Immunolocalization

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Abstract

This chapter presents an overview about the immunocytochemical techniques using in the model legume *Medicago truncatula*. After a short introduction into the basics of immunocytochemistry, detailed protocols that can be used to perform immunolabelling on light, confocal and electron microscopical level are listed. These protocols are successfully applied in the author's laboratories to obtain information about the localization of various proteins in a range of tissues from *Medicago truncatula*. Depending on facilities to perform sectioning and the microscopical equipment, modifications might be required to suit individual demands. Therefore, please use these protocols as a starting point and adjust them after consulting experienced personnel at your local facilities.

1 Introduction into immunocytochemistry

The goal of immunocytochemistry, a combination of immunochemistry and morphology, is to define the cellular location of biochemically defined antigens. Immunocytochemical techniques can be applied to all types of cells. Plant cells, however, require the development and application of specific techniques to optimise their handling and observation, especially for light microscopy. The presence of the cell wall, hindering the penetration of antibodies, and other natural barriers such as cuticles, are features of plants that make working with plant tissues more difficult than with animal tissue. Moreover, abundant carbohydrates which can interfere non-specifically with antibodies, and specific compounds (chlorophylls, diferulic acid or tannins) which have autofluorescence may mask a specific signal. Therefore, the so-called post-embedding labelling is the most favourable procedure, thereby using an indirect immunostaining consisting of two successive immunoreactions: the antigen being first detected by the specific antibody, and this primary antibody itself being recognized by a second antibody to which is attached a visual marker. The choice of the marker depends on the question which has to be answered: cell- and tissue-specific occurrence of an antigen in an overview/large section is easier to visualize by bright field microscopy, whereas the location of a certain protein within a cell is better to resolve by fluorescence microscopy. In the first case, secondary antibodies coupled with an enzyme which leads to the formation of a diachrome, are mostly used. Here, the signal is amplified by the enzymatic reaction. The main disadvantage of such an enzyme is, however, that the reaction product does not always remain much localized. This can be circumvented by the use of fluorescence-labelled antibodies, which remain highly restricted at the site of binding. To get information about sub-organellar location of proteins, the resolution of light microscopy is not high enough; here electron microscopical techniques using gold-labelled antibodies are recommended (Figure 1).

Principally, to observe, localize and identify the structural and molecular components of the cell in their *in vivo* position, the following points have to be taken into account:

1. characteristics of immunoreaction and properties of antibodies
2. preservation of cells in a close to live state
3. chemical reactions and physical treatments which could lead to masking of epitopes, thereby preventing successful immunoreactions

2. Critical points in immunocytochemistry: antibodies and fixation

2.1 Antibodies

One of the main criteria for the use of immunocytochemical techniques is the availability of antibodies, which are specific and show a high-affinity against a cellular antigen. Therefore, it is very important to verify that the antibody does not show any irrelevant cross reactivity. This may occur when the antigen of interest shares a common epitope with an apparently unrelated protein or the antigen used for immunisation was not pure enough. These problems come up mainly due to the use of polyclonal antibodies. In common, polyclonal antibodies are relatively quick, easy, and cheap to make. In the most cases, they are very successfully used in immunocytochemistry, because within this mixed population of antibodies one or two will recognize epitopes even after the antigen has been denaturated by fixation or other processing procedures. In some cases, however, the specificity of polyclonal antibodies will be not high enough (e.g. for different highly similar proteins of one family), so monoclonal antibodies or antibodies raised against peptides have to be used. These antibodies do not need be treated

differently to any other immunogen. However, the likelihood to get successful labelling with anti-peptide antibodies is very difficult to predict.

In all cases, the simplest way to demonstrate antibody specificity is the immunoblot (Western blot). After separation of total proteins in an SDS polyacryl amide gel, proteins were transferred to a membrane followed by immunoreaction with the antibody. Additionally, beside the demonstration of specificity, immunoblots can be used to find the best dilution for the respective antibody. After loading of 10 µg of total plant protein and using secondary antibodies conjugated with alkaline phosphatase followed by staining reaction with *p*-nitroblue tetrazoline chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP), the specific band should appear within 5 min. As rule of thumb: start the first experiments in immunocytochemistry with a two-fold higher antibody concentration as used for the blot. However, sometimes further optimisation of both primary and secondary antibodies dilutions will be necessary. There are, however, exceptions: sometimes clearly detectable bands occurring in immunoblots are not reflected by signals in immunocytochemistry and *vice versa*. For successful immunolocalization, the actual local concentration and specific localisation of the antigen within a cell or organelle is of main importance.

For immunocytochemistry, it is necessary to perform negative controls. This can be done by (i) the use of pre-immune serum (not recommended for purified antibodies); (ii) omitting the first antibody; and (iii) the use of tissues from mutants which lack the respective protein.

2.2 Fixation

The ultimate aim of fixation is to “freeze” cell and tissue organization in a particular time frame so that every molecule in that cell or tissue remains in its original location during visualization. Since cryofixation methods require a high input in specific equipment, we focus on chemical fixation. Chemical fixation using aldehydes is easy to perform and does not require special equipment. However, physical loss of epitopes, steric hindrance of epitopes by fixative-induced cross-links and significant alterations of epitopes by direct reaction with fixatives may occur. Therefore, a “weak” fixation has to be achieved. Formaldehyde and paraformaldehyde are the most frequently used aldehydes in immunocytochemistry, whereas glutaraldehyde may induce autofluorescence and should either be avoided or used in very low concentrations (0.01-0.1 %).

Finally, it is recommended that a large volume of fixative is used compared to the volume of the tissue to be fixed in order to maximize the buffering capacity, and also to avoid uncontrolled dilution of fixative with cell material. Moreover, the fixation should be done with immediate concentrations of aldehydes (3-5 %) and not longer than few hours to avoid ‘over fixation’.

3. Immunocytochemistry at light microscopical level

3.1 Coating of slides

For immunolabelling of sections done with embeddings in PEG1500 (3.2.) we use always normal glass slides and coat them by our self with poly-L-lysine. Alternatively, the slides can be silanized and coated with egg white (see second protocol). Moreover, commercial coated

slides are available: Superfrost+, Menzel-Gläser, Germany, or Poly-Prep slides (Sigma or Agar Scientific).

Coating with poly-L-lysine:
- Label the slides with numbers by using a pencil with diamond pin
- clean slides by incubation with ethanol for 30 min
- dry slides by wiping
- put 60 µl of 0.1 % poly-L-lysine (SIGMA) on one slide and place a second slide on top (arranged "face-by-face"). The solution should be distributed evenly between both slides without any air bubbles.
- incubate in a humid chamber for 30 min
- separate the slides by dunking them in distilled water
- wash slides three times with water, remove access of water by shaking and let them dry at root temperature (dust-free area)
- store slides at –20 °C, before use warm them up and let them dry again!

Coating with egg-white:
- silanize clean glass slides by dipping in 3 % (v/v) (3-aminopropyl)triethoxysilane in pure acetone for one min
- wash slides in pure acetone for one min
- dip slides into ethanol/petroleum ether (3:1) and let dry. These slides can be stored for months at room temperature.
- add 3-4 µl of filtrated chicken egg white to one slide and distribute uniformly over the surface. Slides are immediately ready for use.

3.2 Fixation, embedding and sectioning

The method we prefer is the embedding in poly-ethylene glycol (PEG). This method was first described by Van Lammeren et al. (1985). PEG is available in different molecular weights which causes different hardness of the solid compound. By various mixtures of PEG1500 and PEG4000 various degrees of hardness of the wax-like embeddings can be achieved. This depends on the tissues embedded and the desired thickness of the sections. However, the original published mixture of PEG1500 and PEG400 (2:1) is quite difficult to handle. Therefore, we switched to the use of pure PEG1500, which turned out to be useful for thick (20 µm) as well as semithin (2 µm) sections. Another advantage is the low melting temperature of PEG1500, which allows performing infiltration at 45 °C leading to better preservation of epitopes. If not otherwise indicated, all steps are performed at room temperature. Alternatively, 'Steedman's wax', a mixture of PEG400 distearate : 1-hexadecanol (purity >99%) 9:1 (w:w), heated at 65 °C and stirred for 3-4 hours, can be used. This method adapted to plant material was first described by Brown et al. (1989).

- prepare sufficient amounts of fixative: 3% to 4 % paraformaldeyhde/0.1 % Triton X-100 in phosphate buffered saline (PBS) or in microtubule-stabilizing buffer (MSB) for labelling of cytoskeletal components)
- cut small pieces of the plant material (about 3 mm x 5 mm for leaves, 3-4 mm in length for roots) within the fixative. Use razor blade or fresh scalpel.
- perform vacuum infiltration for 5 up to 10 min;
  attention: due to Triton X-100, solution starts to foam, stop with vacuum if a lot of foam rises, aerate the samples, start a second (if necessary a third) time with vacuum,

stop with vacuum if no more air bubbles are coming out of the tissue; alternatively you can use PFA without Triton X-100 and use vacuum for 1 to 3 h depending on size of material (when appropriately fixed, samples should be located near the surface of the fixing solution).

- fix material for 2 h with gentle agitation
- wash material two times with PBS or MSB for 15 min each
- perform dehydration in graded series of ethanol (diluted with distilled water):

  30 min 10 % (v/v) ethanol, 60 min 30 % (v/v) ethanol, 60 min 50 % (v/v) ethanol, ON 70 % (v/v) ethanol at 4 °C (the samples can be stored at 4 °C for several days)

  60 min 90 % (v/v) ethanol, 60 min 100 % (v/v) ethanol, 30 min 100 % (v/v) ethanol at 45 °C

  alternatively: use a 30%, 50%, 70%, 90%, 97% ethanol serie, 30 min each step)

Embedding in PEG1500:

- stepwise infiltration of PEG: perform all following steps at 45 °C, PEG can easily melted in the microwave:

  60 min ethanol : PEG = 3:1

  60 min ethanol : PEG = 1:1

  90 min ethanol : PEG = 1:3

  two times 60 min pure PEG

  - embedment in PEG (use embedding moulds made from silicon, e.g. from Sigma
- hardening of embedded material at RT ON
- store embedded material at 4 °C within closed tubes until use
- sectioning: fix blocks containing the specimen on holder using melted PEG; perform sectioning by use of a microtom. Sections can be collected in paper boxes and stored for few hours only. Perform immunolabelling at the same day!

Embedding in Steedman's wax:

- one volume of wax is added at 42 °C; 2 phases are formed and tissues initially at the ethanol:wax interface progressively fall into the wax layer. After an ON incubation, first ethanol, then wax is removed and replaced by 100% fresh wax and further incubated ON at 42 °C. At this stage, material (such as roots) can be oriented, taking care of avoiding bubbles; wait 1hour before transferring at RT.
- sections are directly collected on coated slides, which can be dried at 37 °C ON and stored a few days at 4°C before use.

Buffers:

PBS (phosphate buffered saline):

135 mM NaCl
3 mM KCl
1.5 mM KH_2PO_4
8 mM Na_2HPO_4, pH 7.0-7.2

MSB (microtubule-stabilizing buffer):

0.1 M PIPES
10 mM EDTA
10 mM $MgSO_4$, pH 6.9

3.3 Immunolabelling and counter staining

Sections from embedding in PEG1500:

- transfer sections () to poly-L-lysine-coated slides *via* "hanging drop-method". Usually we use a loop made from wire and fill it with 45 % (w/v) PEG6000 in PBS. Likewise, thick sections (20 µm) can be transferred directly into a well of a 24-well-plate containing a tissue culture insert with an 8 µm mesh (Nunc, Wiesbaden, Germany). All further steps are principally the same. In the case of 'in-sieve' labelling, sections have to be transferred to poly-L-lysine coated slides by using a Pasteur pipette after finishing the labelling. Due to the solubility of PEG in water, there are no dewaxing and rehydratation steps necessary.
- incubation of slides in PBS for 10 min (removal of PEG from sections)

Sections from embedding in Steedman's wax:

- use an ethanol : PBS serie (v:v): 97% ethanol, 3x10min; 90% ethanol, 10min, 50% ethanol, 10min; 30% ethanol, 10 min; PBS, 2x10min

Immunolabelling:

- blocking of free aldehydes by incubation in 0.1 M NH_4Cl (in PBS) for 5 min
- wash with PBS, 5 min
- incubate in 5 % bovine serum albumin (BSA) in PBS for 30 min to block unspecific binding sites
- incubate in primary antibody diluted in 5 % BSA/PBS at 4 °C over night, or alternatively 1-2h at RT (per slide about 200 µl solution, incubation without cover slip and within a humid chamber)
- wash with 0.1 % BSA in PBS, 3 x 10 min
- wash with 1 % BSA in PBS, 1 x 10 min
- (- alternatively: wash 3x10 min in PBS, only)

The following steps have to be performed, if an antibody conjugated with fluorescent dye is used. Protect the slides from strong light to prevent fading of the dye:

- incubate in secondary antibody, diluted in 5 % BSA/PBS, at 37 °C for 120 min or 1h at RT (humid chamber)
- wash with PBS, 3 to 4 x 10 min
- counterstaining:
  - with DAPI (1 µg/ml in PBS) or propidium iodide (1 µg/ml in PBS) to stain DNA (15 min)
  - alternatively: toluidine blue 0,01% in PBS, 10 min, can be used
  - with fluorescence-labelled WGA (µg/ml in PBS) to stain fungal structures (30 min)
- wash with PBS twice, 10 min each
- mount section in anti-fading reagent e.g. Citifluor (in glycerol-PBS); use nail-varnish to seal the cover slip on the slide

The following steps have to be performed, if an antibody conjugated with alkaline phosphatase is used:

- equilibration of sections by incubation with staining buffer
- incubation with staining solution: NBT/BCIP (4.5 µl/3.5 µl) in staining buffer, 100 µl per slide, cover slip (without spacer), incubation for 20 – 30 min. in humid chamber in inverse position (upside-down), control of staining reaction by microscope!
- removal of cover slip by floating in TE-buffer

- washing twice with TE, 5 min each
- mount sections with glycerol/PBS, use nail-varnish to seal the cover slip on the slide

Buffers:

PBS:	see 2.2.6.3.2.
staining buffer:	0.1 M Tris/HCl, pH 9.5 0.1M NaCl, 0.05 M $MgCl_2$
stock solutions: NBT:	75 mg/ml 70 % dimethylformamide
BCIP	50 mg/ml 100 % dimethylformamide
TE:	10 mM Tris/HCL, pH 8.0 1 mM EDTA

3.4 Microscopical evaluation

To examine semithin sections (2-5 µm) after immuno labelling with fluorescence-labelled antibodies, a regularly available epifluorescence microscope is commonly used. Usually, all standard filter combinations for the most common dyes are available (see Table 1). For thicker sections (20 µm) the use of a confocal laser scanning microscope is recommended to avoid blurring from the non-in-focus levels. Here, multi track mode is useful to prevent false-positive signals due to the autofluorescence of plant tissue, or when multiple immunostaining are done using dyes with overlapping excitation and/or emission spectra. A fluorochrome database is available for example at: http://microscopy.bio-rad.com/fluorescence/fluorophoreDatab.htm. Staining by alkaline phosphatase results in a purple precipitate. This should be visualized by bright field microscopy. In the most cases, the comparison with control labelling (e.g. pre-immune serum) is crucial due to unspecific binding of the secondary antibody.

Table 1: Survey about the most important dyes used in fluorescence immunocytochemistry (label of antibodies and counterstaining) and the respective filter combinations

fluorochrome	**labelling of**	**excitation (nm)**	**beam splitter(nm)**	**emission (nm)**
fluorescein isothiocyanate (FITC)	antibodies	490	510	525
BODIPY	antibodies	503	510	512
AlexaFluor488*	antibodies, WGA	488/490	510	520
Trimethyl rhodamin isothiocyanate (TRITC)	antibodies, WGA	540-560	580	580
Texas Red	antibodies, WGA	590	600	615
AlexaFluor546	antibodies, WGA	546	580	580
Cy3	antibodies	575	580	605
Cy5	antibodies	640	645	705
DAPI	DNA	359	395	441
propidium iodide	DNA	520	580	610
aniline blue	callose	380	395	515
calcofluor white	cellulose	380	395	440

* AlexaFluor-dyes are available in a broad range of excitation/emission wavelengths. The number after the name always refers to the excitation wavelength.

4. Immunocytochemistry at electron microscopical level

4.1 Coating of grids

Nickel grids for electron microscopy have to be coated with a film to mount the ultrathin sections on it. We usually use formvar films, which are prepared using normal, cleaned glass slides.

- solve Formvar (e.g. from Serva) in water free chloroform to get a 0.5 % (w/v) solution
- fill a separating funnel with an appropriate amount of formvar solution, so that a slide dips in to 2/3 of its length
- let the solution run down very slowly – the speed determines the thickness of the film! (slow – thin film, fast – thick film)
- let coated slides dry at least for 30 min
- remove the film at the edges of the slide by scratching, dip it slowly into a beaker filled with distilled water. The film floats on the water surface, whereas the slides dunk in.
- place several grids on the film swimming on the water surface (glossy side up)
- place a filter paper on grids and film, after soaking with water take the filter paper together with grids and film out, let it dry in a dust-free environment for at least one day.

4.2 Fixation, embedding and sectioning

Here, we describe the embedding in London Resin (LR) White, a methacrylate compound. This is used for embedding at normal (room) temperature and after chemical fixation and is available in different hard grades. After use of other fixation methods (e.g. cryo fixation) and/or cryo-substitution, other embedding materials like buthyl methyl methacrylate or Lowicryls are recommended.

- prepare sufficient amounts of fixative: 3 % paraformaldeyhde/0.25 % glutaraldehyde in PBS
- cut small pieces of the plant material (about 1 mm x 6 mm for leaves, 3-5 mm in length for roots) within the fixative. Use razor blade or fresh scalpel.
- perform vacuum infiltration for 5 up to 10 min
- fix material for 2 h with gentle agitation
- wash material two times with PBS for 15 min each
- perform dehydration in graded series of ethanol (diluted with distilled water):
  30 min 10 % (v/v) ethanol, 60 min 30 % (v/v) ethanol, 60 min 50 % (v/v) ethanol, ON 70 % (v/v) ethanol at 4 °C (samples can be stored at 4 °C for several days)
  60 min 90 % (v/v) ethanol, 60 min 100 % (v/v) ethanol, 60 min 100 % (v/v) ethanol
- stepwise infiltration of LR White (diluted in ethanol, in 10 % steps till 100 % LR White, one hour each)
- exchange pure LR White at least twice within 24 h
- fill gelatine capsules with LR White, put in the plant material, close the capsules properly and let them polymerise for 12-24 h at 60 °C

- sectioning: trim the specimen properly; use an ultramicrotom to get sections of 80 nm thickness. Sections have to be collected on coated grids and stored in a dust-free box until use

Buffers:

PBS: see 3.2.

4.3 Immunolabelling and contrasting

Principally, the method is similar to the labelling of semithin sections on glass slides (3.3.). Using ultrathin sections on grids, grids were floated on 10 – 20 µl of the respective solution (see Figure 2). **Attention**: avoid dunking of grids into solution, they have to swim always on the droplet! For all steps use fresh or sterile filtrated solutions to avoid contaminations with small particles or microbes.

- incubate in 1 % acetylated BSA in PBS (BSA_{ac}/PBS) for 15 min to block unspecific binding sites
- incubate in primary antibody diluted in BSA_{ac}/PBS at 4 °C over night (per grid at least 10 µl solution, incubation within a petri dish containing wet filter paper)
- wash with BSA_{ac}/PBS, 3 x 5 min
- incubate in secondary antibody conjugated with colloidal gold, diluted in BSA_{ac}/PBS, at RT for 60 min (humid chamber)
- wash twice with distilled water, 5 min each
- dry grids carefully

Contrasting:

- incubate sections in saturated aqueous uranyl acetate solution for 10 min
- wash three times with distilled water, 5 min each
- dry grids carefully
- incubate sections in 0.2 % (w/v) aqueous lead citrate solution for 5 min
- wash five times with distilled water, 4 min each

Buffers:

PBS: see 3.2.

4.4 Transmission electron microscopy

Immuno-labelled ultrathin sections are usually examined in a conventional transmission electron microscope. Labelled antigens are marked by gold particles visible as black dots in the micrograph. In common, there is only low degree of amplification of the signal. Using a secondary antibody coupled to gold particles, each antigen can be labelled by a group of gold particles. Nevertheless, to decide where in a cell a specific antigen is located, statistics have to be performed. The number of gold particles has to be counted in even-sized areas of organelles in different sections to obtain a signal-to-noise ratio, both in immunolabelled sections as in controls performed by using pre-immune serum. Please, take into account that the number of gold particles within one cell is often very low. It should always be remembered that the amount of antigen in an ultrathin section is relative small compared to biochemical samples.

For further reading the following books are highly recommended:

G. Griffiths (1993) Fine Structure Immunocytochemistry, Springer-Verlag, Berlin Heidelberg
C. Hawes, B. Satiat-Jeunemaitre (2001) Plant Cell Biology, 2nd edition, University Press, Oxford

References:

Brown, R.C., Lemmon, B.E., Mullinax, J.B. (1989) Immunofluorescent staining of microtubules in plant tissues: Improved embedding and sectioning techniques using polyethylene glycol (PEG) and Steedman's wax. *Bot. Acta* **102**: 54-61

Hans, J., Hause, B., Strack, D., Walter, M.H. (2004) Cloning, characterization and immunolocalization of a mycorrhiza inducible 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in arbuscule-containing cells of maize. *Plant Physiol.* **134**: 614-624

Hawes, C., Satiat-Jeunemaitre, B. (2001) Plant Cell Biology, 2nd edition, University Press, Oxford

Van Lammeren, A.A.M., Keijzer, C.J., Willemse, M.T.M., Kieft, H. (1985) Structure and function of the microtubular cytoskeleton during pollen development in *Gasteria verrucosa* (Mill.) H. Duval. *Planta* **65**: 1-11

Figures:

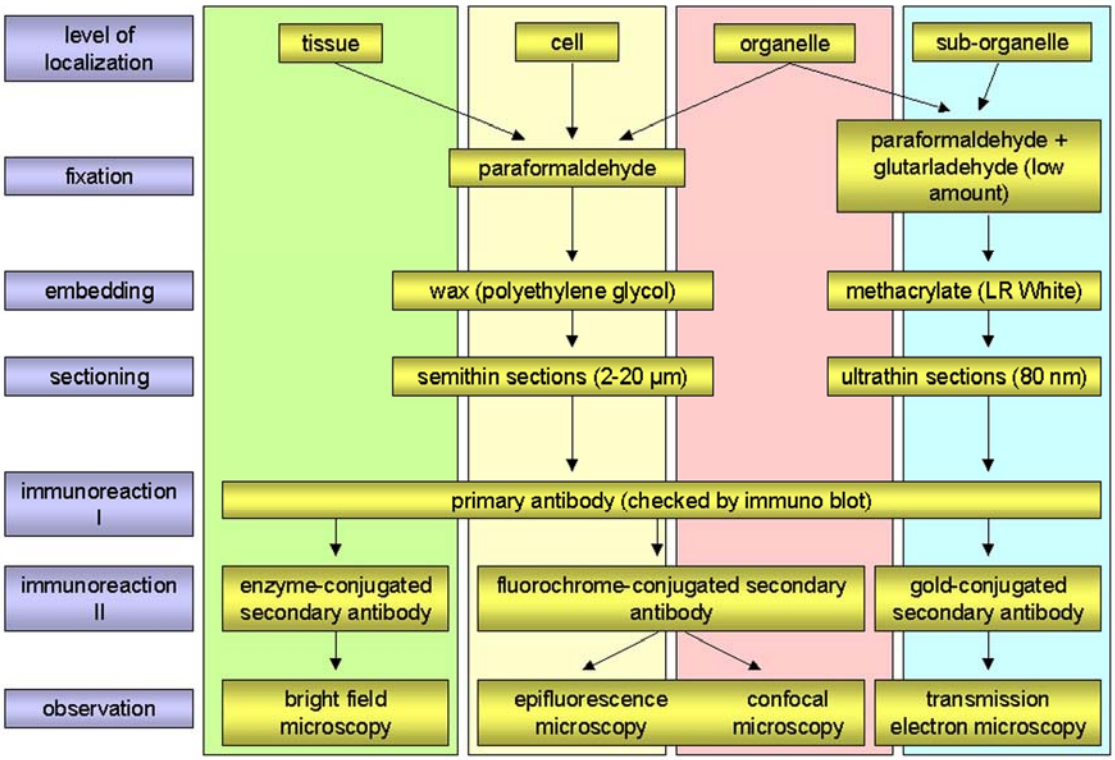

Figure 1:
Flow chart for immunocytochemistry (adapted from Hawes and Satiat-Jeunemaitre, 2001)

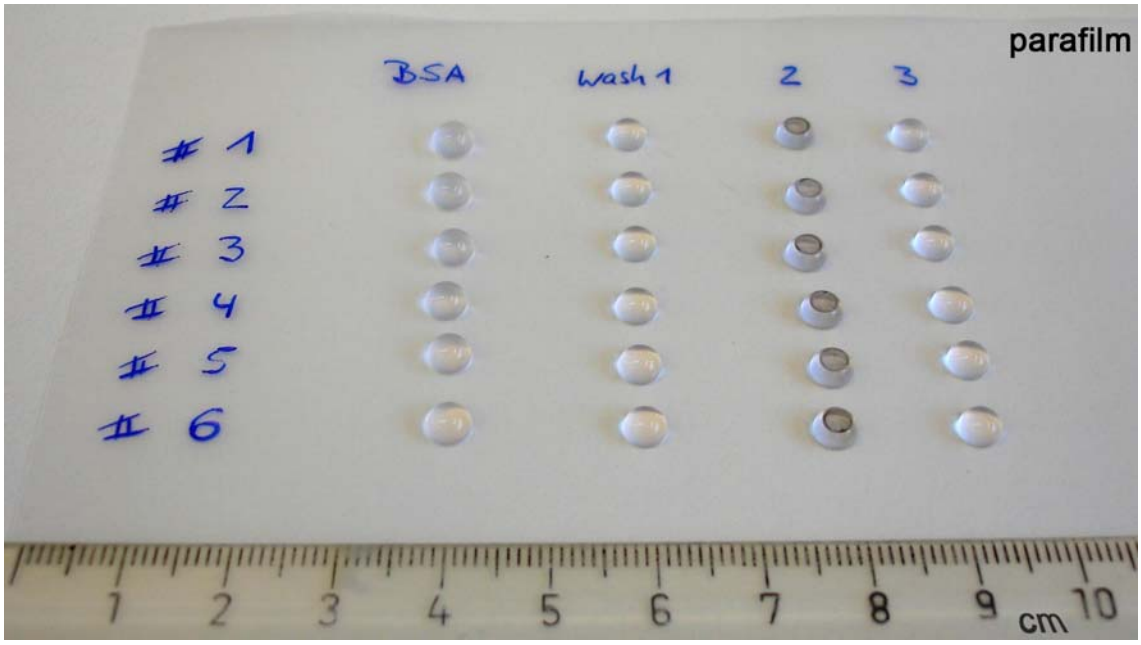

Figure 2:
Set-up for incubation of grids for transmission electron microscopy