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Comparison of TEM sample preparation methods for immunogold labeling

Bachelor Thesis

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Biological Chemistry

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Annotation

The tracking of antigens in biological samples can be achieved by labeling them with gold-conjugated antibodies (immunogold labeling). This thesis gives an overview of several sample preparation methods for TEM and investigates their effects on the binding efficiency of antibodies to certain antigens. For this reason, protein samples were processed according to four different protocols, cut into ultra-thin sections and labeled with primary and secondary antibodies. The micrographs, taken during observation with a TEM, were analysed quantitatively and the obtained data was statistically evaluated.

Affirmation

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I. Abbreviations

AB = antibody

BA = blocking agent

BSA = bovine serum albumin

dH_2O = deionized H_2O

EDC = 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide

EM =electron microscopy

FA = formaldehyde

FITC = fluorescein isothiocyanate

FS = freeze substitution

FSG = fish skin gelatine

GA = glutaraldehyde

GAM = goat anti-mouse IgG

HPF = high pressure freezing

HRP = horseradish peroxidase

Hgb = haemoglobin

LD = labeling density

LN_2 = liquid nitrogen

LRW = London Resin White

MWI = microwave irradiation

ND = not determined

NHS = N-hydroxy-succinimide

NP = nanoparticle

PB = phosphate buffer

PBS = phosphate-buffered saline

PEG = polyethylene glycol

PLT = progressively lower temperatures

RAG = rabbit anti-goat IgG

SQFS = super quick freeze substitution

TEM = transmission electron microscopy

Tfn = transferrin

UA = uranyl acetate

pA = protein A

1 Introduction

Despite of various techniques that have been developed more recently, transmission electron microscopy (TEM) is still one of the most sophisticated technique for morphological and immunological studies of biological specimens nowadays. One major advantage of TEM is its flexibility, which allows the imaging of a wide variety of specimens at high magnification. Consequently, there are also a lot of different sample preparation techniques available for scientists in EM laboratories. In general, the purpose of immunological studies is the detection of a certain antigen in a certain region of the sample. The tracking of antigens can be achieved by labeling them with antibodies (immunolabeling). This labeling process can either be done before embedding of the sample into a polymerized resin (pre-embedding), or afterwards (post-embedding). The preparation of cryosections, also referred to as Tokuyasu technique, is another possibility. In this approach, the dehydration and resin embedding steps are omitted. The post-embedding technique includes putting the sample into polymerized resin, cutting ultra-thin sections and finally the localization of antigens with labeling probes (in most cases IgG molecules) on the section surface. The antibodies used for labeling are conjugated to electron-dense markers (e.g. gold nanoparticles).

First, an appropriate technique of TEM must be chosen, which not only preserves the morphology of the sample, but also maintains good antigenicity of the protein to be found. The processing time should also be kept as short as possible, because antigens tend to be extracted out of the sample even at very low temperatures.

In the last years, researchers have published their efforts in reducing this relatively long procedures to shorter, more convenient time periods. One major progress was made by the introduction of microwave-assisted processing of biological specimens. Experiments involving the application of microwaves resulted not only in reduced processing times, but also in improved morphology and increased antigenicity of some biological antigens. [1]

Another example of these efforts is the development of so-called rapid processing methods for cryo-fixed biological specimens. One recent publication shortens a protocol of several days [2], recommended in 1984, to “3 hours or less” [3] without making any trade-off concerning the fine structure preservation of the sample. The freeze substitution (FS) method, which takes about 3 hours, was called “Quick

Freeze Substitution” (QFS). The same author also presents a protocol, which only takes approximately 90 minutes, referred to as “Super Quick Freeze Substitution”. During these rapid-processing methods the temperature is gradually increased to room temperature, which also increases the speed of FS. QFS and SQFS have both resulted in very good structural preservation, as demonstrated in the provided pictures of the publication. [3] Nevertheless, the effects on antigenicity of proteins and on the labeling efficiency (the number of gold particles per target antigen) still need to be assessed and compared to other, more conventional sample processing methods.

2 Literature Research

2.1 Fixation

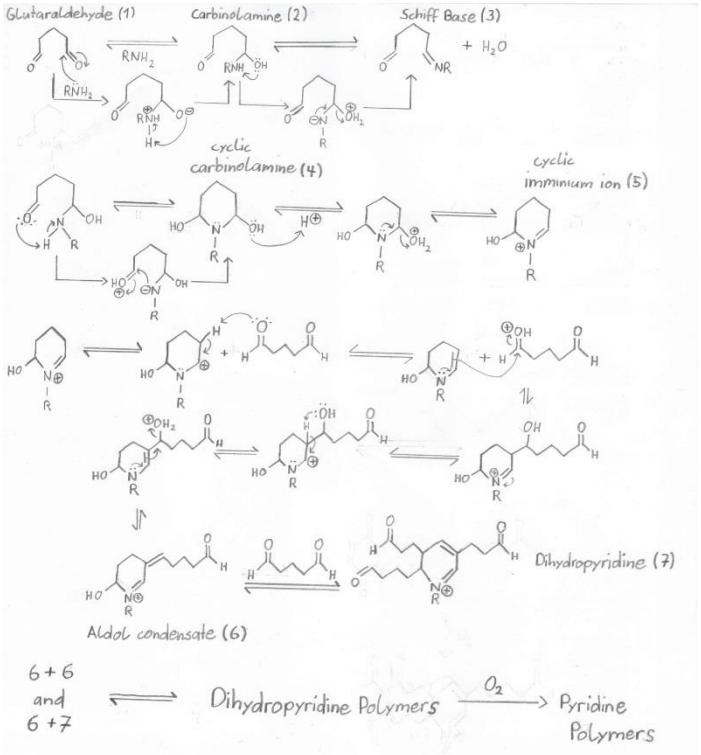
Fixation of biological samples is the first step of sample processing for EM. The purpose of fixation is to preserve the structure of cells with minimum alteration from the living state regarding volume, morphology, and spatial relationships of organelles and macromolecules. [4] In other words, through fixation a minimum loss of tissue constituents and the protection against subsequent treatments (rinsing, dehydration, staining, and exposure to both vacuum and the electron beam) is obtained. [4] There are several methods available for fixing a biological specimen. A major distinction is made between chemical fixation and cryo-fixation, whereas both have advantages and disadvantages. The best technique for preserving the properties of the living state currently is the CEMOVIS imaging procedure combining cryo-immobilization, followed by cryo-sectioning and observation of the hydrated sections under the electron microscope working at the cryogenic temperature. This approach can be alternatively combined with the pre-embedding immunolocalization techniques, but cannot be used in combination with immunolabeling on sections.

2.1.1 Chemical Fixation

For primary chemical fixation, aldehydes such as glutaraldehyde and formaldehyde are most often applied. [5] Non-aldehyde reagents like imidoesters and peroxy-disulfate are alternatives, but are used rarely. [6] Primary fixation usually employs buffered aldehydes forming an inter- and intramolecular network of cross-links (mostly between amino groups) that stabilizes the sample. [6] In any case, the fixative needs to fully penetrate the sample to obtain optimal cross-linking. The appropriate time of fixation, temperature and concentration of fixative are therefore a crucial factor for successful stabilization of the sample. Microwave-assisted sample processing can shorten the time of fixation considerably by increasing the rate of cross-linking in the sample. [1] MWI also increases the rate of diffusion of chemicals such as fixatives into the sample. [7] The diffusion and cross-linking rates are probably accelerated by an increase in vibrational energy or polarization of molecules, even without temperature changes. [8]

2.1.1.1 Glutaraldehyde

Glutaraldehyde is used for primary fixation of all kinds of biological samples. It reacts very rapidly and irreversibly with amino groups of proteins. [6] Furthermore, GA can react with phospholipids of membranes, if they possess a primary amine (e.g. phosphatidylserine, phosphatidylethanolamine). [9] Nucleic acids and carbohydrates are considered to be trapped in the matrix of cross-linked proteins. [10] The reaction with amino groups results in the formation of pyridine polymers. [6] In the first step of the cross-linking process, GA attacks the amino group of a protein and forms a Schiff's base. [6] Afterwards a cyclic iminium ion is formed, which in turn can be attacked by two GA molecules. [6] A so-called condensation reaction finally causes the formation of the pyridine polymer. A more basic environment generally increases the rate of a condensation reaction and therefore also the rate of cross-linking. [6] The result is a gel-like structure, which is resistant to protein denaturation agents, such as urea, and high temperatures. [6] There are several studies discussing the alternating effect of GA on secondary and tertiary structures of proteins, which consequently could affect their antigenicity. However, this effects seem to be relatively small compared to other, more influential ones described in chapter 2.1.1.3.



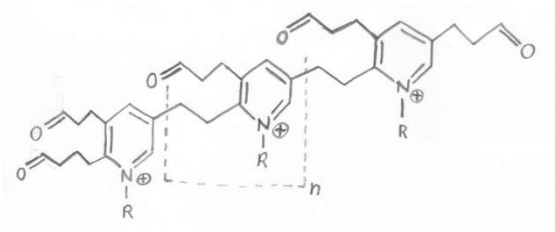


Figure 1: Suggested reaction mechanism of primary cross-linking with glutaraldehyde. [6]

2.1.1.2 Formaldehyde

Since FA has only one carbonyl group, it is very ineffective as a cross-linking agent compared to GA. An advantage of FA is its much faster diffusion rate. FA has its boiling point at -19 °C [11] and is therefore a gas at room temperature. Aqueous solutions are prepared from paraformaldehyde, which is the polymerized, solid form of FA. [6] In aqueous solution FA reacts with water to form methylene glycol, which is able to react with an amino group of a protein accompanied by the formation of water as a by-product. [6] As condensation continues, linear cross-links are formed between proteins and ultimately several proteins can get cross-linked to a cyclic compound [12]. Methylene glycol generally reacts only with non-protonated amino groups, which is the reason for better cross-linking of FA in alkaline solutions. [6] Furthermore, the concentration of FA and amines should be nearly equal to obtain good fixation results. [6]

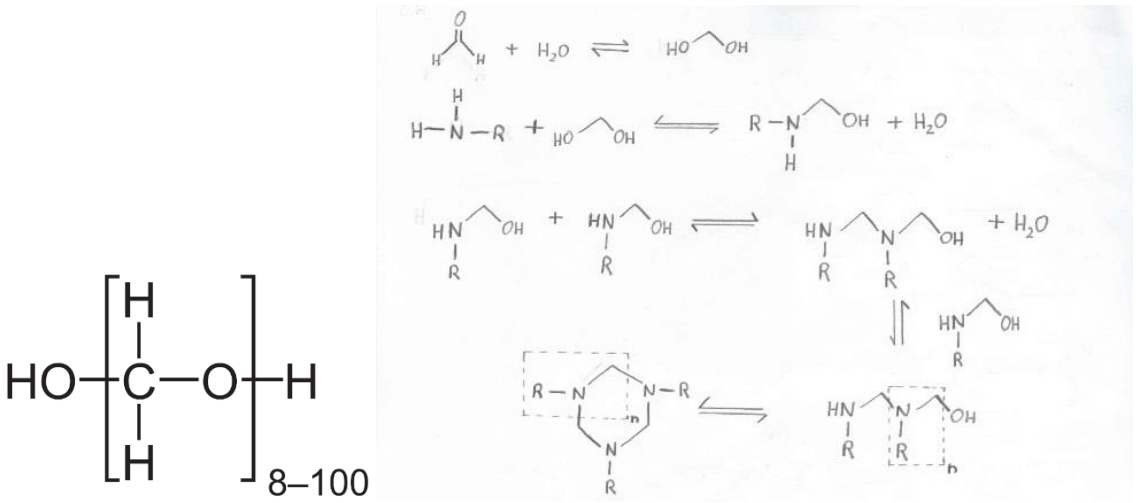


Figure 2: Structure of paraformaldehyde (left); Suggested reaction mechanism of primary cross-linking with formaldehyde (right). [6]

2.1.1.3 Fixation for Immunocytochemistry

The main purpose of fixation is, as mentioned already before, fine structure preservation in biological samples. Fixatives are also necessary to protect proteins against denaturation and to prevent their extraction from the sample. In contrast, fixation can have also negative consequences on immunoreactivity due to the following reasons:

- The process chemically alters the secondary and tertiary structure of proteins. [6]
- Cross-linking sterically hinders the access of antibodies to the antigen even on a thin section. [6]

As shortly mentioned in chapter 3.1.1.1 on the example of glutaraldehyde, the influence of aldehydes on the secondary and tertiary structure of proteins are only minor and differ from one antigen to another. The steric factor, on the other hand, has a major negative effect on immunoreactivity. Although the access to the epitopes is probably more influenced by the presence of resin, the steric hindrance by the fixative should not be underestimated. Formaldehyde should be preferred for immunological analysis, because, unlike glutaraldehyde it forms mainly linear cross-links. [6] Nevertheless, mixtures of different compositions of glutaraldehyde and formaldehyde are often used in practice. The most important factor of fixation for immunocytochemistry is certainly the proper concentration of the fixation solution, which facilitates fine structure preservation and simultaneously avoids excessive unnecessary cross-linking.

Finally, microwave-assisted fixation not reduces processing times, but can also increase antigenicity in biological specimens. [1] The reason for this is probably the increase in molecular motion or the increase in vibrational energy and polarization of molecules. [8]

2.1.2 Cryo-immobilization

Nowadays, cryo-immobilization is one of the most effective means of preserving cellular structures close to the native state. The liquid in and surrounding the specimen is frozen at such a low temperature and in such a short time, that no crystals are formed and the redistribution of cellular components is prevented. [13] The most important condition is the formation of amorphous ice, because the formation of ice crystals would damage the sample irreversibly. [13] The vitrification

temperature of water is at -130 °C at 1 bar. [14] There are several techniques for achieving vitrification. Samples with diameters up to 20 µm can be frozen already at ambient pressure and with very high cooling rates (e.g. plunge freezing). [15] Samples with diameters up to 200 µm are frozen rapidly under very high pressure with liquid nitrogen. [15] High pressure inhibits the expansion of water during freezing, which prevents the formation of crystal structures. [15] In the EM PACT2 system, the specimen in the container is pressurized with a transmission fluid (methylcyclohexane) and is subsequently cooled with a stream of liquid nitrogen at 10 bars. [16] This process is called high-pressure freezing (HPF). An optimum pressure of 2100 bar should be applied within milliseconds, followed by immediate lowering of temperature. [16]

The addition of a cryo-protectant improves the quality of freezing directly by suppressing the formation of extracellular ice crystals and indirectly by reducing the amount of heat released by the exothermic solidification process, thereby increasing the overall rate of cooling of the sample. [17] Regularly used cryo-protectants are for example polyvinylpyrrolidone, hydroxyethyl starch, dextrane, 1-hexadecene and BSA. [17]

Cryo-immobilization followed by FS (see Chapter 3.4.) has some major advantages compared to chemical fixation. Cryo-immobilization occurs at a much faster rate than chemical fixation, in which the fixative must penetrate the sample before cross-linking can take place. The speed of penetration is thereby dependent on the diffusion rate of the fixative. Some samples may also have special surface structures (e.g. shells), which prevent the penetration of chemical fixatives. Furthermore, during cryo-fixation, all components of the sample are immobilized simultaneously, whereas only some elements are cross-linked during chemical fixation, depending on the type of fixative. The almost instant immobilization of all components makes cryo-immobilization especially useful for the study of dynamic events in cells.

Another advantage of physical cryo-immobilization is the smaller chemical influence (e.g. through buffers, fixatives) upon sensitive biological materials. The antigenicity is therefore retained more effectively and the cell constituents stay in their original chemical structure. [18]

2.2 Post-fixation

Osmium tetroxide (OsO_4) is often applied as a secondary fixative after primary fixation. It preferably binds to phospholipid heads of membrane structures. [19] OsO_4 is also a widely employed staining agent in TEM, because it provides contrast to the image due to its high electron density (atomic number = 79). [19]
The post-fixation step was omitted in this experiment, because OsO_4 has very negative effect on antigenicity, even at low concentrations.

2.3 Embedding

To be able to cut biological samples into ultrathin sections, they need to be stabilized by some sort of embedding medium. There are different options of stabilizing the sample, but they have all the same six requirements [6]:

- They must easily infiltrate tissues and then be uniformly hardened without significant swelling or shrinkage.
- The blocks should combine hardness with plasticity so they can be sectioned smoothly and thin (ultrathin sections 70-90 nm).
- The polymerization process should not adversely affect the ability of antigens to recognize antibodies, or modify fine structure.
- The sections and the structures contained in the media must be resistant to irradiation by the electron beam.
- It must be possible to adequately contrast and recognize the structures present in the sections.

2.3.1 Resin-free Sections

2.3.1.1 Tokuyasu Technique [20]

The samples are first chemically fixed with low-concentrated solutions of formaldehyde and glutaraldehyde the same way as for resin samples and are then infiltrated by a 2.3 M sucrose solution, which acts as a cryo-protectant. [6] Additionally, UA is used as a staining agent. [6] As mentioned in chapter 2.1.2 the cryo-protectant prevents the formation of crystals during cryo-fixation through, for

example, plunge freezing. By using cryoprotectants that can penetrate the sample, larger samples can also be frozen successfully even with lower cooling-rates (e.g. plunge freezing). Cryo-fixed samples are then ready to be cut into ultra-thin sections with a cryo-ultramicrotome. After cutting the sections are thawed at room temperature and subsequently labeled with primary and secondary antibodies. Finally, the samples are stained and stabilized by a mixture of UA and methyl cellulose. [6] The methyl cellulose film supports the sample during the drying step. [21]

The Tokuyasu technique is the most sensitive post-sectioning technique for immunolabeling, since the initial aldehyde fixation is the only potential chemical denaturation step for antigens. [6] On top of that, the results in fine structure preservation at the molecular level are at least as good as in other techniques. [6]

2.3.2 Water-miscible Embedding Media

The advantage of hydrophilic embedding media is, that they can be applied without any dehydration prior to infiltration and embedding. [6] Dehydration is usually done with denaturing solvents like ethanol or acetone. The effect of these solvents on antigenicity differs from one antigen to another. The dehydration step also facilitates the extraction of antigens from the sample. Water-miscible embedding media are rarely applied in electron microscopy laboratories due to some disadvantages.

One example of hydrophilic embedding media is the use of a 20 % BSA solution, which is cross-linked with glutaraldehyde and then dried before sectioning. [6] The major problem with this approach is the severe shrinkage occurring when the cross-linked BSA blocks are dried, prior to sectioning. [22] Further examples of water-miscible embedding media include a mixture of melamine and formaldehyde, polyvinyl alcohol (PVA) or Immunobed A, a water-soluble acrylic compound. [6]

2.3.3 Acrylic Resins

Acrylic resins are not water-soluble, but at least have some hydrophilic character that favours immunochemical reactions. They were designed for low temperature infiltration and polymerization. Low temperatures are very advantageous for the preservation of ultrastructure and the retention of soluble components in the sample. [23] Acrylic resins are not as tightly cross-linked than epoxy resins, which improves the accessibility to the antigens. [24] In addition, LRW can be polymerized in presence of water (up to 12% of total volume). [25] Therefore, the complete

dehydration of unosmicated tissue with concentrated organic solvents, which facilitates the extraction of antigens, can be avoided. [25]

2.3.3.1 Lowicryl Resins

These resins were designed to be able to polymerize at temperatures below 0 °C. During dehydration, the removal of the outer water shell leads to the denaturation of proteins and therefore to a loss of antigenicity. [26] This effect can be reduced or completely eliminated by dehydrating and polymerizing at lower temperatures. Lowicryl resins consist of acrylates, methylacrylates respectively, with alcohol or hydrocarbon side chains and triethylene glycol dimethylacrylate (TEGDMA), acting as a cross-linker and determining the hardness of blocks after polymerization. [6] Lowicryl resins have low viscosities, which ensures fast infiltration, and can be polymerized at low temperatures with ultraviolet light. [6] The polymerized blocks have very good plasticity for ultra-thin sectioning. [6] The sections show also high stability under the electron beam and very low electron scattering properties. [6] The most prominent Lowicryl resins are K4M and HM20, whereas K4M is more polar/hydrophilic and also more viscous. [6] K4M can be used only at temperatures above -35 °C, whereas infiltration by HM20 is possible down to a temperature of -70 °C. [6]

A Lowicryl resin kit consists of the monomer, a crosslinker and an initiatior. Handling of this chemicals should be done with caution and appropriate protective measures.

	Monomer	**Crosslinker**	**Initiator**
Lowicryl K4M Kit	H314, H318, H400, H312, H317	H315, H319, H317, H335	H301, H332, H315, H319
Lowicryl HM20 Kit	H226	H317, H335	H332, H315, H319

Table 1: Hazards of Lowicryl resin kits [27] (Hazard statements in the Appendix)

2.3.3.2 LR White

LRW is a polyhydroxy aromatic acrylic resin with a viscosity slightly higher than Lowicryl K4M. [6] Its hydrophilic character allows immunocytochemistry reagents (e.g. antibodies) to easily penetrate into the section. [28] It can be polymerized by heat, UV-light or chemically by an aromatic tertiary amine accelerator. [6] Polymerization is mostly done at room temperature, but infiltration is possible down to a temperature of -20 °C. [6] As for Lowicryl resins, it is recommended to avoid the use of high concentrations of OsO_4 prior to UV-polymerization. [6] Due to the dark

colour of the stained regions, heat can accumulate and damage the stained areas including their surroundings. Furthermore, LR White resins are far less toxic than Lowicryl resins, because the monomers have longer chains. [6]

2.3.4 Epoxy Resins

Epoxy resins are unsuitable for immunocytochemical analysis, because of their hydrophobic character. Infiltration can only be done above 0 °C, which probably has severe effects on the protein conformation. [6] In addition, the necessary heat-induced polymerization may cause further loss of antigenicity. The extensive cross-linking of the polymerized resin strongly inhibits the accessibility of the antigen, which results in a low labeling efficiency. [24] Epoxy resins are very useful for morphological analysis. [29]

2.4 Freeze Substitution

During freeze substitution, the amorphous ice in the cryo-immobilized sample is dissolved into the organic solvent (ethanol or acetone). In the beginning the temperature is usually kept between -80 to -90 ° to prevent the recrystallization of water, which would alter the original fine structure of the sample. During that time, the specimens are simultaneously infiltrated with fixatives. After dehydration, the temperature can be raised to enable the infiltration by a resin, which is designed for low temperature embedding (e.g. acrylic resins). Although effective covalent cross-linking can only occur at significantly higher temperatures (see Figure 3), experiments have shown improved fine structure preservation in the specimens, when aldehydes such as glutaraldehyde were added. [30] Chemical fixation with glutaraldehyde is usually done at 4 °C. [31] After fixation and dehydration, the sample is infiltrated by mixtures of solvent with increasing percentage of resin. Depending on the type of resin, a certain temperature and irradiation wavelength must be chosen to facilitate an optimal polymerization process.
The suggested length of infiltration by solvent and resin greatly differs between plenty of freeze substitution protocols. According to a protocol recommended by Hunziker EB & Schenk R in 1984 (see Table 2), it would take more than one week to get a sample ready for sectioning using this technique.

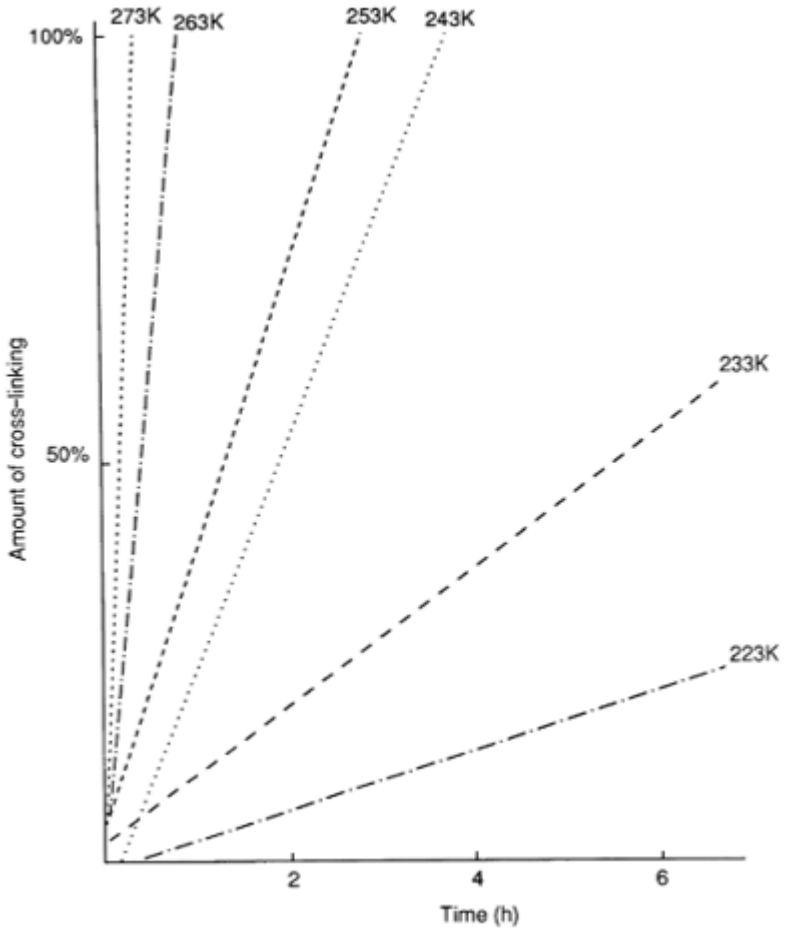


Figure 3: Changes in the cross-linking properties of 3% glutaraldehyde in methanol as a function of temperature. [32]

Process	**Time**	**Chemicals**	**Temperature**
Dehydration and Fixation I	17 hours	3 % GA + 0.5 % UA in MeOH	-90 °C
Dehydration and Fixation II	13 hours	3 % GA + 0.5 % UA in MeOH	-60 °C
Dehydration and Fixation III	12 hours	3 % GA + 0.5 % UA in MeOH	-35°C
Infiltration	4 days	solvent-resin mixtures with increasing percentages of resin	-35°C
Polymerization	2 days	HM20	RT (UV irradiation)

Table 2: Summary of an early freeze substitution protocol. [2]

2.5 Immunology

2.5.1 The Immune Response

The immune response involves the recognition and subsequent destruction of foreign substances such as viruses, bacteria, toxins or tumour cells. [6] It can be divided into two major classes. The antibody-mediated immune response involves the production of antibodies induced by a foreign antigen. [6] Antibodies, present in the blood, specifically bind to the antigen, which is either neutralized or destroyed. The cell-mediated immune response involves the induction of cells capable of destroying host cells bearing the antigen. [6] The cells mostly responsible for the immune response, called lymphocytes, are found in blood, lymph and lymphoid tissues such as bone marrow, thymus, spleen and lymph nodes. [6] Lymphocytes are produced from multipotent stem cells (hemocytoblast) present in the bone marrow and can further mature either to T-cells or B-cells. [33] T-cells are responsible for the cell-mediated immune response, whereas B-cells produce antibodies. [34] The activation of a B-cell in turn requires a subset of T-cells, called T-helper cells. [18] The primary immune response, triggered by the very first contact with a certain antigen, needs several days to develop, whereas the secondary immune response is accomplished by so-called memory cells, which are developed from B-cells of the primary immune response. [6] Memory cells rapidly differentiate into antibody secreting plasma cells and therefore the secondary response can occur much faster. [6]

2.5.2 The Structure of Antibodies

In the late 1950s and early 1960s, Rodney Porter from Great Britain and Gerald Edelman from the United States elucidated the chemical structure of immunoglobulin G (IgG). [35] Edelman treated IgG with reducing agents to break disulphide bonds and obtained two subunits in equimolar concentrations. He designated the larger subunit (50 kDa) as the heavy, or H-chain and the smaller subunit (23 kDa) as the light, or L-chain. By knowing the original weight (approximately 150 kDa), he concluded that an antibody consists of two heavy and two light chains connected by disulphide bonds and noncovalent interactions. [36] In contrast to the light chains, the heavy chains are glycosylated. [6]
Porter treated IgG with a proteolytic enzyme and obtained 3 fragments. Two of them were still able to bind to antigens and were therefore called Fab fragments

(fragments of antigen-binding). The third fragment crystallized readily under cold conditions and was named Fc fragment (fragment crystallisable).
Every light chain can be divided into a constant region (C_L-region) and a variable region (V_L-region). The heavy chain can be separated into one variable region (V_H-region) and 3 constant regions (C_H1-/C_H2-/C_H3-region). Furthermore, the heavy chain contains the hinge region between C_H1 and C_H2. This is a flexible segment of the polypeptide chain and allows variation in the angle between the two antigen binding sites, which improves the ability of the antibody to bind two antigens. [36] The C-regions of different antibodies have very similar amino acid sequences, whereas the V-regions are unique for every type of antibody.

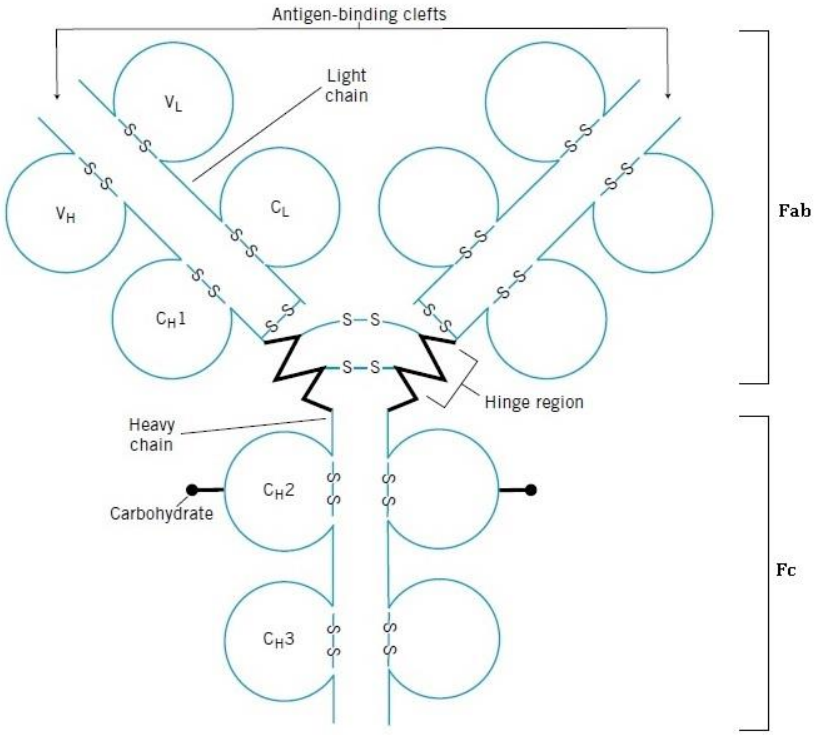


Figure 4: General IgG structure [35]

The V-regions of heavy and light chains contain the so-called hypervariable regions or complementarity-determining regions (CDRs). [37] There are 3 CDRs (CDR1, CDR2 and CDR3) in each V-region, which come in direct vicinity to the antigen binding site. [37] The amino acid sequences of the CDRs define the spatial and ionic properties in the antigen-binding clefts and are therefore responsible for the specificity of an antibody. [6]

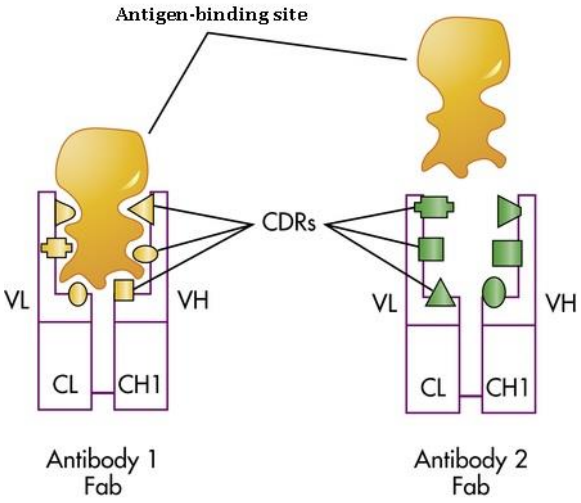


Figure 5: Schematic depiction of antibody binding specificity through CDRs. [38]

Mammals possess 5 types of immunoglobulins in the serum characterized by their heavy chains. [6] IgG is the most abundant immunoglobulin and coats microorganisms to speed up their uptake by phagocytic cells. [35] IgG is predominantly used for immunolocalization. IgA is mostly present in body fluids and protects the entrances into the body. IgM plays a major role in the primary response. [35] IgD is found on the surface of B-cells and is responsible for their activation. [35] IgE is only present in trace amounts in the serum and attaches itself to specialized cells, where it triggers the symptoms of allergies. [35] The extent of glycosylation varies greatly between all types of immunoglobulins, but generally is restricted to the Fc region. [6] IgG is the least glycosylated type, whereas IgE is the most glycosylated immunoglobulin. [6] Also the structures vary between the immunoglobulin types. For example, IgM forms mostly a pentameric structure resulting in 10 antigen binding sites. [6]

Another type of immunoglobulin usable for immunolocalization is IgY, which is produced by birds and reptiles. IgY is also present in the yolk of chicken eggs, which makes it a cheap and non-invasive alternative to IgG from mammals

2.5.3 Preparation of Antibodies

Polyclonal antibodies are produced by injecting an antigen repeatedly into an animal (mouse, rabbit, rat, …) to activate a secondary immune response. [6] The response can be enhanced by initial injection of the antigen directly into the lymph node. [6] After immunization a set of antibodies is present in the plasma, which can be

purified by ammonium sulphate precipitation and further by ion exchange chromatography or affinity chromatography. [6] Polyclonal antibodies are generally easier to produce than monoclonal antibodies and can bind to multiple epitopes of an antigen. [39] This results in a high affinity even after minor antigen changes (e.g. glycosylation or denaturation), but also makes them inappropriate for quantitative analysis. [39] Monoclonal antibodies recognize only one specific epitope of an antigen and should therefore be used for quantitative experiments. [39] On the other hand, the affinity to the antigen is very low after the loss of epitopes through chemical treatment of the antigen. [39] They can be produced *in vitro* by culturing B-cells fused with myeloma cells (lymphoid tumour cells), which are necessary due to the very short lifetime of B-cells in cultures. [6] The resulting hybridoma is both capable of growth and antibody production. [6] Purification can be done in the same ways as mentioned for polyclonal antibodies.

2.6 Non-immunological High-affinity Interactions – Protein A

Protein A was initially discovered in the cell wall of *Staphylococcus aureus*. [40] The cylindrical shaped molecule is water-soluble, can be easily purified and possesses high affinity to the Fc fragment of IgG. [6] Protein A has five IgG binding domains, but it can only bind two antibodies at the same time due to steric hindrance. [6] The protein is predominantly used for the secondary labeling step, because it allows a more precise localization due to its smaller molecule size compared to the IgG. Furthermore, if protein A is bound to a gold particle, it is unlikely that it can bind more than one IgG molecule. [6] The protein specifically binds to CH_3 and CH_2 groups of the Fc domain of the antibody. [6] Protein A is also specific for some species. For example, it binds excellent to IgG of rabbit, human, pig and guinea pig and poorly to IgG of goat, mouse, rat, horse and bovine. [6]

2.7 Gold Nanoparticles – Markers for Immunoelectron Microscopy

Gold colloids, which are suspensions of well-divided gold particles are produced by the reduction of a gold salt (e.g. gold chloride) conducted in aqueous solution. [6] Citrate (Frens 1983), a mixture of tannic acid and citrate (Slot and Geuze 1985), or

thiocyanate (Baschong et al. 1985) can be used as a reducing agent. [6] During the reaction gold ions are reduced to elemental gold (Au^0), which precipitates in form of particles from a supersaturated solution. [6] The particle size, ranging from 2 to 150 nm, can be controlled by varying the reaction conditions, whereas gold colloids with particle a size between 3 and 80 nm (red colour, λ_{max} = 515 to 540 nm) are most often used for immunoelectron microscopy. [6]

Under appropriate conditions, high-affinity macromolecules (e.g. protein A, antibodies, lectins) can be conjugated with gold particles by physical or chemical interactions. Physical interactions include ionic attraction between the negatively charged gold and the positively charged antibody, hydrophobic attraction between the antibody and the gold surface, and coordinate covalent bonds between the gold conducting electrons and amino acid sulphur atoms of the antibody. [41] Chemical interactions can be achieved through chemisorption (via thiol derivatives), bifunctional linkers (e.g. EDC/NHS) and adapter molecules (streptavidin and biotin). [41]

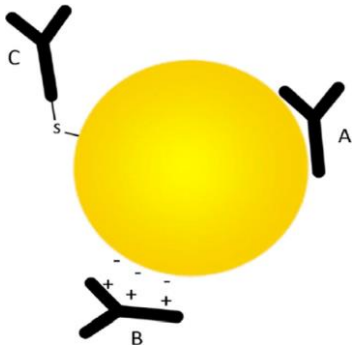


Figure 6: Physical interactions between antibody and gold nanoparticle (A: hydrophobic interaction; B: ionic interaction; C: coordinate covalent bond) [41]

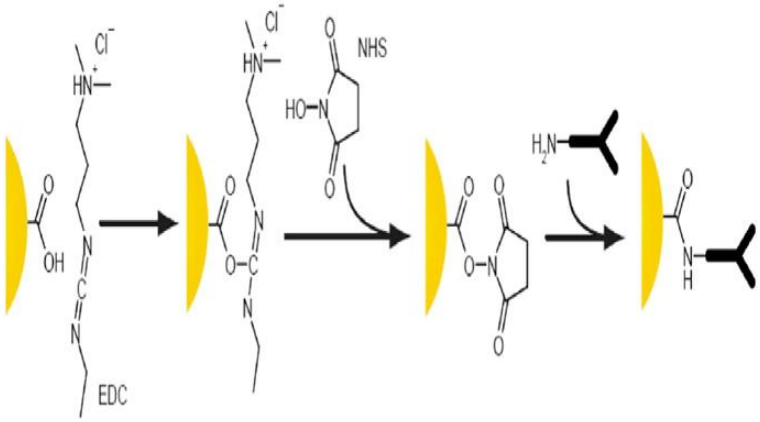


Figure 7: EDC/NHS chemistry (bifunctional linkers) [41]

3 Experimental

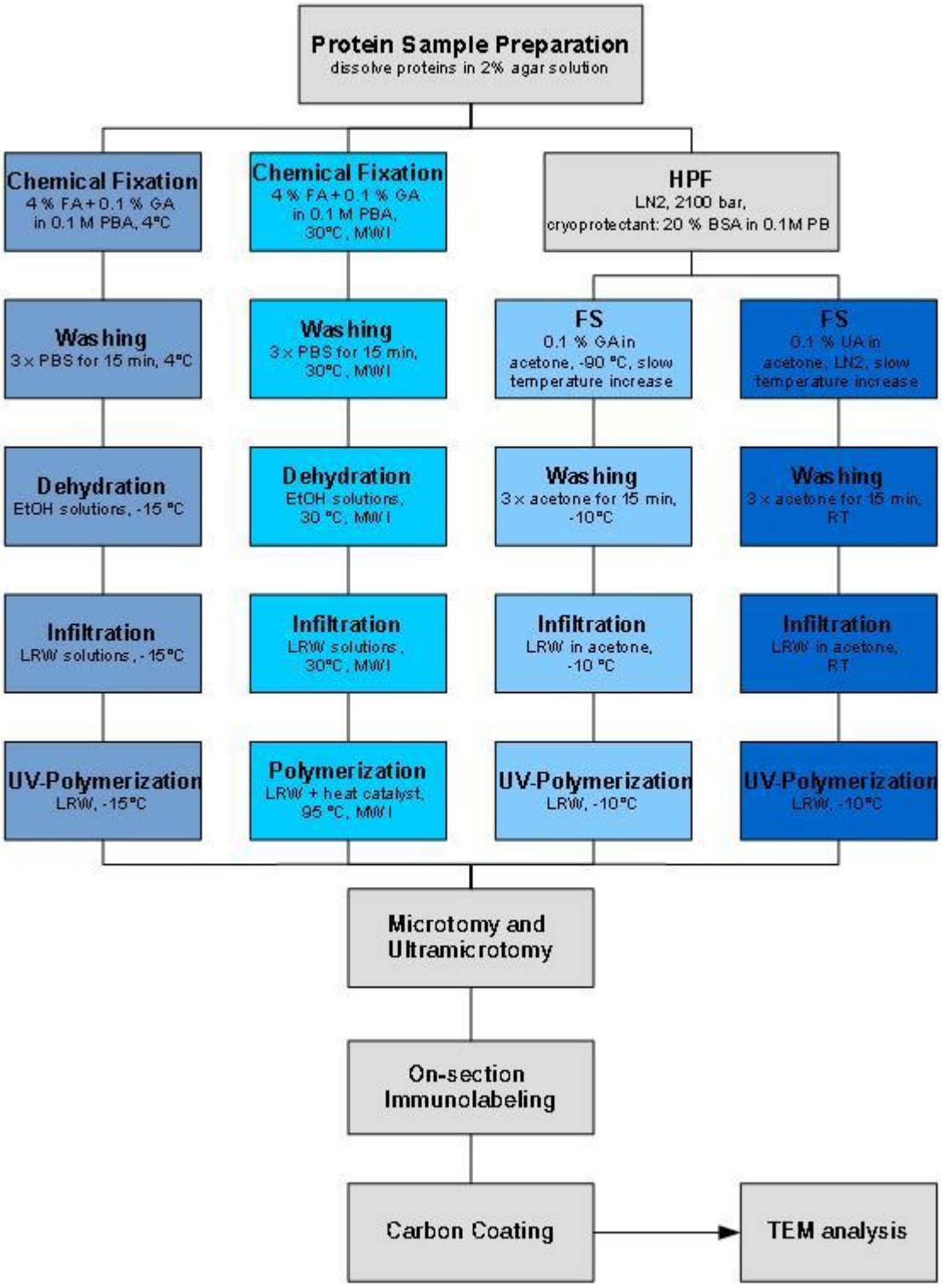


Figure 8: General overview of the experimental procedures.

3.1 Chemicals and Solutions

- **0.2 M PB, pH 7.2:** Dissolve 14.32 g Na_2HPO_4*12 H_2O in 200 ml dH_2O and1.36 g KH_2PO_4 in 50 ml ddH_2O. Mix both solutions. Adjust pH to 7.2 and filter. [42]
- **0.01 M PB + 0.15 M NaCl, pH 7.2 (PBS):** Dissolve 0.023 g NaH_2PO_4 (or 0.03 g NaH_2PO_4*2 H_2O), 0.144 g Na_2HPO_4*2 H_2O (or 0.29 g Na_2HPO_4*12 H_2O) and 0.9 g NaCl in 100 ml ddH_2O. Adjust pH to 7.2 and filter. [42]
- **4 % FA with 0.1 % GA in 0.1 M sodium phosphate buffer (Standard chemical fixation)**: Warm 0.8 g of paraformaldehyde powder with 9.92 mL of distilled water to 65-70 °C. Add drops of Sodium hydroxide solution until the solution becomes clear. Cool the solution to room temperature. Mix the solution with 10 mL of 0.2 M phosphate buffer and 0.08 mL of 25 % GA. Check the pH and adjust it to 7.2 - 7.4.
- **Dehydration**: ethanol or acetone (p.a., 100%)
- **Coating of HPF sample holders:** 1 % lecithin in 100% chloroform
- **Cryo-protection**: 20 % BSA solution in 0.1 M PB
- **Fixation before freeze substitution**: 0.01 % GA in 100% acetone
- **Embedding:** LR White (Polysciences); UV polymerization catalyst: benzoin methylether (0.025 g/5g of resin)
- **Staining solution for semithin sections:** 1 % toluidine blue in 1 % sodium borate solution (Dissolve 1 g toluidine blue and 1 g sodium borate in 100 ml distilled water and filter.)
- **Coating of specimen grids:** 0.3 % formvar in 100% chloroform
- **Blocking agent for labeling:** 1 % FSG (Sigma-Aldrich) in PBS + 0.01 M glycine
- **Proteins:** dissolved in a 2 % agar solution (see Table 3)

Protein	**Molecular weight**	**Calculated mass**	**Weighed mass**	**Calculated concentration**
Albumin–fluorescein isothiocyanate (FITC) conjugate (bovine serum albumin) [43]	66 kDa	0.003 g	0.0029 g	0.437 mmol*L^{-1}

Transferrin from Human Serum, Texas Red® Conjugate [44]	80 kDa	0.0036 g	0.0033 g	0.413 mmol*L^{-1}
Haemoglobin from bovine blood, lyophilized (freeze-dried) powder [45]	64.5 kDa	0.0029 g	0.0031 g	0.4813 mmol*L^{-1}
Peroxidase from horseradish, Type VI, essentially salt-free, lyophilized powder [46]	44 kDa	0.002 g	0.0019 g	0.432 mmol*L^{-1}

Table 3: Proteins used for the experiment

- **Labeling solutions:** Antibodies were diluted in BA (for dilutions see Table 8) just before use, concentrated antibodies were stored at -20°C (see Table 4)

Antigen	**Primary AB**	**Conc.**	**Clone**	**Secondary AB**	**Conc.**	**Clone**
BSA	Anti-BSA IgG from rabbit in buffered aqueous solution [47]	1.4 mg/mL	Polyclonal	pA10 in buffered aq. sol. + 1 % BSA + 15 mM NaN_3 [48]	10-20 µg of specific protein/ml	-
Hgb	Anti-Hgb from rabbit (whole antiserum, 15 mM NaN_3) [49]	ND	polyclonal	pA10 in buffered aq. sol. + 1 % BSA + 15 mM NaN_3 [48]	10-20 µg of specific protein/ml	-
Tfn	Anti-Tfn from goat (whole antiserum, 15 mM NaN_3) [50]	ND	polyclonal	Rabbit anti Goat (RAG) IgG in buffered aq. sol. [51]	2.4 mg/mL	polyclonal
HRP	Anti-HRP from mouse (ascites fluid, 15 mM NaN_3) [52]	ND	P6-38, monoclonal	Goat anti Mouse (GAM) IgG in buffered aq. sol. [53]	2.4 mg/mL	polyclonal

Table 4: Primary and secondary antibodies used for labeling of the corresponding antigens. The secondary antibodies were conjugated with 10 nm gold particles.

3.2 Protein Sample Preparation

All proteins were weighed into an Eppendorf tube and were mixed with 100 µL of a 2 % agar solution. The masses of Transferrin, Haemoglobin and Peroxidase were calculated to obtain in the same molar concentration as a 30 mg*mL^{-1} BSA solution (0.45 mmol*L^{-1}).
For complete dissolution of the proteins, the mixture was heated to 100 °C. After cooling down to 62 °C the solution was filled into 50 µL glass microcapillaries (Hirschmann Lab., cat. no 9600150; see Figure 9). Finally, the agar mass was removed from the capillaries and cut into approximately 2 mm^3 sample pieces.

Figure 9: 50 µL glass microcapillaries used in the experiment.

3.3 Processing at progressively lower temperatures (PLT)

Pieces of agar that contained proteins of interest were immersed into a solution of 4 % FA plus 0.1 % GA in 0.1 M sodium phosphate buffer for at least 1 hour at 4 °C. The samples were washed 3 times in PB for 15 minutes and stored in PB at 4 °C overnight. The next steps involved the dehydration of the samples by 30, 50, 70, 80, 90, 95 and finally 100 % solutions of EtOH. The samples were put 30 minutes into each solution at -15 °C in the freeze substitution unit. Suppliers of LR White do not recommend acetone as dehydration agent, because residual amounts of it can interfere with the polymerization process later. [54]

Afterwards the samples were infiltrated by LR White resin using mixtures of LR White and ethanol with increasing resin concentrations (see Table 5). Infiltration was

done for one hour by each solution at again -15 °C in the freeze substitution unit. For the last step, the sample were embedded in 100 % LR White resin overnight and then UV-polymerized for 2 days at -15 °C in the freeze substitution unit. All steps were performed in 1.5 mL Eppendorf tubes.

Solution	Time	Temperature
4 % FA with 0.1 % GA in 0.1M buffer	1 h	4 °C
Washing solution	3 x 15 min	4 °C
30 % EtOH	30 min	-15 °C
50 % EtOH	30 min	-15 °C
70 % EtOH	30 min	-15 °C
80 % EtOH	30 min	-15 °C
90 % EtOH	30 min	-15 °C
95 % EtOH	30 min	-15 °C
100 % EtOH	30 min	-15 °C
LRW + 100 % EtOH 1:2	1 h	-15 °C
LRW + 100 % EtOH 1:1	1 h	-15 °C
LRW + 100 % EtOH 2:1	1 h	-15 °C
100 % LRW	overnight	-15 °C

Table 5: Overview of TEM standard procedure; chemical fixation and resin infiltration

3.4 Microwave-assisted Processing at room temperatures (MW-RT)

Microwave-Assisted Processing and Embedding of the samples was done according to Paul Webster in “Electron Microscopy: Methods and Protocols, Methods in Molecular Biology, vol. 1117” with few modifications. Chemical fixation, washing, dehydration, infiltration and polymerization was all done automatically in the laboratory microwave processor (Leica EM AMW, Leica Microsystems GmbH), following the protocol in Table 6.

For primary fixation, the samples were immersed again in 4 % FA with 0.1% GA in 0.1 M sodium phosphate buffer. After chemical fixation, the samples were washed, firstly with PBS for five minutes and secondly with deionized water for 30 seconds. Both washing steps were conducted without any microwave irradiation. The

samples were dehydrated using solutions with increasing concentrations of EtOH. The last two dehydration steps were conducted with 100 % EtOH. Each dehydration step lasted for 40 s at a temperature of 30 °C.
For resin infiltration the samples were put first into a 1:1 EtOH - LRW mixture and afterwards twice into pure LRW resin. The samples were heated to 30 °C and irradiated 15 minutes for each infiltration step. To facilitate polymerization of the LRW resin, a heat catalyst (benzoyl peroxide, 1,98 w%) had to be added in advance. The samples were heated to a temperature of 90 °C and irradiated for 45 minutes at 30 watts.

Solution	**Time**	**Irradiation energy (Watt)**	**Temperature**
4 % FA with 0.1 % GA in 0.1 M buffer	10 s	30 W	30 °C
4 % FA with 0.1 % GA in 0.1 M buffer	20 s	-	30 °C
4 % FA with 0.1 % GA in 0.1 M buffer	10 s	30 W	30 °C
Washing solution	5 min	-	30 °C
Water	30 s	-	30 °C
30 % EtOH	40 s	30 W	30 °C
50 % EtOH	40 s	30 W	30 °C
70 % EtOH	40 s	30 W	30 °C
80 % EtOH	40 s	30 W	30 °C
90 % EtOH	40 s	30 W	30 °C
95 % EtOH	40 s	30 W	30 °C
100 % EtOH	40 s	30 W	30 °C
100 % EtOH	40 s	30 W	30 °C
LRW + 100 % EtOH 1:1	15 min	30 W	30 °C
100 % LRW	15 min	30 W	30 °C
100 % LRW	15 min	30 W	30 °C
100 % LRW	45 min	30 W	90 °C

Table 6: Overview of microwave-assisted processing and embedding for TEM

3.5 High-pressure Freezing (HPF) and Freeze Substitution (FS)

Some of the samples were cryo-immobilized with a high-pressure freezer Leica EM PACT2 equipped with a rapid transfer system. The samples were put into a specimen holder (flat specimen carrier, gold plated, 0.5 mm thick, 1.2 mm in

diameter, 200 µm deep, Leica Inc.), which were coated with a solution of 1 % lecithin in chloroform (see Figure 10). Just before freezing, the specimen holders were filled-up completely to avoid inclusions of air, that would damage the sample during high-pressure freezing. A 20 % BSA in 0.1 M PB solution was used as a “filler” and additionally acted as a cryo-protectant. [55]

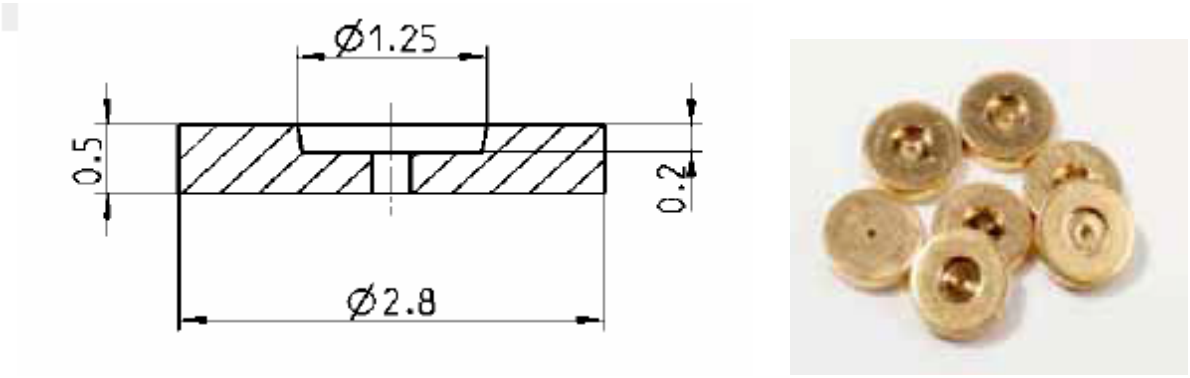


Figure 10: HPF specimen holder profile (left) and illustration (right)

Frozen specimens were transport under liquid nitrogen to the FS unit precooled to -90°C. Here, coating the top piece of the HPF carriers with lecithin made it possible to remove it cleanly under liquid nitrogen, leaving the biological material in the bottom piece. The specimens were immersed into a solution of 0.01 % GA in 100% acetone and left for 96 hours at -90 °C. Afterwards the solution was gradually warmed to +5 °C over a 14-hour period. The freeze substitution medium was exchanged, and specimens were incubated at -20 °C for 36 hours and warmed gradually to +10 °C over a 10-hour period.

After washing the samples three times for 15 minutes in pure acetone, resin infiltration was performed at -10 °C using acetone solutions with increasing LR white concentrations (see Table 7). After infiltration, LR white was polymerized under UV-light for 48 hours at -10 °C in the FS unit.

Solution	Time	Temperature
0.01% GA in acetone	96 hrs	-90°C
	14 hrs	Slow increase to 5°C
0.01% GA in acetone	36 hrs	-20°C
	10 hrs	Slow increase to 10°C
Acetone	3 x 15 min	-10°C
10% LRW in acetone	1 hr	-10°C
20% LRW in acetone	1 hr	-10°C
40% LRW in acetone	1 hr	-10°C
60% LRW in acetone	2 hr	-10°C
80% LRW in acetone	2 hr	-10°C
100% LRW in acetone	Overnight	-10°C

Table 7: Overview of freeze-substitution and subsequent resin infiltration

3.6 High-pressure Freezing (HPF) and Super Quick Freeze Substitution (SQFS)

Some protein samples were again high-pressure frozen exactly with the same conditions described in 3.5. This time SQFS was conducted according to McDonald and Webb (2011) [3] with few modifications.

The sample holder, a metal heating block, was placed in a Styrofoam® box (see Figure 11). The box was filled with LN_2, until the block was fully immersed. As a fixative, 10 mL of 0.1 % Uranyl-acetate in acetone were prepared by diluting 0.2 mL of a 5 % stock solution. Approximately 1 mL of the solution was filled into every cryo-tube, which were then put into LN_2. After some time, the high-pressure frozen samples were added to the fixative at LN_2-temperature. The samples and temperature probe (in a cryo-tube filled with 1.5 mL acetone) were placed in the holes of the heating block as fast as possible. After temperature recording was started, LN_2 was poured off and the block was rotated 90°. It is important to have the cryo-tubes horizontal, or nearly so, to ensure good mixing of the FS solution. [3] The block was agitated at 100 rpm until the temperature reached 0 °C. At 0 °C the samples were removed from the block and placed on the shaker again at 100 rpm until reaching room temperature.
The samples were washed 3 times in pure acetone for 5 minutes and thereby rotated at 100 rpm. Resin infiltration was facilitated by adding mixtures with

increasing concentration of LR white in acetone. For each infiltration step (25, 50, 75, 100,100 and 100 % resin) the samples were put into a centrifuge for 30 seconds at 6000 rpm. LR white was polymerized under UV-light for 48 hours at -10 °C in the FS unit.

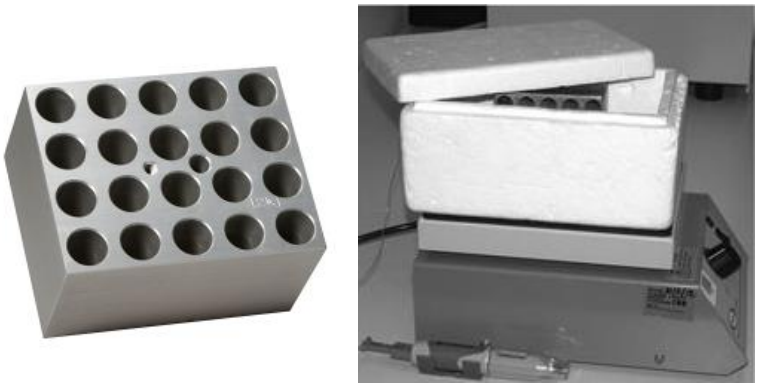


Figure 11: Benchmark Scientific® BSW13 Digital Dry Bath Heating Block for 20 x 12 mm or 13 mm Test Tubes (left); heating block placed in a Styrofoam box on a shaker (right) [3]

3.7 Microtomy and Ultramicrotomy

A polymerized resin block containing a sample was mounted on a holder and trimmed with a sharp razor blade until the protein sample was exposed on the upper surface. The block was also trimmed on lateral sides to obtain a small quadratic upper surface. The sample was mounted on a Leica UCT ultramicrotome, equipped with a glass knife. At first, dry sectioning was done with a thickness of 200 nm at a speed of 1 mm/s. A glass knife was replaced by a knife equipped with a so-called “boat” and adjusted properly. The boat was filled with distilled water exactly to the level of the glass-edge. Now cutting of thick sections (500 nm) was started until several sections could be seen floating on the water surface. The sections were transferred onto a glass slide with a drop of water on it and circled with a permanent marker on the opposite side of the glass to mark their positions. The glass slide was dried on a hot plate at 90 °C. Afterwards the dried sections were stained by adding a drop of 1 % toluidine blue in 1 % sodium borate solution using a filtered syringe. The glass slide was again placed on the hot plate for about 1 minute. Excess staining

solution was washed away with distilled water and the dry, stained sections were examined under the light microscope.

Ultrathin sectioning (70 – 100 nm) with diamond knife was performed by the co-supervisor, because this technique affords more practical experience. After cutting, the ultrathin sections were transferred onto the electron microscopy grids optionally coated by formvar film.

3.8 Coating of TEM Grids

First, TEM grids were washed 5 min in 50 % aqueous acetic acid, 5 minutes in dH_2O with one drop of detergent, 5 minutes in dH_2O and finally 5 minutes in 100 % acetone. The clean grids were coated by formvar film as follow: A clean and dry glass slide was dipped into a solution of 0.3 % formvar in chloroform for about 30 seconds and was then withdrawn carefully. The slide was put to dry for 2 minutes and a glass vessel, filled with dH_2O, was warmed up to the room temperature. The edges of the dry glass slide were scratched with a diamond pen on all sides. The coated glass slide was dipped slowly into the water in a 90° angle. The formvar film should detach itself from the glass and swim on the water surface. The clean and dry specimen grids were transferred onto the formvar film with their shining side facing up. The grids and formvar film were collected with a piece of parafilm and dried.

Figure 12: TEM grids on a formvar film floating on distilled water.

3.9 Immunolabeling for TEM

Immunolabeling was conducted by placing the grids specimen´s section-side down on 40 µL drops of either blocking, antibody or washing solutions (see Table 8). The drops were placed on a clean piece of parafilm (see Figure 13). Primary and secondary antibodies were used for immunolabeling of the corresponding antigens (see Table 4). Primary antibodies specifically targeted certain antigens, whereas secondary antibodies bound to the primary antibodies. The secondary antibodies were conjugated to gold particles of 10 nm in diameter.

Firstly, the grids were incubated with a blocking agent for 1 hour. Secondly, the grids were put on solutions of primary antibody diluted in blocking agent for 3 hours. After primary labeling the grids were washed in several steps with blocking agent and distilled water. Afterwards the sections were incubated for one hour in solutions of secondary antibodies. In the end, all sections were washed again several times with blocking agent and dH_2O. In addition to the conventionally labeled sections a negative control section was prepared for every sample preparation method to investigate the presence of non-specific secondary-antibody binding. Instead of primary antibodies pure blocking agent was used in the first step of the procedure. After washing, the control sections were treated with a solution of protein A.
After immunolabeling the grids were coated with a thin layer of carbon using a vacuum evaporator. Coating of samples is required to enable or improve the imaging of the samples.

Antigen	**BA (1 h)**	**Prim. AB (3 h)**	**Washing (3 min)**	**Sec. AB (1 h)**	**Washing (3 min)**
BSA	1 % FSG	1:20 AB in BA	3 x 1 % FSG in	1:40 AB in BA	3 x 1 % FSG in
Hgb	in PBS +	1:30 AB in BA	PBS + 0.01 M		PBS + 0.01 M
Tfn	0.01 M	1:40 AB in BA	glycine		glycine
HRP	glycine	1:30 AB in BA	3 x dH_2O		3 x dH_2O

Table 8: Overview of the labeling procedure.

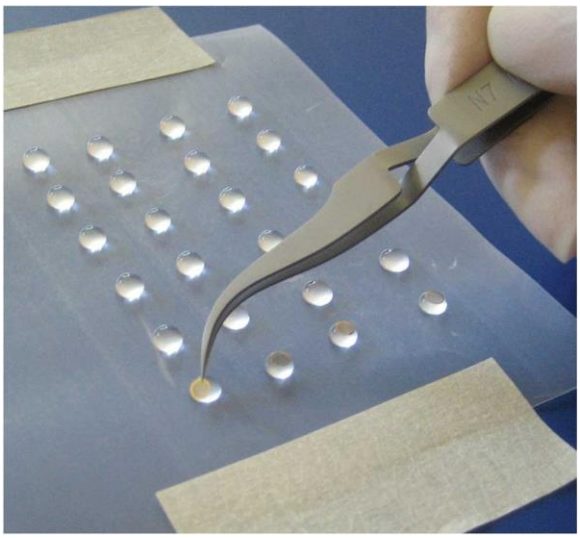


Figure 13: 40 µL drops of either block, antibody or washing solutions on parafilm.

3.10 Evaluation Procedure

The labeled ultra-thin sections were observed with a JEOL JEM – 1010 TEM. The quantitation of gold particles was made by simple random sampling. Additionally, the control groups were checked for unspecific binding or contamination by antibodies. Approximately 50 micrographs were taken for each section at the same magnification of 60,000 (60k). The micrographs were evaluated by using the Java-based image processing program “ImageJ”. [56]

First, the area of one micrograph was determined using “ImageJ”. After setting the scale (see Figure 14), the area of one micrograph was processed by the “Measure” function (see Figure 15). The area of one micrograph, which was determined to be 3.7061 μm^2, was valid for all micrographs, because all of them were taken at the same magnification. Now it was possible to calculate the labeling density (LD) for all tested groups. The labeling density for a protein is defined as Σ gold nanoparticles / Σ area (μm^2). Afterwards the expected labeling density for a protein was calculated by total Σ gold nanoparticles / Σ area (μm^2) of all 4 methods. In the next step, the expected number of gold particles was calculated by multiplying this ratio (gold nanoparticles per μm^2) with the area observed. By means of a two-sample Chi squared analysis with two columns (observed and expected gold counts) and c compartments (arranged in rows), the two distributions were compared. The total

and partial “Chi squared” values were calculated to determine whether to accept or reject the null hypothesis for c - 1 degrees of freedom. The null hypothesis (H_0) of the statistical test was defined as: “There is no difference in distributions between groups.” Additionally, the relative labeling index (RLI) was determined by dividing the number of observed particles by the number of expected particles of one group. RLI indicates the degree to which a compartment is preferentially labelled in comparison to the theoretical situation of random labelling. [57] The criteria for deciding on preferential labelling of a compartment are twofold: First, the value of RLI must be greater than 1, and second, the partial χ^2 value must account for a significant proportion (10% or more) of the total χ^2 value. [57]

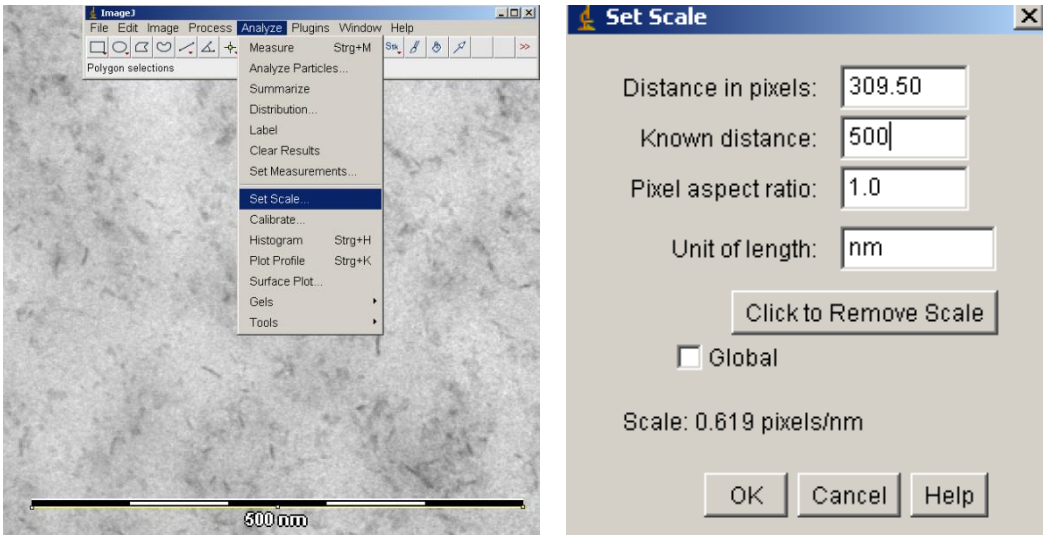


Figure 14: Setting the scale in “ImageJ”.

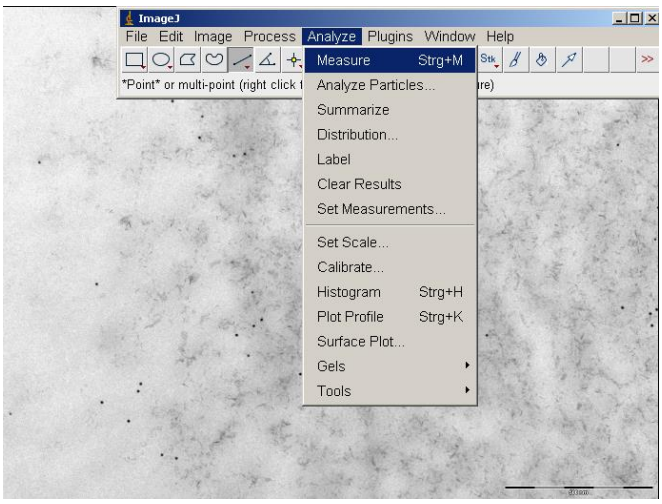


Figure 15: Measuring the area of one micrograph.

4 Results

First, the control sections were searched for gold particles to evaluate the proportion of unspecific antibody binding. However, the control sections were almost free of gold particles. Unspecific antibody binding was therefore not included in the evaluation procedure. The labeling density is calculated by dividing the sum of observed particles (see Appendix) by the total observed area of one method. The labeling of Hgb and Tfn resulted in very low labeling densities compared to the other two proteins. Except for the HPF/SQFS method, where the LD of HRP was slightly higher, BSA had the highest labeling densities of all four proteins (Table 9, Figure 16).

	HPF/FS	**MW-RT**	**HPF/SQFS**	**PLT**	**Expected LD**
BSA	11.867 [2243/183.01]	8.248 [1559/189.01]	2.285 [381/166.77]	9.931 [1877/189.01]	8.26 [6060/733.81]
HRP	1.836 [347/189.01]	6.382 [1159/181.60]	2.489 [452/181.60]	5.322 [1006/189.01]	4.00 [2964/741.22]
Hgb	1.04 [200/192.72]	1.18 [228/192.72]	0.86 [162/189.01]	0.98 [181/185.30]	1.01 [771/759.75]
Tfn	0.74 [134/181.60]	0.85 [161/189.01]	0.58 [112/192.72]	0.855 [149/174.19]	0.75 [556/737.51]

Table 9: Calculated observed labeling densities and expected labeling densities given in gold particles per µm². Written in brackets: Sum of all counted gold NPs/sum of counted areas

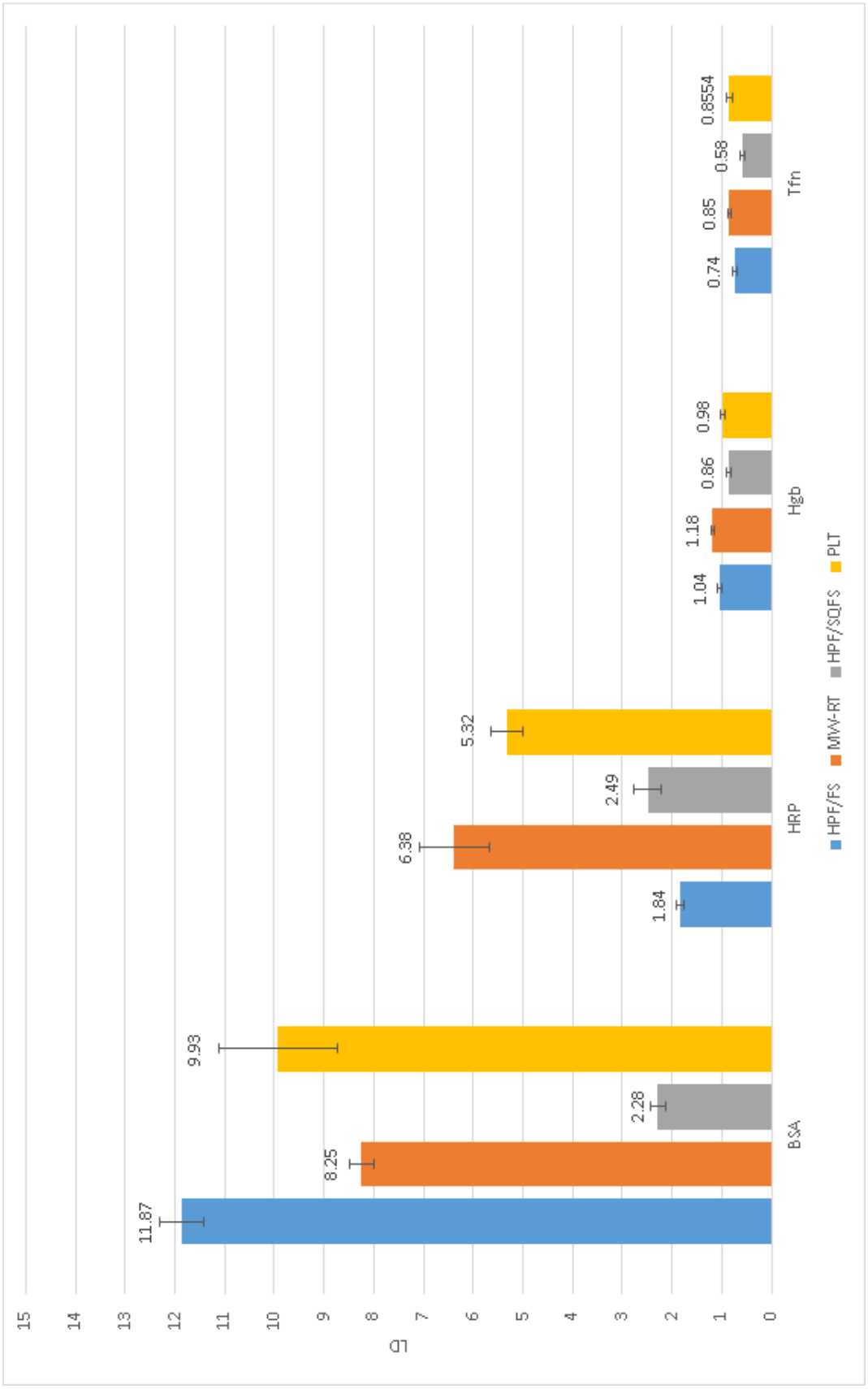


Figure 16: Graphical illustration of calculated <u>labeling densities</u> of the different methods. LD (y-axis) is again given in observed gold particles per µm². Error bars represent the standard error of the mean.

The calculated expected number of particles was compared to the observed number of particles.

		HPF/FS	MW-RT	HPF/SQFS	PLT
BSA	**expected no. of particles**	1560.91	1560.91	1377.27	1560.91
	observed no. of particles	2243	1559	381	1877
HRP	**expected no. of particles**	755.82	726.18	726.18	755.82
	observed no. of particles	347	1159	452	1006
Hgb	**expected no. of particles**	195.57	195.57	191.81	188.05
	observed no. of particles	200	228	162	181
Tfn	**expected no. of particles**	136.91	142.49	145.29	131.32
	observed no. of particles	134	161	112	149

Table 10: Expected and Observed number of gold particles.

	HPF/FS	MW	SQFS	LRW	χ2 Total
BSA	298.061 (27.53 %)	0.002 (< 0.01 %)	720.668 (66.56 %)	64.009 (5.91 %)	1082.741 (100 %)
HRP	221.129 (33.23 %)	257.971 (38.77 %)	103.521 (15.56 %)	82.811 (12.44 %)	665.431 (100 %)
Hgb	0.100 (0.97 %)	5.378 (51.83 %)	4.633 (44.65 %)	0.264 (2.55 %)	10.375 (100 %)
Tfn	0.062 (0.50 %)	2.405 (19.28 %)	7.628 (61.15%)	2.380 (19.08%)	12.474 (100%)

Table 11: χ^2 values of the Chi-squared test and percentages of the total χ^2 values.

df \ p	0.995	0.975	0.9	0.5	0.1	0.05	0.025	0.01	0.005	df
1	.000	.000	0.016	0.455	2.706	3.841	5.024	6.635	7.879	1
2	0.010	0.051	0.211	1.386	4.605	5.991	7.378	9.210	10.597	2
3	0.072	0.216	0.584	2.366	6.251	7.815	9.348	11.345	12.838	3
4	0.207	0.484	1.064	3.357	7.779	9.488	11.143	13.277	14.860	4
5	0.412	0.831	1.610	4.351	9.236	11.070	12.832	15.086	16.750	5
6	0.676	1.237	2.204	5.348	10.645	12.592	14.449	16.812	18.548	6
7	0.989	1.690	2.833	6.346	12.017	14.067	16.013	18.475	20.278	7
8	1.344	2.180	3.490	7.344	13.362	15.507	17.535	20.090	21.955	8
9	1.735	2.700	4.168	8.343	14.684	16.919	19.023	21.666	23.589	9
10	2.156	3.247	4.865	9.342	15.987	18.307	20.483	23.209	25.188	10
11	2.603	3.816	5.578	10.341	17.275	19.675	21.920	24.725	26.757	11
12	3.074	4.404	6.304	11.340	18.549	21.026	23.337	26.217	28.300	12
13	3.565	5.009	7.042	12.340	19.812	22.362	24.736	27.688	29.819	13
14	4.075	5.629	7.790	13.339	21.064	23.685	26.119	29.141	31.319	14
15	4.601	6.262	8.547	14.339	22.307	24.996	27.488	30.578	32.801	15

Table 12: Critical values of the χ^2 distribution.

	HPF/FS	**MW-RT**	**HPF/SQFS**	**PLT**
BSA	1.437	0.999	0.277	1.203
HRP	0.459	1.596	0.622	1.331
Hgb	1.023	1.166	0.845	0.963
Tfn	0.979	1.130	0.771	1.135

Table 13: Calculated Relative Labeling Index (RLI)

The hypothesis is rejected if the P-value is below the significance level of to α = 0.05. Based on the statistical evaluation by the Chi squared test (see Table 12) the following conclusions can be drawn:

- **BSA:**

  For a total $\chi^2 = 1082.741$ and 3 degrees of freedom, $P < 0.005$. The null hypothesis of no difference in distributions between groups must be rejected. Examination of the partial χ^2 values shows that the major contributors to the differences resided in the HPF/FS and HPF/SQFS groups. HPF/FS has significantly more-than-expected gold particles (RLI = 1.437) and HPF/SQFS has significantly fewer-than-expected gold particles (RLI = 0.277). The partial

χ^2 value of the MW-RT method indicates that, there is no significant difference between the expected and observed number of particles.

- **HRP:**

  For a total $\chi^2 = 665.431$ and 3 degrees of freedom, $P < 0.005$. The null hypothesis of no difference between group distributions must be rejected. Examination of the partial χ^2 values shows that HPF/FS (RLI = 0.459) and HPF/SQFS (RLI = 0.622) resulted in significantly less-than-expected numbers of HRP specific binding antibodies, whereas the MW-RT (RLI = 1.596) and PLT (RLI = 1.331) methods resulted in significantly more-than-expected numbers of antibodies.

- **Hgb:**

  For a total $\chi^2 = 10.375$ and 3 degrees of freedom, $P < 0.025$. For the HPF/FS (RLI = 1.023) and the PLT (RLI = 0.963) method, there is no significant difference between number of observed particles and number of expected particles, which is verified by the partial χ^2 values. In contrast, the sample processed by the MW-RT (RLI = 1.166) method shows a significantly higher-than-expected number of antibodies, whereas the sample processed by the HPF/SQFS (RLI = 0.845) method shows a significantly lower-than-expected number of antibodies.

- **Tfn:**

  For a total $\chi^2 = 13.119$ and 3 degrees of freedom, $P < 0.005$. The null hypothesis of no difference in distributions between groups is rejected. Considering the partial χ^2 values of the methods HPF/FS, MW-RT and PLT, there was no significant difference between the observed and expected number of antibodies. Only the HPF/SQFS (RLI = 0.771) method resulted in significantly fewer-than-expected gold particles.

5 Discussion

In general, BSA and HRP yielded relatively high labeling densities, whereas Hgb and Tfn had a very low affinity to antibody-binding. The very minor response of these two proteins made sampling rather difficult. The high differences in labelling densities can have several reasons. It may result from using antibodies of different quality and binding avidity. In this case, the LD of Hgb and Tfn could possibly be increased by replacing the respective antibodies.

Unfortunately, some of the protein sample blocks dissolved partly into the non-polymerized resin. The dissolved proteins precipitated afterwards in the resin and a clear boundary between the sample and resin could not be observed anymore. The precipitation can be seen very markedly in MW-RT samples (see Figure 17), because of the high temperature used for resin polymerization (90°C). The agar sample blocks already melt at 60 °C and the proteins are therefore carried into the resin before polymerization. An almost complete absence of gold particles on the control sections and the presence of gold particles solely on darker protein areas indicates, that the antibody binding was in fact very specific towards the corresponding antigen.

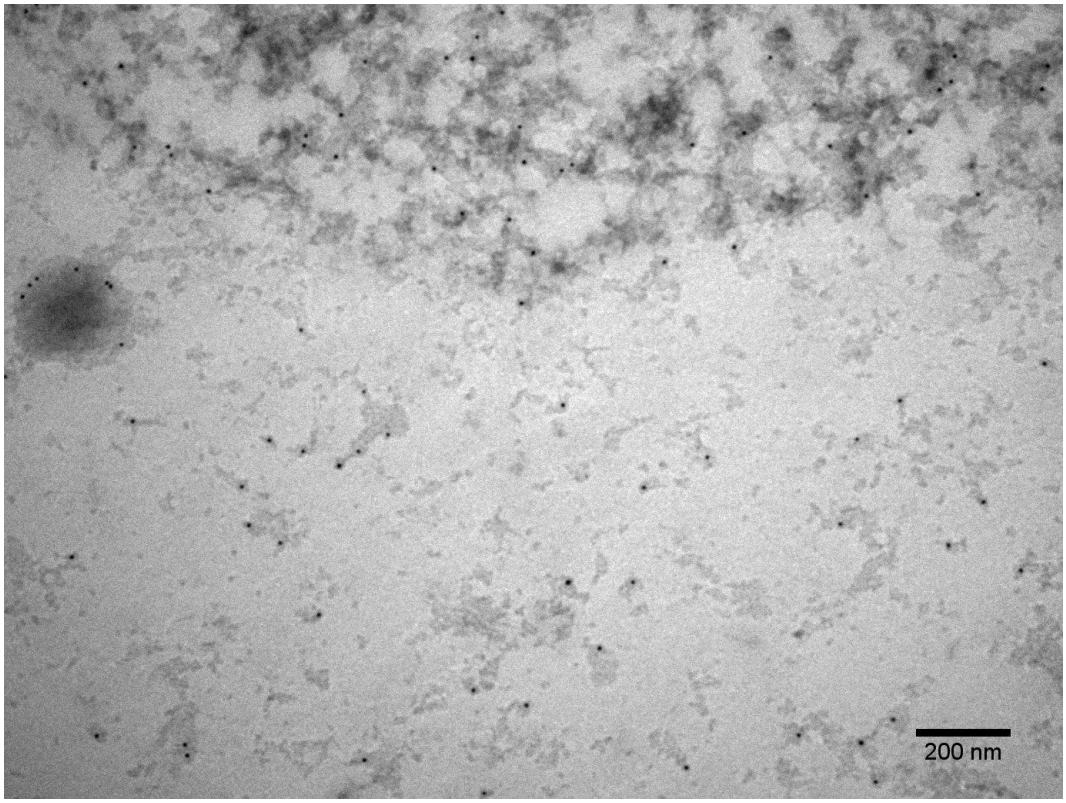


Figure 17: The result of dissolving protein sample blocks followed by precipitation in the resin. Method: MW-RT; Labeled protein: BSA

The microwave-assisted procedure led to satisfying results in terms of labeling densities. The number of observed particles was higher than expected for HRP and Tfn, which supports the positive effects of MWI on antigenicity, suggested in several literature sources [1] [7] [58]. Especially for the labelling of HRP, this method seems to be very suitable. However, the positive effects of MWI on antigenicity could not be repeated in the case of BSA and Hgb, where no significant difference to the expected number of antibodies was observed. This implies, that antigens are not positively affected to the same extent by MWI. Furthermore, the density of labeling may be further increased by lowering the temperature of polymerization, which was with 90 °C certainly too high. LRW can already be polymerized at 60 - 65 °C with a heat catalyst. [59]
In summary, it can be stated that the microwave-assisted protocol gives good labeling results and additionally saves a lot of time and trouble, especially when a professional laboratory microwave is available.

The literature source suggests that after HPF/SQFS, the results of on-section immunolabeling were positive with very low background labeling. [60] McDonald also supposes that long FS procedures may actually have a negative effect on antibody labeling and extraction of cell components. [60] However, in this experiment, HPF/SQFS led to very poor labeling densities and significantly fewer-than-expected gold particles in all four tested proteins. The initial purpose of SQFS was to reduce the processing time and to avoid the extraction of antigens. The results, in contrast, indicate that the antigens were extracted even faster compared to conventional FS. This was probably due to insufficient primary fixation. Furthermore, freeze substitution was done until reaching room temperature, which also contributes to the extraction of antigens. HPF/SQFS is therefore, although it is very time-saving, not very suitable for immunological studies.

High-pressure freezing (HPF) followed by freeze substitution is most often regarded as the ideal method to preserve cellular structures closely to the living state and is therefore beneficial for preserving morphology [23] [29] [17]. Furthermore, chemical fixatives, necessary prior to room temperature-dehydration, often have a negative influence on antigenicity. [61] Bittermann et al. (1992) [62] for example, compared different sample preparation methods with the highest labelling efficiency obtained by cryofixation, followed by freeze-substitution in methanol (without fixatives) and embedding in hydrophilic Lowicryl resins at low temperatures. The presence of

different chemical fixatives always reduced the labelling density. Methods involving fixation at temperatures higher than 0 °C, such as "progressive lowering of temperature" (PLT), always resulted in lower labeling efficiencies.

In the case of BSA, high-pressure freezing followed by freeze substitution had, as expected, a very positive effect on the efficiency of antibody-binding, which might be the result of the minor alterations by chemical fixatives. In contrary to SQFS, the proteins were efficiently retained in the sample during freeze substitution. However, the results of the other three proteins did not reflect this positive effects. In the case of Hgb and Tfn, the observed numbers did not differ from the expected numbers of particles. In addition, freeze substitution protocols, regardless of conventional FS or SQFS, seem to be unsuitable for immunological analysis of HRP. The reason, why the MW-RT and the PLT method gave better LD, could not be explained and needs to be discussed in future experiments. In conclusion, HPF followed by conventional freeze substitution perhaps is the best method in terms of morphology, but it may give poor labeling results depending on the protein to be visualized. Due to the varying effects on different antigens, the indeed very time-consuming procedure may not pay-off in the end.

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II. Appendix

BSA				HRP				Hgb				Tfn			
HPF/FS	MW-RT	HPF/ SQFS	PLT	HPF/FS	MW-RT	HPF/ SQFS	PLT	HPF/FS	MW-RT	SQFS	PLT	HPF/ FS	MW-RT	HPF/ SQFS	PLT
56	28	16	46	8	11	9	16	7	6	3	5	2	4	4	2
53	29	8	50	13	15	8	10	6	5	4	4	3	3	2	2
88	28	2	17	9	10	5	18	4	4	3	4	3	4	3	2
56	32	15	47	8	6	8	8	3	4	3	4	2	5	2	2
44	34	11	41	7	13	8	14	4	3	3	5	2	4	2	2
35	32	7	24	7	22	49	16	3	5	3	7	3	4	2	6
65	27	8	46	8	6	9	10	4	5	3	2	2	4	2	2
40	34	12	27	5	12	5	34	3	4	5	4	3	4	1	5
46	32	11	28	8	15	7	53	5	3	4	3	2	3	1	2
51	26	15	42	6	12	10	29	4	5	4	4	2	2	1	2
51	26	5	27	5	10	8	23	5	4	4	5	5	4	2	3
63	38	5	38	9	14	7	23	4	4	6	4	2	3	2	2
52	28	7	40	8	21	4	14	5	4	4	3	2	3	6	2
54	29	9	46	5	12	10	11	3	5	2	3	2	5	1	3
50	33	6	41	7	14	4	23	3	5	3	2	4	4	5	5
42	21	5	32	9	11	8	19	4	6	4	4	2	4	1	4
56	25	7	31	7	41	5	15	3	3	3	5	2	5	2	2
50	24	5	49	11	53	8	18	3	4	3	3	2	2	1	2
30	35	6	64	5	34	5	18	5	4	2	3	2	3	2	3
34	29	6	33	5	87	5	17	6	4	2	3	5	2	3	2
35	30	4	39	5	67	11	18	3	3	2	3	2	2	1	3
28	32	9	31	5	74	8	15	3	5	3	2	2	4	1	2
45	30	14	33	7	43	19	15	3	3	2	2	2	3	1	2
30	22	12	32	6	10	9	18	4	4	3	4	2	2	3	2
40	30	8	40	7	10	4	12	4	6	4	4	5	2	2	2
59	34	6	40	5	57	8	22	4	5	3	3	3	2	5	3
46	35	7	35	6	13	19	25	3	5	3	6	3	2	3	5
42	25	4	36	5	36	6	23	3	6	3	2	2	2	2	2
34	25	8	51	4	24	8	22	3	4	3	8	2	3	2	3
38	39	5	38	7	31	6	17	7	5	3	3	2	2	2	2
33	30	7	40	9	15	7	19	2	5	3	2	3	5	2	3
42	37	10	42	7	28	16	8	2	6	1	2	2	2	4	3
31	26	5	33	8	35	6	16	3	3	3	3	2	2	4	2
33	29	5	26	8	18	8	12	3	4	2	4	3	3	1	2
34	25	5	33	4	9	8	17	3	3	3	4	3	4	2	4
52	25	15	29	5	27	6	16	3	3	2	4	3	3	2	2
31	36	13	31	11	36	5	14	3	4	3	3	3	3	1	4
32	31	8	42	6	15	5	13	2	4	4	4	3	2	2	5
47	22	8	32	8	11	17	29	3	4	3	3	5	3	4	2
37	23	7	33	5	16	12	16	3	6	3	4	2	2	1	2
38	31	8	36	7	15	22	32	5	3	3	3	3	6	1	6
32	44	15	47	8	16	15	24	3	4	6	2	2	3	1	3

43	29	9	42	6	31	6	25	4	4	4	3	3	3	1	8
50	36	7	44	5	8	7	15	4	3	3	1	5	3	1	3
50	56	16	27	5	14	5	43	6	4	4	5	4	3	1	7
42	27		44	6	12	8	20	6	4	3	4	2	3	3	7
33	33		26	7	16	5	20	5	4	2	4	4	2	2	5
42	25		27	7	16	5	24	3	5	4	4	3	3	1	
28	27		28	7	37	9	22	3	5	3	6	2	3	2	
58	30		37	6			24	4	5	3	2		4	3	
42	45		34	5			21	3	5	3			3	1	
								6	7					5	
2 2 4 3	**1 5 5 9**	**3 8 1**	**1 8 7 7**	**3 4 7**	**1 1 5 9**	**4 5 2**	**1 0 0 6**	**2 0 0**	**2 2 8**	**1 6 2**	**1 8 1**	**1 3 4**	**1 6 1**	**1 1 2**	**1 4 9**

Table 14: Data collected from micrographs taken at a magnification of 60k. The numbers represent the counted gold particles per image. Outliers specified by the General ESD test with a significance level of α = 0.05 are labeled in grey. The bottom numbers represent the sum of particles observed.

Hazard statements:

H226: Flammable liquid and vapour

H301: Toxic if swallowed

H312: Harmful in contact with skin

H314: Causes severe skin burns and eye damage

H315: Causes skin irritation

H317: May cause an allergic skin reaction

H318: Causes serious eye damage

H319: Causes serious eye irritation

H332: Harmful if inhaled

H335: May cause respiratory irritation

H400: Very toxic to aquatic life