr>
<td>Naphthol AS-D chloroacetate esterase stain (Moloney et al. 1960) (enzyme-histochemical stain; abbreviated to ASD)</td>
<td>Neutrophil myeloid cells with all preliminary stages stain wine red</td>
<td>Mostly selective detection of neutrophil granulocytes in purulent inflammation of all kinds (phlegmons, abscesses)</td>
</tr>
</tbody>
</table>
2.1 Conventional Histological Staining

Immunohistochemical evidence can be found in formalin-fixed tissue, depending on the antigen, as is the case for viral antigens (Lozinski et al. 1994), but also in other molecular genetic investigations (Miething et al. 2006). Antigen-conserving methods are also discussed in order to overcome antigen loss or difficult detectability due to autolysis (Pelstring et al. 1991). Microwave pretreatment can accelerate fixation with formaldehyde (Login et al. 1987). In addition to conventional histology, which has long been common practice, immunohistochemical techniques have also found their way into forensic diagnostics (Bratzke and Schröter 1995).

### Table 2.1 (continued)

<table>
<thead>
<tr>
<th>Staining</th>
<th>Presented structures</th>
<th>Examples from forensic practice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nissl stain</td>
<td>Detects cell nuclei and tigroid bodies in nerve cells; cell nuclei and Nissl substance violet, nerve cells light blue, the rest is colorless</td>
<td>Detection of nervous tissue</td>
</tr>
<tr>
<td>Orcein stain</td>
<td>Detection of elastic fibers, used to identify the Australia HBsAG</td>
<td>Hepatocellular single cell necrosis in the case of active hepatitis B – detection of hepatitis B surface antigen; result should be checked immunohistochemically</td>
</tr>
<tr>
<td>Papanicolaou stain</td>
<td>Cells are blue to black, nucleoli are black to red, cytoplasm is blue-green (cyanophil) to pink-red (eosinophil); erythrocytes are bright red</td>
<td>Standard stain for vaginal wet mount</td>
</tr>
<tr>
<td>PAS (periodic acid-Schiff’s reagent)</td>
<td>Stains carbohydrates, in particular glycogen, purple-red (magenta) and epithelial mucin</td>
<td>Glycogen positive Armanni–Ebstein cells in the renal tubules in the case of diabetic coma</td>
</tr>
<tr>
<td>Periodic acid – silver</td>
<td>Stains basal membranes, Alzheimer’s plaques, and fungi black</td>
<td>Detection of basal membranes, for example, in the kidney</td>
</tr>
<tr>
<td>PTAH</td>
<td>Phosphotungstic acid-hematoxylin according to Mallory</td>
<td>Used to differentiate between smooth and striated muscle fibers, detects fibrin; suitable in the case of muscle damage, also in the myocardium</td>
</tr>
<tr>
<td>Prussian blue</td>
<td>Blue: hemosiderin, Fe III</td>
<td>Siderosis of the lung, hemosiderin macrophages full of pigments</td>
</tr>
<tr>
<td>Reticulin stain</td>
<td>Silvering of fine (pre-) collagen reticulin fibers</td>
<td>Basal membranes, newly formed fibers</td>
</tr>
<tr>
<td>Sudan III</td>
<td>Fat stain; lipids stain yellowish-red; Sudan IV stains more orange-red</td>
<td>Fat embolisms, fatty liver</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Detects striation of muscle fibers and метachromatic substances</td>
<td>Striated muscle tissue, mast cell granules</td>
</tr>
<tr>
<td>Silvering</td>
<td>Black: reticular fibers, nervous fibers; Brown: collagen fibers</td>
<td>Hepatic peliosis, glomeruli</td>
</tr>
<tr>
<td>Ziehl–Neelsen stain</td>
<td>Acid-resistant rods, mycobacteria (also lepra bacteria) stain bright red</td>
<td>In particular tuberculosis; microscopy x1000, oil immersion</td>
</tr>
</tbody>
</table>

There are numerous other simple and combined staining methods that are described in the relevant literature.

2.1.1 Background Staining and Artifacts in Conventional Staining Methods

In order to assess the quality of a tissue section, impurities and disturbing artifacts should be defined:

- **Displaced tissue not belonging on the microscope slide** (e.g., displaced splenic tissue, which can simulate a lymphocytic inflammatory infiltrate) (Figs. 2.1 and 2.2)
- **Excessive formalin pigment**
- **Over-staining due to a coloring agent in the case of dye combinations**
- **Slice artifact with partly missing or torn tissue** (Figs. 2.3 and 2.4)
- **Wave formation in histological sections with insufficient staining** (Fig. 2.5)
- **Artificially modified tissue due to incorrect treatment** (Fig. 2.6)

2.2 Immunohistochemical Techniques

The ability to produce monoclonal antibodies (Köhler and Milstein 1975) resulted in numerous highly specific antibodies becoming available on a commercial basis. This enables microscopic representation of specific antigenic proteins or molecules in a section or cell specimen (immunohistochemistry, immunocytochemistry). The range of immunohistochemically displayed cell and tissue proteins includes, e.g., collagens, basal
2.2 Immunohistochemical Techniques

membrane components, hormones, cytoskeleton proteins, glycoproteins of cell membranes, viral and bacterial antigens, cytokines, and complement factors.

Unlike conventional histological staining methods, immunohistochemical techniques are based on antigen–antibody bindings, which can be affected by inappropriate fixative selection and duration. Microwave-based fixation of tissue in formaldehyde may also have negative consequences (Login et al. 1987).

Fixative selection must be considered individually for each antigen and each antibody. Manufacturers state, however, whether an antibody – following formaldehyde fixation – can be used on a paraffin section or not (Noll and Schaub-Kuhnen 2000).

In practice, formaldehyde has been acknowledged as a fixative for conventional routine staining methods for decades and can also be used for fixation in certain immunohistochemical techniques.
The compatibility of different concentrations of these solutions with specific immunohistochemical techniques has only been partially investigated.

Note: The current recommendation for immunohistochemical techniques is a maximum of 4% neutral buffered formaldehyde solution and for some antibodies a maximum fixation time of 48 h.

Tissue can then be dehydrated with various concentrations of alcohol in ascending order, and can be embedded in paraffin according to Peterfi’s methyl-benzoate
2.2 Immunohistochemical Techniques

Finally, 3- to 5-μm slices are prepared as unstained sections.

With longer fixation times, proteins are cross-linked more intensely due to the fixative, so that the antigen-binding sites are masked and the added primary antibodies cannot dock (Mason and O’Leary 1991), resulting in false negative findings. To avoid this, various methods of antigen unmasking can be used, e.g., enzyme autodigestion or steeping in citrate solution. The antigen reactivity of proteins cross-linked due to fixation can be rebuilt (antigen-retrieval).

Note: Temperatures of > 60°C cause a denaturation of the proteins or antigens, and thus can also result in false negative results. A temperature of approximately 58°C is recommended, which must be considered when mounting tissue sections on microscope glass slides in a water bath.

Polyclonal and monoclonal antibodies are distinguished:

- Polyclonal antibodies bind to different parts of a macromolecular antigen.
- Monoclonal antibodies recognize only a single epitope of an antigen.

The binding of antigen and antibody (the antigen–antibody precipitate) in the tissue section must be made visible in further steps. For this purpose, an enzyme-labeled detection system is used: a secondary antibody (bridge antibody) reacts with the primary antibody, which is already specifically bound in the tissue. This leads to a local enrichment of attached enzymes. After adding a substrate solution, these enzymes become active and lead to a dye formation, which is also reflected locally. Horseradish peroxidase and alkaline phosphatase have proven successful as enzymes for this purpose. As a rule, one of these two enzymes is typically used with different coloring agents (chromogens). Even if few specific antigen quantities are visualized in this way, counterstaining of the cell nuclei is done with Haemalaun (hematoxylin), so that a microscopic orientation is possible in the tissue section.

In order to label defined antigens, two methods have been established, which can vary in individual cases: the ABC method and the APAAP method. Depending on the enzyme, substrate, and chromogen used, a different color marking is made (Table 2.2). The various immunohistochemical methods have in part been compared and tested (Sabattini et al. 1998).

In many cases, better results are achieved when tissue sections are pretreated for antigen unmasking.

### 2.2.1 Methods of Antigen Demasking

Even if only a few antigens are detected immunohistochemically, a loss of antigenic reactivity is expected due to the use of fixative, fixation duration, and paraffin embedding (excessively high temperatures). Additionally, tissue extracted during autopsy can be autolytically modified at extraction (see Chap. 19). It still applies that a particular procedure must be determined for every antigen to be detected immunohistochemically and for every antibody (fixative choice, fixation duration, temperature, incubation period, etc.). Not all commercially available antibodies can be used on a paraffin section; some can only be used after appropriate pretreatment (Imam 1995), one reason being the strong cross-linking of proteins due to formaldehyde (Mason and O’Leary 1991). In this context, different methods have proven helpful to retrieve antigenic reactivity, i.e., to break up the proteins cross-linked due to fixation (antigen retrieval) (Table 2.3). Some antigens cannot be detected immunohistochemically without antigen retrieval (Merz et al. 1995a, b). The demand for better standardization, including methods of antigen unmasking, seems to be reaching its limit due to the fact that every tissue type is different, the duration before taking a tissue sample varies (at autopsy), and the duration of formalin fixation and paraffin embedding also

<table>
<thead>
<tr>
<th>Table 2.2</th>
<th>Chromogen-dependent color marking in immunohistochemistry or immunocytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Substrate</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Hydrogen peroxide (H₂O₂)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Naphthol phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
varies considerably (Taylor et al. 1996). On the other hand, immunohistochemical visualization should be possible even with only a small number of antigens and when it is useful to strengthen their signal.

### 2.2.2 ABC-Method

Immunohistochemical staining according to the avidin–biotin complex method (ABC) is done according to the procedure of Hsu et al. (1981a, b) (Table 2.4). This procedure has more recently been modified to the LAB or LSAB method (labeled avidin/streptavidin biotin, secondary antibodies with covalently linked biotin and enzyme-marked avidin or streptavidin). When using this method, the unconjugated primary antibody initially binds to the appropriate antigen. The avidin-biotin-peroxidase complex then binds to the biotin on the secondary antibody. The added chromogen reacts with the enzyme and is deposited where the antigen is located. Contrasting cell structures are presented through counterstaining with Haemalaun. In doing so, antigens which are localized, e.g., at the cell surface can be specifically identified (cell adhesion molecules). Color intensity may vary depending on the number of antigens.

### 2.2.3 APAAP-Method

The APAAP immunohistochemical staining method is performed according to the method described by Cordell et al. 1984 (Table 2.5).

### 2.2.4 Background Staining and Artifacts in Immunohistochemical Staining

Undesirable changes to the tissue section may occur when conventional histological staining is used, as well as certain immunohistochemical techniques (see above). Artifacts in the histological section are predominantly caused by unprofessional work, incorrect fixation and embedding (e.g., tears), improper tissue cutting or mounting of the tissue section, or during staining (e.g., lighter or darker spots, etc.).

The above-mentioned technical errors while preparing tissue sections are also possible when preparing tissue sections for immunohistochemical techniques. However, in immunohistochemistry, attention should be paid to other changes or artifacts, especially in the area of unspecified, marginal background stains or undesired dye deposits (Fig. 2.7). For this reason, positive and negative controls should be conducted parallel to examination of the compound. Nevertheless, an inexperienced examiner may confuse artifacts with a positive stain (Fig. 2.8). Excessively thick tissue sections or folded tissue sections may result in an

---

**Table 2.3** Methods of antigen unmasking (antigen retrieval) in order to allow immunohistochemical staining on paraffin-embedded tissue (selection)*

<table>
<thead>
<tr>
<th>Method</th>
<th>Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic autodigestion (trypsin, pronase, pepsin, etc.)</td>
<td>Incubate tissue section with the enzyme. Note: an extremely intensive autodigestion can lead to undesired destruction of tissue structure.</td>
</tr>
<tr>
<td>Cooking in citrate buffer</td>
<td>Cook tissue sections briefly in citrate buffer in the microwave; varying concentrations and cooking times apply (Brown and Chirala 1995; Cuevas et al. 1994; Gown et al. 1993; Leong 1996).</td>
</tr>
<tr>
<td>Cooking in aluminum chloride</td>
<td>Less-known method: the tissue sections are cooked in aluminum chloride in the microwave; varying concentrations and cooking times applied.</td>
</tr>
<tr>
<td>Wet-autoclaving</td>
<td>Influence of wet heat, e.g., 120°C with citrate buffer pH 6.0 (Bankfalvi et al. 1994a, b; Dreller et al. 1998); relatively simple handling, special microscope slides may be necessary to prevent detachment of the tissue section.</td>
</tr>
<tr>
<td>Cooking in urea solution</td>
<td>Cook tissue sections in urea solution of various concentrations (Shi et al. 1994, 1995, 1997).</td>
</tr>
</tbody>
</table>

*Compare Williamson et al. (1998); Pileri et al. (1997); Werner et al. (1996); von Wasielewski et al. (1994); Dookhan et al. (1993); Leong and Milios (1993); Shi et al. (1991)
### Table 2.4  Procedure when using the ABC method according to Hsu et al. (1981a, b)

<table>
<thead>
<tr>
<th>Method</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of tissue sections</td>
<td>Mount 3- to 5-μm thin slices onto special microscope slides in order to prevent a detachment of tissue sections; water bath of maximum 58°C</td>
</tr>
<tr>
<td>Deparaffining</td>
<td>Put tissue section into xylol (2 × 10 min), then 3 min into 100% alcohol</td>
</tr>
<tr>
<td>Blockage of endogenous peroxidase activity</td>
<td>0.5% Hydrogen peroxide solution (H₂O₂)/methanol solution in order to block endogenous peroxidase, then 3 min into 100% alcohol</td>
</tr>
<tr>
<td>Rehydration</td>
<td>Rehydrate with various concentrations of alcohol in descending order, then washing in distilled water</td>
</tr>
<tr>
<td>Antigen unmasking</td>
<td>Optional: pretreatment with various methods, e.g., enzymatic autodigestion with pronase, pepsin, trypsin, or cooking in citrate solution or aluminum chloride solution, autoclaving; then washing in PBS buffer (10–20 min), incubate with normal serum (approximately 15–20 min)</td>
</tr>
<tr>
<td>Primary antibodies</td>
<td>Incubate with the desired polyclonal or monoclonal primary antibody (e.g., from mouse); incubation period varies depending on the primary antibody; then wash with PBS buffer for approximately 5 min, may be mixed with Brij solution (4–1,000 mL of PBS buffer)</td>
</tr>
<tr>
<td>Secondary antibodies (bridge antibodies)</td>
<td>Incubate the tissue section with a biotinylated secondary antibody (incubation period varies); then wash in PBS buffer or Brij solution (approximately 5 min)</td>
</tr>
<tr>
<td>ABC reagent</td>
<td>Incubate with ABC reagent (duration varies)</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>Add the substrate solution with the coloring agent consisting of: 30 mg AEC (3-amino-9-ethyl-carbazole) dissolved in 12 mL dimethyl sulfoxide, adding 200 mL 0.1 M sodium acetate buffer (pH 5.2) and 10 μL of 30% hydrogen peroxide (H₂O₂) – incubation period varies</td>
</tr>
<tr>
<td>Rinse</td>
<td>Rinse for 10 min with running tap water</td>
</tr>
<tr>
<td>Counterstaining and covering</td>
<td>Counterstain with Haemalaun (stains cell nuclei blue) and fix cover slips with glycerol gelatin</td>
</tr>
</tbody>
</table>

### Table 2.5  Procedure when using the APAAP method according to Cordell et al. 1984

<table>
<thead>
<tr>
<th>Method</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of tissue sections</td>
<td>Mount 3- to 5-μm thin slices onto special microscope slides in order to prevent a detachment of the tissue section; water bath of maximum 58°C</td>
</tr>
<tr>
<td>Deparaffining</td>
<td>Put tissue section into xylol (2 × 10 min), then into 100% alcohol for 3 min</td>
</tr>
<tr>
<td>Blockage of endogenous peroxidase activity</td>
<td>0.5% Hydrogen peroxide solution (H₂O₂)/methanol solution in order to block endogenous peroxidase, then into 100% alcohol for 3 min</td>
</tr>
<tr>
<td>Rehydration</td>
<td>Rehydrate with different concentrations of alcohol in descending order, then water in distilled water</td>
</tr>
<tr>
<td>Antigen unmasking</td>
<td>Optional: pretreatment with various methods, e.g., enzymatic autodigestion with pronase, pepsin, trypsin, or cooking in citrate solution or aluminum chloride solution, autoclaving; then wash in PBS buffer (10–20 min), incubate with normal serum (approximately 15–20 min)</td>
</tr>
<tr>
<td>Primary antibodies</td>
<td>Incubation with the desired polyclonal or monoclonal primary antibody (e.g., from mouse); incubation period varies depending on the primary antibody; then wash with PBS buffer (or Tris buffer) for approximately 5 min</td>
</tr>
<tr>
<td>Secondary antibodies (bridge antibodies)</td>
<td>Incubation of the tissue section with a biotinylated secondary antibody (incubation period varies); then wash again in PBS buffer (approximately 5 min)</td>
</tr>
<tr>
<td>APAAP complex</td>
<td>Incubation with the APAAP complex at room temperature (incubation period varies)</td>
</tr>
<tr>
<td>Wash</td>
<td>Wash in Tris buffer (5 min)</td>
</tr>
<tr>
<td>APAAP complex</td>
<td>Optional: repeat incubation with the APAAP complex at room temperature (incubation period varies)</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>Add the substrate solution with the coloring agent consisting of: 2 mg naphthol AS-MX phosphate dissolved in 0.2 mL dimethylformamide with 9.8 mL, 0.1 mL Tris buffer, 10 mL levamisole; add and filtrate 10 mg fast red TR salt prior to use</td>
</tr>
<tr>
<td>Wash</td>
<td>Wash in Tris buffer (5 min)</td>
</tr>
<tr>
<td>Counterstaining and covering</td>
<td>Counterstain with Haemalaun (approximately 20 s, stains cell nuclei blue), annealing in H₂O, fix cover slips with glycerol gelatin</td>
</tr>
</tbody>
</table>
accumulation of reagents with a false positive reaction. During immunohistochemical representation of amorphous necrotic areas or those with cell detritus, non-specific staining occurs regularly. This is also the case for strongly hemorrhagic imbibed compounds. If the desired antigen is also found in the serum following insufficient rinsing, partially intensive background stains will result.

The standardized blockade of endogenous peroxidase activity and preceding incubation with normal serum will help avoid contamination and artifacts. Non-specific binding of primary and secondary antibodies to tissue structures, which may lead to false positive results, should be avoided by increased diluting of the antibodies, which should be done in a separate procedure for each individual antibody.

In forensic medicine, the dilutions prescribed by the manufacturer can be utilized initially, but often, variations are needed for the distinct autolytic tissue to be examined.

In addition, a specificity control must be made even if immunohistochemical staining occurs on the anticipated structures microscopically. Here, positive and negative controls are critical; tissue sections containing the antigen to be detected should be stained parallel to the withdrawal trials. Specific tissue probes may be used for positive controls, e.g., tonsil tissue to detect lymphatic cells or epidermis to show cytokeratin. For the representation of individual cells, a control of identical tissue should be used, e.g., when qualifying and quantifying leukocytes in the renal glomeruli or in the myocardial interstitium. Non-specific stain deposits may be mistaken for a positive reaction during a superficial observation (Fig. 2.9), a mistake that can be clarified by using magnification while making the observation (Fig. 2.10). For qualification and quantification purposes of defined cell types, control and observation under high magnification (×400) are essential.

In immunohistochemistry, background staining can have different causes (Feiden 1995).

- It can be frequently caused by blocking of endogenous peroxidase activity; for this reason, H2O2 block (or alternatively use of the APAAP method), as well as incubation with normal serum, is part of the standard protocol for immunohistochemical staining.
- When antibodies show non-specific binding, the most effective way to counteract this is by significantly diluting the antibodies. This process must be repeated individually for each antibody. In general, the manufacturer’s dilution ratio is valid.
- Increased activity of alkaline phosphatase can be counteracted by adding levamisole to the substrate solution.
- Drying of the compound or complete deparaffinization should be avoided.
- When disruptive electrostatic binding forces are present, the ion concentrations in the dilution buffer should be increased.
- When antigen diffusion is followed by a false negative or an increasingly weak reaction, tissue or cell fixation must be examined.
- In the case of polyclonal antibodies and cross-reactivity of the antibody, one should consider

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Fig. 2.7 Non-specific stain deposit at the margin of the tissue section – ABC method (×100)
2.2 Immunohistochemical Techniques

absorption; changing to a monoclonal antibody is better.
- Tissue necrosis and advanced autolysis may lead to immunohistochemical staining which should not be regarded as specific.

It should be taken into consideration that interpretation of immunohistochemical stains presumes that the results of conventional histological stains are known. Immunohistochemical findings that do not fit within this context should be examined critically; in the case of ambiguity, findings should be limited to histological routine staining. Erroneous evaluations may occur when a finding is based on only one immunohistochemical stain. A spectrum of several antibodies should be used.

Fig. 2.8 Non-specific false positive staining of obviously intravascular, agglutinated structures with an antibody for macrophages (CD68 ×200)

Fig. 2.9 False positive detection of intramyocardial CD45R0-positive T-lymphocytes with minimal enlargement (×100)
2.3 Selection of Antigens and Antibodies

The selection of antigens to be detected or the antibodies to be used depends on the questions being asked. Thus, in the case of a newborn found dead, aspirated epidermal cells floating in the amniotic fluid of the fetus may be immunohistochemically shown under the microscope with an antibody against cytokeratin, proving amniotic fluid aspiration (see Chap. 11). A spectrum of immunohistochemical markers (antibodies) is recommended as ischemia markers for the myocardium to prove acute death following stenosing coronary sclerosis (clinical: acute lethal coronary insufficiency) (see Chap. 13), as well as to determine the age of injuries or skin lesions (see Chap. 10). The recommendation to use a spectrum of immunohistochemical markers is also valid when determining the age of brain or myocardial infarcts.

Numerous functionally relevant surface molecules of immunocompetent cells previously discovered have been given multiple descriptions. For simplification, CD nomenclature was introduced (CD, cluster of differentiation). The molecules are named with a prefix, “CD,” and they are assigned a number. The basis for assigning a CD number to a surface molecule is the availability of monoclonal antibodies that clearly define the respective surface molecule.

After an antibody has been selected, the manufacturer’s specifications for the antibody must be verified, especially in terms of whether the antibody is only to be used for a frozen section or also for a paraffin section, thus whether it is “paraffin-compatible.” The term paraffin-compatible may be misunderstood since formaldehyde, which is the most frequently selected fixative, can hinder immunohistochemical detection of antigens. Formaldehyde results in a relatively intensive interlacing of proteins such that — initially also according to manufacturer’s specifications — a procedure for antigen unmasking may be needed (see above). If sufficient reproducibility of antigen detection is ultimately achieved, modification of the antigen demasking pretreatment may be established in one’s own laboratory; different methods, solutions, and incubation times (microwave pretreatment, damp autoclave treatment, etc., see also above) are possible.

There is a differentiation between antigens of the extracellular matrix and membrane-bound antigens, e.g., of the cell or basal membranes. For example, it is feasible to select the immunohistochemically detectable basal membrane components collagen IV and laminin as representative intact basal membrane antigens. Fibronectin and complement C5b-9(m) antibodies are indicated to prove prior myocardial necrosis in the myocardium. However, in each case, the goal of immunohistochemical techniques is to gain knowledge in addition...
to conventional histological staining. Thus, in conventional myocarditis diagnosis according to the Dallas criteria, significant diagnostic insecurity exists due to interobserver variability. Immunohistochemical qualification and quantification of interstitial inflammatory cells leads to the confirmation of a high quota of inflammatory cardiac myopathies (chronic myocarditides) with dilative cardiomyopathies (see Chap. 13).

Immunohistochemical examination of injuries, in particular skin and soft tissue lesions, may lead to an approximate age determination of the lesion, which is helpful and may be significant in criminal investigations. However, in many cases, caution should be taken when basing conclusions solely on immunohistochemical findings, even if this may be possible for an individual case. Table 2.6 contains a list of current antibodies with reference to forensic medical problems. However, the number of available antibodies is so high that only selected antibodies can be listed. In the area of neurotraumatology, antibodies are used against glial and neuronal cells, as well as to determine the age of brain injuries (please see the specialized literature for general and forensic neuropathology).

Table 2.6 List of selected immunohistochemical primary antibodies (according to bibliographical references) frequently used in forensic medicine

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Destination structure/localization</th>
<th>Problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion molecule, e.g., ICAM-1, VCAM-1</td>
<td>Surface membranes, especially on endothelial cells for cell–cell interaction</td>
<td>Activation of leukocyte invasion with inflammatory processes</td>
</tr>
<tr>
<td>Anti-C5b-9&lt;sub&gt;im&lt;/sub&gt; complement</td>
<td>Complement factor C5b-9</td>
<td>Early necrosis marker, e.g., with myocardial infarct</td>
</tr>
<tr>
<td>Anti-fibrinogen</td>
<td>Fibrinogen</td>
<td>Early necrosis marker, e.g., with myocardial infarct</td>
</tr>
<tr>
<td>Anti-fibronectin</td>
<td>Fibronectin</td>
<td>Early myocardial necrosis</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>Immunoglobulin type IgG</td>
<td>Immunoglobulin deposit in glomerulus loops with heroin-associated nephropathy</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>Immunoglobulin type IgM</td>
<td>Immunoglobulin deposit in glomerulus loops with heroin-associated nephropathy</td>
</tr>
<tr>
<td>Anti-myoglobin</td>
<td>Myocardial and skeletal muscle cells</td>
<td>Myoglobin-containing protein cylinder with rhabdomyolysis</td>
</tr>
<tr>
<td>CD3</td>
<td>T-lymphocytes</td>
<td>Viral infections</td>
</tr>
<tr>
<td>CD68</td>
<td>Macrophages</td>
<td>Cellular histiocytic reaction when determining age of lesion</td>
</tr>
<tr>
<td>CD45R0</td>
<td>Activated T-lymphocytes</td>
<td>Viral infections</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Enterochromaffin-like cells, neuroendocrine tumors</td>
<td>Pheochromocytoma</td>
</tr>
<tr>
<td>Collagens</td>
<td>Basal membrane component</td>
<td>Intact basal membranes</td>
</tr>
<tr>
<td>Cytokeraatin</td>
<td>Epithelial cells, amongst others keratinizing squamous epithelial cells</td>
<td>Amniotic fluid embolism in pregnant women or amniotic fluid aspiration in newborns</td>
</tr>
<tr>
<td>Cytokines – generic term for peptide mediators with biological effect on cells, especially interleukins, interferons, chemokines, TNF-α, TGF-β, colony-stimulating factors (CSFs)</td>
<td>In part many somatic cells, amongst others vascular endothelial cells, different types of leukocytes, including T-lymphocytes, monocytes, macrophages, T-helper cells, stroma cells, etc.</td>
<td>For example: emphasized expression in inflammatory processes, activation factors for natural killer cells etc.; thus, TNF-α is produced by monocytes/macrophages in particular</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>Infected cells</td>
<td>CMV sialadenitis in particular with SIDS, CMV pneumonia</td>
</tr>
<tr>
<td>Desmin</td>
<td>Smoothly and horizontally striped muscle cells, myocardial structure protein (Paulin and Li 2004)</td>
<td>Absent in the case of myocardial necrosis</td>
</tr>
<tr>
<td>Heat shock proteins (HSP)</td>
<td>Different proteins which help other proteins maintain their secondary structure; this means protecting cellular proteins from denaturation (Javid et al. 2007; Hasday and Singh 2000)</td>
<td>Increased expression following cellular stress caused by heat, radiation, toxins, etc</td>
</tr>
</tbody>
</table>

(continued)
The following antibodies or markers have been intermittently available for the group of infectious agents: Chlamydia pneumoniae (Dettmeyer et al. 2006), cytomegalovirus (Dettmeyer et al. 2007), Cryptococcus neoformans, Epstein-Barr virus (EBV), Helicobacter pylori, hepatitis antigens HBs and HBc, HIV (p. 24), herpes simplex, human papilloma virus (HPV), Pneumocystis carinii, and Toxoplasma gondii.

The following is valid for the evaluation of immunohistochemical stains:

1. Methodical errors and artifacts must be excluded. Both positive and negative controls must yield expected results. An “internal positive control” is conceivable [e.g., thrombocytes and megakaryocytes show constitutive expression of P-selectin (Ortmann & Brinkmann 1997)].

2. When cellular antigens are specifically detected, this results in a stained cell (e.g., leukocytes, T-lymphocytes, B-lymphocytes, macrophages, etc.); at low cell counts, quantification may be done by counting cells per visual field (high power field = ×400) or per surface (mm²).

3. Cell-bound antigens may also show different intensities of expression, which correlate with color intensity. A graduation of the extent of expression is possible.

4. In the case of non-cell-bound antigens, which can be found in the intra- and extra-cellular matrix, a graduation of color intensity is normal, for example to evaluate the expression of MHC class I and II molecules. The following graduation is used:

   0 = No staining
   + = Minimal
   ++ = Moderate
   +++ = Intense
   ++++ = Extreme

   Such a semi-quantitative analysis of the staining results can be found in published forensic medicine studies and may be included in statistical analysis (Dettmeyer et al. 2004; Ortmann and Brinkmann 1997; Nwariaku et al. 1995). Microscopic evaluation of the compounds should be carried out in a timely manner, since – also according to own experience – depending on the antibody selected and storage of the tissue section, a reduction in color intensity can be possible after only a few months, which directly affects the quantification of immunohistochemical findings (Dettmeyer et al. 2009).

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Table 2.6 (continued)

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Destination structure/localization</th>
<th>Problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>Basal membrane component</td>
<td>Intact basal membranes</td>
</tr>
<tr>
<td>LCA (CD45)</td>
<td>Pan-leukocyte marker (leukocyte common antigen)</td>
<td>Inflammatory processes</td>
</tr>
<tr>
<td>MHC molecules</td>
<td>MHC molecules function in different cells as binding and presentation</td>
<td>More emphasized expression of certain MHC molecules with, e.g., viral infections</td>
</tr>
<tr>
<td>(major histocompatibility complex = MHC complex)</td>
<td>molecules for intracytoplasmic and endocytic antigens</td>
<td></td>
</tr>
<tr>
<td>Myosin</td>
<td>Cells of the skeletal musculature</td>
<td>Rhabdomyolysis; myosin cylinder in renal tubules</td>
</tr>
<tr>
<td>Selectins (E-, P-, and L-selectin)</td>
<td>E-selectin in plasma membranes of endothelial cells, P-selectin in endothelial cells and thrombocytes, L-selectin is made by all leukocytes: surface molecules to organize leukocyte invasion: rolling, trapping, diapedesis</td>
<td>Pro-inflammatory marker, in inflammatory processes</td>
</tr>
<tr>
<td>Tenascins</td>
<td>Extracellular matrix glycoproteins (Chiquet-Ehrisman and Chiquet 2003)</td>
<td>Repair processes surrounding healing lesions, including myocardial necrosis</td>
</tr>
<tr>
<td>Troponin I</td>
<td>Myocardial structure protein which builds the contractile part of the muscle cell with myosin and actin</td>
<td>Absent in the case of myocardial necrosis</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Intermediate filament of mesenchyme cells (e.g., fibroblasts, endothelial cells, smooth muscle cells)</td>
<td>Wound healing in skin lesions</td>
</tr>
</tbody>
</table>

*There are numerous other antibodies which have not been checked for suitability in connection with forensics but which are used in individual forensic studies for defined problems.
2.4 Special Examination Techniques

A number of special examination techniques are used in forensic medicine, mainly in the context of scientific studies, including: TUNEL assay, in situ hybridization, confocal laser scanning microscopy, electron microscopy, and laser microdissection.

2.4.1 TUNEL Assay

The TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) is used to detect cell nuclei in apoptotic cells. “TdT” describes an enzyme, “terminal deoxynucleotidyl transferase,” which is needed for an intermediate step. The enzyme TdT causes marked nucleotides to be added to the hydroxyl groups (3′-OH groups) released on the fragmented DNA string when apoptosis occurs. These hydroxyl groups can be made visible with the help of fluorescence microscopy. The method was first described in 1992 (Gavrieli et al. 1992). Critics find fault with the fact that reliable differentiation between apoptotic and necrotic cells is not possible (Grasl-Kraup et al. 1995). Improvements to the method have since been reported (Labat-Moleur et al. 1998). Currently, the TUNEL assay is not relevant for routine forensic medicine diagnostics, but it is used within the scope of scientific studies. Tumor tissue may be used as a positive control, since it contains many apoptotic cells, e.g., a malignant lymphoma (Fig. 2.11).

2.4.2 In Situ Hybridization

In situ hybridization is a molecular biological method used to detect nucleic acids, RNA or DNA in tissue, single cells or metaphase chromosomes. To this end, an artificial nucleic acid probe is used. The probe hybridizes (binds) to the nucleic acid of interest with the help of base pairing. The description “in situ” means that the analysis occurs directly in the cell or tissue and not in a test tube. The probes involved are generally DNA probes that are more stable than RNA probes. Marking of the probe can be done directly with haptens (e.g., digoxigenin, biotin, or 2,4-dinitrophenol) or with fluorescing molecules (fluorescence in situ hybridization, FISH). Hybridization may take from 1 h to several days depending on the probe material and
destination sequence. Probe molecules which are not specifically bound are washed out. The method used depends on the problem, e.g., proving cytomegaloviruses in the parotid gland in cases of assumed sudden infant death (Fig. 2.12). In principle, in situ PCR and PCR in situ hybridization are also possible in paraffin-embedded tissue (Schiller et al. 1998).

### 2.4.3 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) uses two channels, e.g., laser line 1 (argon ion 488 nm) and laser line 2 (krypton 568 nm) and allows detection of two fluorescent signals (double markers) from the same specimen scanned simultaneously and digitally converted into an image. This technique of microscopic imaging has transformed the field of biology, and forensic histopathology in particular (Wyss and Lasczkowski 2008; Turillazzi et al. 2007; Lucitti and Dickinson 2006). By allowing greater resolution, optical sectioning of the sample and three-dimensional reconstruction, CLSM has found a wide field of application (e.g., sudden cardiac death, neonatal hypoxic-ischemic lesions, electrical and explosion injuries). For example, CLSM was used to investigate the vitality and age of conjunctival petechiae by investigating the expression of the endothelial adhesion molecule P-selectin (Wyss and Lasczkowski (2008), Fig. 2.13).

### 2.4.4 Electron Microscopy

The development of electron microscopy has opened new horizons for medical and physical research (Biro et al. 2010). The interior of an object, or its surface, can be displayed with the help of an electron microscope. While the optical microscope only reaches a
resolution of approximately 200 nm, the current resolution of the electron microscope is approximately 0.1 nm. There are different types of electron microscope. When creating a picture, the raster electron microscope (REM) (scanning electron microscope) is differentiated from the still-life microscope. In view of the geometry of the arrangement, scanning transmission electron microscopy is considered to be a technical variation of still-life microscopy.

With the scanning electron microscope (SEM), a thick electron ray is guided over the object. During this process, emitted or backscattered electrons, including other signals, are synchronously detected; the intensity of the pixel is determined by the current. When working with the transmission electron microscope, electrons travel through the object, which need to be correspondingly thin. The object should be embedded in the fixative glutaraldehyde for electron microscopic evaluation.

SEM with energy dispersive microanalysis (EDX) provides valuable information in forensic medicine about the morphology of injuries and injury implements. The use of SEM is not limited by autolysis to the same degree as transmission electron microscopy, for example. SEM can be used for the study of various types of wounds and particularly for the study of bullet wounds (Havel 2003; Havel and Zelenka 2003; Kage et al. 2001; Torre et al. 2002; Fechner et al. 1990; Brinkmann et al. 1984). Also, other authors concluded that SEM, together with EDX, can provide explicit information in bullet wound investigations (Cardinetti et al. 2004), and can be useful for diagnosis in cases of electrocution (Kinoshita et al. 2004).

Electron microscopy plays an important role in forensic medicine for the detection of metallic particles, but otherwise it is used foremost within the scope of scientific studies. In certain cases, SEM together with EDX enables the determination of projectile parameters in firearm wounds, as well as an approximate determination of firearm distance (Biro et al. 2010; Dubrovin and Dubrovina 2003).

### 2.4.5 Laser Microdissection

Laser microdissection is a technique to isolate certain cells from microscopically analyzed smears, tissues, and/or organs. The tissue or single cells are cut open with a laser without damaging their morphology. This technique is used to collect cells for specific DNA or RNA analyses, e.g., sperm following a sexual offense (Vandewoestyne et al. 2009).

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