CHAPTER 2

IMMUNOLABELING AND AUTOFLUORESCENCE OF FINGERMARKS
1. INTRODUCTION

In this chapter an overview will be given about the techniques used and described in this thesis. Firstly, the immunolabeling of fingermarks will be explained. Secondly, the autofluorescent properties of fingermarks are discussed, followed by a short description of thin layer chromatography and fluorescence spectroscopy.

2. IMMUNOLABELING OF FINGERMARKS

Immunological techniques are often used in the (bio)medical field to aid in diagnosis, therapy and prognosis of diseases. Immunological techniques are based on the specific binding of antibody to antigen. Immunohistochemistry is a commonly used immunological technique, which can be used for the detection and localization of a specific epitope of a protein in a cell or tissue [1-2]. Also, in the forensic field immunological methods have been explored for the detection of specific components in traces, like blood, urine and fingermarks [3-6]. The name immunohistochemistry implies the use of antibodies (immuno) and tissue (histos). In this thesis we have applied the immunohistochemistry technique on fingermarks, as fingermarks cannot be described as tissue we will call this method immunolabeling, the specific labeling or detection of components in fingermarks using antibodies.

One of the first groups that used antibodies to detect specific components in fingermarks was the group of Ishiyama et al [7]. In 1977, they described an immunogenic method which was used to determine the blood group type of the donor of the fingermark [7]. They used a mixed cell agglutination reaction (MCAR) technique, which is based on the agglutination reaction that occurs between blood group antigens, antibodies and indicator cells [7]. Other research groups started using immunolabeling methods to determine blood group types in fingermarks [8-9]. However, in the nineties and early zeroes, not many new publications were available on the use of immunolabeling for the detection of specific components in fingermarks. Currently, the use of immunolabeling has received new attention in the forensic field and has also attracted the attention of our research group. In this chapter, a short description will be given about the basic principles of antibodies and immunolabeling.

2.1 Antibodies

Antibodies play a role in the immune system in the clearance of pathogens and they are present in blood and tissue [10]. When foreign material enters the human body, the immune system responds by activating the bone marrow by producing B-cell or B-lymphocytes, which in their turn synthesize antibodies. Antibodies, also called immunoglobulins, are able to localize and bind to epitopes present on the foreign material. This foreign material is called an antigen, each antigen can have multiple epitopes that can be recognized by different antibodies. Besides foreign material, antibodies are also able to recognize disease-causing agents and altered body cells, such as tumor cells and autoimmune events. Most antibodies are Y-shaped and consist of four polypeptides, two
identical light chains and two identical heavy chains. The antigen binding site (Fab) can be found at the light chain of the antibodies as shown in figure 1-A. This site is able to detect and bind to antigens. Antibodies are highly specific for their antigen and therefore the binding between antigen-antibody is known as one of the most specific non-covalent reactions. On the heavy chain the constant fragment (Fc) region can be found, this region plays an important role in the breakdown of the antigen [10]. Once an antigen is bound to the antibody, a conformational change will occur, which activates the Fc-region. Now, Fc-receptors are able to bind to the antibody, which may lead to clearance of the antibody-bound antigens. There are different types of classes of antibodies, which are also known as isotypes, including the IgA, IgD, IgE, IgG and IgM. The most common isotype is immunoglobulin G (IgG). In general all isotypes consist of two light and two heavy chains, except for IgM, as they form polymers of the basic structure. The biology activity of the various isotypes is different, whereas isotypes IgG and IgM can activate the complement system, IgE will activate mast cells and is involved in allergy responses [10].

A remarkable feature of antibodies is that they can be produced against almost any kind of macromolecule, which makes them of special interest to the biomedical field. By injecting the antigen of interest in an animal, the specific antibody can be produced [10]. Sometimes the size of the antigen of interest is too small to induce the immune system of the animals, in that case the antigens are bound to carrier proteins, like bovine serum albumin or keyhole limpet hemocyanin to activate the immune system.

Two different types of antibodies can be recognized, polyclonal and monoclonal antibodies. Both types of antibodies have their own advantages and limitations. Polyclonal antibodies are able to recognize many dissimilar epitopes in one antigen and are able to recognize antigens that are presented in different orientations. They are derived from various B-cell lines and therefore their response is heterogeneous. Polyclonal antibodies have thus a high affinity and wide reactivity to the antigen [10]. However, the specificity of polyclonal antibodies is low and batch-to-batch variation can occur, which leads to differences in reactivity. Monoclonal antibodies react only with one specific epitope. They are derived from clones of single B-cells. The clone of one single B-cell is able to produce all identical antibodies, that recognize one specific epitope. Therefore, monoclonal antibodies have a high specificity for their epitope. The batches are homogenous, which makes them extremely relevant for the development of standardized procedures. However, the production costs of these antibodies is higher and more time consuming then the polyclonal antibodies [10].

Antibodies can thus be used to specifically detect molecules, such as proteins, hormones and carbohydrates, and are therefore an interesting tool to use for the detection of specific components in fingermarks. These specific antibodies are produced by injecting animals with human material.
2.2 Immunolabeling and fingermarks

The use of antibodies to detect specific components in fingermarks has been described in literature [4-9, 11-20]. Immunolabeling of fingermarks can serve two purposes: i) to obtain additional information from the donor of the fingermark, ii) to (re)develop fingermarks. In 2007, immunolabeling of fingermarks gained renewed interest of the forensic field. Leggett et al. described the detection of cotinine in fingermarks of smokers using anti-cotinine antibodies conjugated to gold nanoparticles, where they used a secondary antibody, tagged with a fluorophore, to detect the primary antibody [6]. Once ingested or inhaled, nicotine is metabolized in different metabolites, including cotinine. Via the sweat pores in the skin, cotinine is secreted in sweat and therefore will be present in fingermarks of smokers. Leggett et al. were able to detect cotinine using anti-cotinine antibodies in fingermarks of smokers, suggesting that discrimination between smokers and non-smokers was possible in fingermark residues [6].

Hazarika et al. continued on the work described by Leggett et al., instead of the use of gold nanoparticles they conjugated the antibodies with magnetic particles coated with protein A/G [6, 11]. There are two major advantages of using magnetic particles over gold nanoparticles. The first is that no washing steps are necessary, since the access of unbound magnetic particles can be removed with a magnet. Second, the conjugation of antibodies to magnetic particle is much easier, because the purification of the conjugates can be performed with a magnetic separator. Figure 1-B presents a schematic overview of the working mechanism of magnetic particles functionalized with antibodies. Protein A/G has a high affinity to bind to the Fc region of the antibodies, which makes that the Fab-site of the antibody is still available for antigen recognition. Hazarika et al. conjugated anti-cotinine antibodies to magnetic particles. Both, magnetic particles and primary antibodies could be detected in fingermarks of smokers, leading to the conclusion that additional information can be obtained from fingermarks using immunolabeling [7]. Additionally, the immunolabeling of fingermarks from smokers
using anti-cotinine functionalized magnetic particles could also be used for the development of fingermarks as high detailed information about the ridge pattern and even pores could be recognized [7]. The research group of Hazarika et al. continued the immunolabeling of fingermarks using functionalized magnetic particles for the detection of illicit drug metabolites in fingermarks, leading to interesting and inspiring results for the whole forensic field [9, 11-13].

We tried to mimic the results described by the research group of Hazarika et al. Our attempt to reproduce their protocol, including the use of protein A/G coated magnetic particles, resulted in unwanted non-specific binding of the functionalized and non-functionalized magnetic particles to the carrier surface and/or fingermarks, as shown in figure 2 [17].

Figure 2. Fingermarks incubated with functionalized (A) and non-functionalized (B) magnetic particles. Black particles indicate the presence of magnetic particles.

Fingermarks were incubated with anti-glutamate functionalized magnetic particles and non-functionalized magnetic particles. High detail levels could be obtained from fingermarks treated with both the functionalized and non-functionalized magnetic particles. Non-specific binding of the functionalized antibodies is not desired, since the immunolabeling is developed to serve two purposes, firstly, to obtain donor profiling information from the fingermarks and secondly, to redevelop the fingermark pattern.

A somewhat different immunolabeling method was described by Drapel et al., in which they tried to detect general occurring proteins in fingermarks, namely cathepsin D, keratin 1/10 and dermcidin [4]. In their study, unconjugated primary antibodies were used, followed by incubation of tagged secondary antibodies that specifically detect the primary antibodies [4]. Specific immunolabeling was obtained using this method.

In this thesis, we describe an optimized immunolabeling method for the specific application of detecting components in fingermarks based on the research performed by Drapel et al. and on the general immunohistochemistry method. The basics of the immunolabeling labeling method described in this thesis are illustrated in figure 3, including direct and indirect immunolabeling. The direct method is a one-step method, in
which the primary antibody is conjugated with a detection dye, such as a fluorophore, enzyme or other tag. In the indirect method, unlabeled primary antibodies are incubated with the fingermarks [21]. Additionally, secondary antibodies conjugated with a detection dye are applied to the fingermarks. The secondary antibody is raised to bind to the primary antibody. The sensitivity of the detection of the antibodies with the indirect method can be higher, because more than one ‘tagged’ secondary antibody can detect one primary antibody [21].

Figure 3. schematic overview of the direct and indirect immunolabeling method.

Figure 4 shows the steps involved in the indirect immunolabeling method. After placing a fingermark on a surface of interest, the fingermark is incubated with a surplus of proteins. This first step is called the blocking step. Blocking is necessary to prevent the binding of antibodies to other nonspecific proteins with possible reactive sites. Since the antibodies have a higher affinity for the antigen than the blocking proteins, the blocking proteins will be replaced by antibodies at the epitope sites of the antigen. After the blocking step, the primary antibodies are incubated with the fingermark. If the antigen is present, the antibodies will localize and bind to the antigen. To remove unbound antibodies, washing steps are performed. Additionally, the ‘tagged’ secondary antibody, which is raised against the primary antibody, will bind to the primary antibody. A second washing step is needed to wash away the unbound and excess of secondary antibodies. After washing, the sample can be dried and visualized.

In short, immunolabeling can be used to detect specific components in fingermarks. This method can serve two purposes: i) to obtain additional information from the donor of the fingermark, ii) to redevelop fingermarks. Immunolabeling is not limited to fingermarks, but can also be applied to other minimal traces encountered during the crime scene investigation. In this thesis, the main focus is on the detection of components in fingermarks.
3. AUTOFLUORESCENCE OF FINGERMARKS

Chemical components present in fingermarks have intrinsic (auto)fluorescent properties. Fingermarks therefore have the ability to emit fluorescence upon excitation with the proper wavelength. Not much information is available on which components cause this intrinsic fluorescent signal. In this thesis, we have tried to identify the major fluorescent components and use the spectral changes in autofluorescence to estimate the time passed since deposition of the fingermark. This part of the chapter will explain the basics of autofluorescence and the technique we have used to detect and analyze the fluorescent components in fingermarks.

3.1 Fluorescence spectroscopy

Fluorescent molecules have the ability to interact with light. They are able to absorb light at a given wavelength and after a short period of time (pico- or nanoseconds) will release the energy in the form of light generally at a longer wavelength [22-23]. These molecules are called fluorophores or fluorochromes. A schematic representation of the absorption and emission of such a molecule is shown in figure 5. Molecules at rest (ground state or \( S_0 \)) can absorb light, leading to the redistribution of the electron cloud of the molecule. The energy of these molecules is raised to an excited state (\( S_1 \) or \( S_2 \)). The exact vibrational state and electronic levels that will be reached depend on the energy that is absorbed by the molecule. The thick lines in the Jablonski diagram, figure 5, represent the lowest energy levels of the electronic states, whereas the thin lines represent the vibrational modes. Once the excited energy state is reached, molecules will quickly lose their energy to the environment and will revert to the lowest vibrational state of the lowest excited state; this process is called thermalization. After a short period of time, this process is followed by internal relaxation, causing emission of photons.

Figure 4. Steps involved in the immunolabeling process.
and the return of the molecule to its ground state [22-23]. During the transition of the molecule to the ground state, light is emitted, which is observed as fluorescence, as shown in the simplified Jablonski diagram [22].

Figure 5. A simplified Jablonski energy level diagram, describing the Absorption (―) and emission (---) process and thermalization and internal relaxation, figure adapted from Croney et al. [22].

Fluorescence spectroscopy can be used to obtain information about the chemical structure of molecules and unknown samples by measuring their specific excitation and emission spectra [23]. Fluorescent molecules are characterized by two characteristic spectra, an excitation spectrum and an emission spectrum [23-24]. Excitation spectra are obtained by measuring the emission intensity at a fixed wavelength, while scanning the excitation wavelength over the UV/VIS part of the spectrum [24]. This excitation spectrum is in most cases similar to the absorption spectrum of the molecule. The emission spectrum is the relative intensity of emitted light as a function of emission wavelength, which can be demonstrated by measuring light at different wavelengths using a fixed excitation wavelength [23-24]. The emission spectrum is generally at a longer wavelength (lower energy) than the excitation spectrum [22-24]. The difference in wavelength between the excitation and emission maxima, is called Stokes shift. In our work described in chapter 7, 9 and 10, excitation and emission spectra were obtained with the Perkin Elmer LS 55 luminescence spectrometer, a schematic overview of the instrument is given in figure 6.

3.2 Fingermarks and their autofluorescent properties
Components present in the fingermark residue have fluorescent properties, as depicted in figure 7, in which a hand palm left on a white wall was photographed upon excitation using a UV Crime-lite ® torch. Dalrymple et al. was one of the first who described the fluorescent properties of fingermarks [25] after excitation with a 514.5 nm laser. Fresh fingermarks displayed a yellowish green fluorescence signal upon excitation, whereas
Different research groups found a remarkable difference in autofluorescence color and signal in aged fingermarks compared to fresh fingermarks [29-30]. When excited with 280 nm the maximum emission of fresh fingermarks is around 340 nm, after exposure of light a shift in the emission signal was observed to 440 nm [30]. A similar red shift in fluorescence signal was observed in aged fingermarks [31]. This observation is interesting and can potentially be used for the age estimation of fingermarks. In chapter 7 and chapter 9 we have tried to identify the most important contributors to the autofluorescence of fingermarks.

![Figure 6. Schematic overview of fluorescence spectroscopy.](image)

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orescence signal of fresh and aged fingermarks. Thin layer chromatography (TLC) and fluorescence spectroscopy were used to identify these compounds.

![Hand print left on a wall, visualized with an UV light source.](image)

**Figure 7. Hand print left on a wall, visualized with an UV light source.**

### 3.3 Thin layer chromatography
Thin layer chromatography (TLC) is a simple method to separate inorganic and organic substances. The method involves a plate, which is a thin layer, which is able to absorb another material or fluid, also called the stationary phase or solid phase. The solid phase can be made from different materials [32]. The most frequently used solid phase is silica gel, other solid phases can be cellulose, aluminum oxide and polyamide. Samples of interest are spotted on the TLC plate and are separated when they come in contact with the mobile phase. The mobile phase is able to migrate through the plate by capillary action and will migrate through the sample. Different components present in the sample are now allowed to migrate depending on their affinity for the solid phase or mobile phase [32]. When the mobile phase reaches almost the top of the plate, the plates are removed from the mobile phase and left to dry. The separated components that are present in the sample can be visualized using a special light source (of specific wavelength) or a chemical treatment that reacts with chemical groups of interest. An illustration of the thin layer method is shown in figure 8.

In forensic research, TLC has been used to study the compounds present in fingermarks. Duff and Menzel introduced the use of TLC in 1978 to separate compounds present in the fingerprint residue by swabbing fingertips and palms with a cotton swab and spotted the contents of the swabs on a TLC-plate [29]. After running the TLC plates no spots could be observed by eye. Additionally, laser illumination was used to excite possible fluorescent spots applying different wavelengths. Under UV illumination different spots could be observed on the developed TLC plates. However, no statements were made on the origin of these spots. The group of Dikshitulu used TLC in combination with high performance liquid chromatography (HPLC) to examine chemical changes of components as a function of time and environment in fingerprint residues [33].
After development, plates were treated with iodine vapor or sprayed with rhodamine B reagent. Different spots could be observed on the developed TLC plates and different fractions could be observed with HPLC analysis. They concluded that in fingermarks of different individuals similar components were present, but in different proportions. Upon aging of fingermarks disappearance of components was observed, probably caused by degradation, and a change in relative amount of individual components could be noted [33]. Bramble et al and Jones et al. also used the TLC method, whereby fingermarks were placed directly on the TLC plates, which gave a more realistic and representative view of the fingermark residue [28, 34]. They concluded that the fatty components, triglycerides, squalene and bilirubin play a possible role in the intrinsic fluorescence of fingermarks [28, 34].

In chapter 7 and 9 we have used TLC in combination with fluorescence spectroscopy and chemical treatment to obtain more knowledge about the chemical components present in fresh fingermarks and aged fingermarks. Unknown fingermark components were compared with known reference samples. Knowledge on the chemical components present in fingermarks can be used to develop new fingermark development techniques or to aid in the development of a method to estimate the time of deposition of a fingermark.

Currently, no methods exist that are able to estimate the time of deposition of fingermark traces. The need of such a method is high, since age estimation of fingermarks may increase the forensic value of the trace. Knowledge on the time of deposition of fingermarks can help in determining whether a trace is relevant for the crime scene investigation and/or to link a testimony of suspect or witness with the found traces. An important issue that hinders the age estimation of fingermarks is the high inter-
and intravariability in the chemical composition of fingermarks [35-36]. In the past, the quality of the fingermark and the ease of the development with for instance fingermark powder, was used by fingerprint experts to estimate the time of deposition. However, this method was not reliable as it appeared that fingermarks indicated by fingerprint experts as ‘fresh’ were in fact one or several years old [37-38].

Several studies on the aging of fingermarks have been performed to develop a method that is able to estimate the age of fingermarks. Chemical and morphological properties of the fingermarks have been used to find characteristic changes over time to estimate the age of fingermarks, however at this moment, no methods are available that accurately can estimate the time since deposition [35, 39-41]. In chapter 10 of this thesis, we will introduce a new method that uses the autofluorescent properties of fingermarks and the changes in autofluorescent signal over time to estimate the time since deposition of the fingermarks. We approach the fingermark as a lipid-protein emulsion, wherein the oxidation process of the lipid-protein emulsion plays a major role. In food science, the oxidation process between protein and lipids to understand the aging of food is thoroughly studied [42]. Therefore, the interaction between oxidized lipids and proteins is the starting point of the age estimation method.

Additionally, in chapter 8, we have used the intensity of the autofluorescent signal of fingermarks to determine whether there is a relation between the DNA content. We hypothesize that the DNA content is related to the amount of material that is left behind by the fingermark donor and that fingermarks that emit a high fluorescent signal have a high DNA content.

4. CONCLUSION

Immunolabeling, TLC and fluorescence spectrometry can all be used to obtain chemical information from fingermark traces. Immunolabeling is an interesting technique that can be used to specifically target antigens in fingermark residues. In this thesis we explore the use of immunolabeling as forensic technique and the value of immunolabeling to serve as additional tool to obtain donor profiling information and redevelop fingermarks. Additionally, TLC and fluorescence spectroscopy are used to identify important fluorescent components in fingermarks. This information may help in donor profiling and in the end be helpful in the age estimation of fingermarks.

REFERENCES


