CHAPTER 3

SIMULTANEOUS LABELING OF MULTIPLE COMPONENTS IN A SINGLE FINGERMARK

ABSTRACT

A fingermark contains important forensic information of the donor, not only in its ridge pattern, but also in the chemical composition of its secretion. Detection and identification of these secretions can be done by immunolabeling. In this study, we describe for the first time a reproducible immunolabeling method that allows the simultaneous detection of multiple components of interest. This method not only reduces the manipulation of fingermarks, but also different types of information can be obtained about the donor in one labeling session. To prove the concept of this technique, we selected two general components as antigens of interest, dermcidin and the human serum albumin. Conjugation of both antibodies to two different synthetic fluorophores, followed by simultaneous incubation of both conjugated antibodies, resulted in successful multiple immunolabeling of fingermarks left on a porous nitrocellulose membrane and on a non-porous glass slide surface. In order to minimize false positives to prevent non-specific binding of antibodies to fingermarks and surface carriers, careful blocking and washing steps were found crucial. With this reproducible protocol, high quality images could be obtained from the multiple labeled fingermarks. In conclusion; simultaneous multiple immunolabeling of fingermarks can identify specific components in the secretion of the fingermark, including components related to hygiene, diet, time of day, contacts gender and drug use. Multiple immunolabeling therefore has the potential to make a major impact in the forensic field.
1. INTRODUCTION

Fingermarks found at crime scenes and on crime-related objects, contain skin ridge patterns that are used for database searches and identification purposes. Their DNA content allows personal identification as well [1-2]. Secreted components of fingermarks that originate from skin glands have a chemical composition that includes lipids, amino acids, proteins, and also exogenous components like debris and cosmetics, which are affected by personal hygiene, diet, time of the day and type of contact [3-5]. Thus, analysis of the composition could provide intelligence information about the donor, like gender, diet and/or presence of drug metabolites [3, 6-8]. A method to retrieve this information is immunolabeling.

Not all fingermarks found at a crime scene will lead to the identification of the donor. Profiling information will then be of great forensic value, for example to include or exclude potential donors. For forensic purposes antibodies (ABs) can be used to detect specific components of interest and/or to enhance the visibility or develop fingermarks [9-16]. Recently, we have described how immunolabeling is compatible with two common fingermark visualization methods, ninhydrin spraying and powder dusting [10]. This finding is a major advantage, since the fingermark cannot only be used for identification purposes using the ridge pattern, but, when using immunolabeling, also for the detection of antigens of interest. An important issue then is to minimize the manipulation of the fingermark. Therefore, we have sought to develop a method that is reproducible and able to detect two or more antigens in a fingermark simultaneously without physically affecting the fingermarks.

To prove the principle of multiple and simultaneous immunolabeling, single fingermarks were directly incubated with a mixture of two ABs to obtain multiple and simultaneous immunolabeling. Two generally present peptides of endogenous origin were selected as antigens of interest, first dermcidin, an antimicrobial peptide and, second, the human serum albumin (HSA), an abundant carrier protein [13, 17-20]. Both antigens are identified to be present in eccrine sweat. Dermcidin and HSA have been successfully detected in fingermarks by Drapel et al. and Reinholz [9, 13, 17-18]. We emphasize that there are no fundamental reasons to be limited to only two antigens. In this study, fingermarks were left on porous nitrocellulose membranes and on common non-porous glass slides. Positive and negative controls were performed to investigate the specificity of the antibodies used during the experiments.

2. MATERIAL AND METHODS

All experiments were performed under room temperature unless mentioned otherwise. Buffer solutions were prepared according to the description in table 1.
2.1 Fingermark collection.
Fingermarks were collected during working hours. Donors were asked to deposit a fingermark on nitrocellulose membranes (0.45 µm, Millipore, Merck KGaA, Germany) or on glass slides (Superfrost plus, Gerhard Menzel GmbH, Germany). Fingermark deposits were completely natural; no special instructions were given to the donor. Before treatment, samples were left to dry for 24 hours. For every experiment at least eight fingermarks of different donors were included, as shown in table 2.

Table 2. Number of fingermarks used for each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of donors</th>
<th>Fingermarks per donors (minimal)</th>
<th>Total fingermarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single labeling dermcidin</td>
<td>10</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Single labeling albumin</td>
<td>10</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Multiple labeling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrocellulose</td>
<td>8</td>
<td>6</td>
<td>56</td>
</tr>
<tr>
<td>Glass</td>
<td>8</td>
<td>6</td>
<td>65</td>
</tr>
</tbody>
</table>

2.2 Incubation of primary and secondary antibodies on fingermarks left on nitrocellulose membrane.
After one day, fingermarks deposited on nitrocellulose membrane were incubated for one hour in blocking buffer 1 (Table 1). Next, the membrane was incubated with 100 µl primary AB for one hour: Mouse monoclonal IgM anti-dermcidin (G-81) (SC-33656, Santa Cruz Biotechnology, INC, USA) was diluted in blocking buffer 1 at a concentration of 1:20 (v/v). Mouse monoclonal IgG2a anti-HSA (A6884, Sigma Aldrich, Germany) was diluted in blocking buffer 1 at 1:100. After incubation, the membrane was washed three times for two minutes with washing buffer. Next, the membrane was incubated with 100 µl secondary AB, goat anti-mouse IgM FITC (115-095-075, Jackson Lab, Brunschwig, Switzerland) or goat anti-mouse IgG FITC (ab6785, Abcam, UK) both diluted in blocking buffer 1 at 1:100 (v/v) for thirty minutes. After incubation the membrane was washed three times for two minutes with washing buffer. Negative controls included isotype controls: mouse IgM FITC (553474, BD Biosciences, USA) and mouse IgG2a (MCA929F, Bio-connect, The Netherlands) and the exclusion of the primary AB and the exclusion of both primary and secondary ABs.
2.3 Positive control experiment
Hundred µg of dermcidin, DCD-1L (SP2420a, Abgent, USA) was diluted in 100 µl MilliQ (Millipore, Merck KGaA, Germany) to obtain a concentration of one mg/ml. One mg of albumin from human serum (A9511-100 mg, Sigma Aldrich, Germany) was diluted in one ml MilliQ to obtain a concentration of one mg/ml. To obtain two spots for each protein solution, two µl were applied to the nitrocellulose membranes. Immunolabeling was performed according to the protocol described in Section 2.2. Instead of using a secondary antibody, conjugated with a fluorescent dye, a 1:2 (v/v) diluted goat anti-rabbit secondary antibody conjugated to horse radish peroxidase (HRP) (prediluted) was used. After incubation, the membrane was washed three times for two minutes with the washing buffer. HRP was activated with 3,3’-Diaminobenzidine (DAB) (DAB plus, powerDAB, Immunologic, Netherlands). DAB was prepared according the manufacturer’s protocol. After activation, the membrane was washed three times with washing buffer.

2.4 Conjugation of antibodies to fluorophores
Multiple labeling was obtained by conjugation of the ABs to different fluorophores. Anti-dermcidin was conjugated to Dylight®350, anti-HSA to Cy3, using Lightning-Link® Rapid DyLight®350 and Lightning-Link®Rapid Cy3 (Innova Biosciences, UK), respectively. The conjugations were prepared according to the manufacturer’s instructions.

2.5 Multiple labeling performed on fingermarks left on nitrocellulose membrane
One day after deposition, fingermarks on a nitrocellulose membrane were incubated with blocking buffer 1 for one hour. Next, the membrane was incubated with a mixture of both conjugated primary ABs anti-dermcidin 1:20 (v/v), anti-HSA 1:100 (v/v) (dilutions of total multiplex solution) for one hour. After incubation, the membrane was washed three times for two minutes with the washing buffer. Negative controls included isotype controls, the exclusion of the primary ABs and the exclusion of both primary and secondary ABs.

2.6 Multiple labeling performed on fingermarks left on glass slides
One day after deposition, fingermarks on glass slides were fixated in methanol (Sigma Aldrich, Germany) for twenty minutes at -20°C. Next, the slides were rinsed three times for two minutes in MilliQ. Samples were air-dried for 10-15 minutes. A layer of fixogum (Marabu, Germany) was applied around the fingermark and dried for thirty minutes. The fixogum serves as a water-repellent barrier that keeps staining reagents localized on the fingermark sections. MilliQ was added to cover the section for five minutes. After that, PBS was applied to the samples and left for five minutes. Samples and surface were blocked with blocking buffer 2 for one hour. The mixture of both conjugated primary ABs (anti-dermcidin 1:20(v/v), anti-HSA 1:100(v/v)) (dilutions of total multiplex solution) were applied to the fingermark and left overnight in a wet chamber, using moistened tissues. After overnight incubation the samples were washed three times for two
minutes in PBS. Fixogum was removed and a drop of DAKO fluorescent mounting media (Dako, Corp. Denmark) was applied to every glass slide, and cover slips were put on. Samples were dried and analysis was done by fluorescent and bright field microscopy. Negative controls included isotype controls, the exclusion of the conjugated primary ABs and the exclusion of both primary and secondary ABs.

2.7 Imaging.
Samples were dried. The presence of ABs was confirmed by obtaining fluorescence images using a Nikon Eclipse E600 microscope (Dylight®350 (blue channel): Excitation filter 340-380 nm, dichroic mirror 400 nm, barrier filter 435-485 nm; FITC (green channel): Excitation filter 465-495 nm, dichroic mirror 505 nm, barrier filter 515-555 nm; Cy3 (red channel): Excitation filter 510-560 nm, dichroic mirror 575 nm, barrier filter 590 nm and a Nikon Coolpix 990 digital camera. Fluorescent overall images of fingermarks were obtained using the blue/green (Excitation: 460-510 nm) Crime-lite®2 torch (Foster and Freeman, UK). Digital images were taken using the yellow filter (GG495, Foster and Freeman) and a Nikon D40X digital camera (Nikon, Japan). To distinguish fluorescence by the labeling from autofluorescence, the FITC channel was used to monitor the autofluorescence when performing multiple labeling.

3. RESULTS
Single labeling of specific components in fingermarks, left on a nitrocellulose membrane, was possible using immunostaining with anti-dermcidin and anti-HSA, as shown in figure 1. Nitrocellulose membrane binds proteins and amino acids with a high affinity and is normally used to check the specificity of ABs [21]. As depicted in figure 1-A, dermcidin is only spread around the pore-sites of the fingermark and provides therefore information that can help in obtaining third level details from fingermarks. Figure 1-B shows that HSA is found at the pore-sites of the fingermark deposition and along the ridges.

Differences in fluorescence intensity could be observed in and between anti-dermcidin and anti-HSA labeled fingermarks. Lower fluorescence intensity was obtained in all fingermarks when detecting HSA, compared to dermcidin detection. Possible explanations for this observation can be found in (i) the different affinity of the antibodies to their epitope, (ii) the amount of the antigen present in the fingermark deposition, (iii) the isotype of the antibodies (IgM vs IgG) and (iv) antigen presentation. These results in combination with the performed negative and positive controls (supplementary data), show that ABs can be used to detect specific components in fingermarks.

Figure 2 shows the ridge flow and pore positions of an overall image of a complete fingermark left on nitrocellulose membrane in which dermcidin was detected. Due to the low fluorescence intensity arising from the anti-HSA labeled fingermarks, only images could be obtained at higher magnifications (from 2 × magnification).
Figure 1. Detection of dermcidin and HSA in fingermarks left on nitrocellulose membrane (2× magnification). A: Single immunolabeling of dermcidin using anti-dermcidin and a secondary antibody conjugated to FITC. B: Single immunolabeling of albumin using anti-HSA and a secondary antibody conjugated to FITC.

Figure 2. Single immunolabeling of dermcidin in a fingermark left on nitrocellulose membrane using anti-dermcidin and a secondary antibody conjugated to FITC (scale bar represents 0.5 cm).
To obtain multiple immunolabeling simultaneously, both antibodies need to be incubated on the fingermarks in a mixture. We decided not to use a secondary antibody with a fluorescent dye to prevent cross-reaction, because both primary ABs are from the same species. Hence, both monoclonal ABs were coupled to two different fluorophores. An illustration of this conjugation is shown in figure 3.

Successful multiple immunolabeling was demonstrated on fingermarks positioned on nitrocellulose membranes (fig. 4). Dermcidin and HSA could be distinguished when two different fluorophores were conjugated to the primary ABs, Dylight®350 (blue) and Cy3 (red), resulting in a multicolor image (Fig. 4-C). Figure 4-C shows high detail levels and pores can easily be identified in these labeled fingermarks. Figure 4-D shows the autofluorescence of the fingermark in the green channel.

Fingermarks exhibit autofluorescence [8]. The autofluorescence signal is much lower than the fluorescence intensity of the fluorophores used during our experiments. However, autofluorescence can be a problem when higher magnifications are required. To discriminate between the autofluorescence signal and the detection of the antigens of interest, we used a control channel. When autofluorescence is present, the signal shows up in all three available channels, the blue, green and the red channel (supplementary data). Discrimination of autofluorescence from fluorescence from multiple labeling was therefore done by distinguishing the images from the blue (dermcidin detection) and red (albumin detection) channel of one single fingermark, versus the images from the green channel (autofluorescence).

Multiple labeling was also demonstrated to be successful on fingermarks left on glass slides. Figure 5 shows a magnified image of multiple labeled fingermarks left on glass slides. Both antigens, dermcidin (fig. 5-A) and albumin (fig. 5-B) can be detected in fingermarks, resulting in a multicolor image (fig. 5-C). Ridges and pores can be identified. In figure 5-E, pore positions are indicated with white lines, whereby the ones marked with a continuous line most likely represents pores, whereas the pores marked with
Figure 4. Fluorescence images (2× magnification) of multiple detection of dermcidin (A) and HSA (B) in a fingermark left on a nitrocellulose, combined in a multicolor image (C). D: Autofluorescence of the fingermark observed in the green control channel.

Figure 5. Fluorescence images (10 × magnification) of multiple detection of dermcidin (A) and albumin (B) combined in a multi-color image (C) in a fingermark left on a glass slide. D: Autofluorescence of the fingermark observed in the green control channel. E: Multi-color image, white lines indicate sites where there is a strong (continuous line) or weak (dotted line) indication for pore location. A strong indication for pore location is based on the presence of dermcidin in a clear circular pattern.
dotted lines were assumed to be pores. A strong indication for the pore location is based on the presence of dermcidin in a clear circular pattern [9, 12]. In figure 5-E, the assumed pores are numbered with 1, 2 and 5, which express a lot of dermcidin, whereas pores 3 and 4 express relatively more albumin.

Fluorescence imaging of the detection of both antigens simultaneously on glass slides resulted in a more diffuse and indistinct image, compared to the images of labeling on nitrocellulose membrane. This is probably caused by the non-porous characteristics (no absorption) of the surface. Figure 5-D shows the autofluorescence observed in the green channel.

In all labeled fingermarks, high detail levels can be observed and pores are easily identified. Variation of pore-areas can be found in individual fingermarks and also between donors, as shown in figures 1, 2 and 4. Explanations for these findings can be found in the differences of pressure while depositing the fingermark, inter- and intra-variability and/or the highly variability in the reproducibility of the pore area. The results shown in this study combined with the performed control experiments (appendix 1) demonstrate that conjugated ABs can be used to detect specific components in fingermarks. Results are found to be reproducible, at least, fingermarks of eight different donors were tested with our immunolabeling method, and all yielded positive results.

4. DISCUSSION
We have shown for the first time, to the best of our knowledge, that simultaneous and specific multiple immunolabeling in a single fingermark is possible without physically dividing the fingermark into different parts. This reduces manipulation of fragile fingermarks, but importantly, can provide additional donor information such as gender, lifestyle and drug consumption, by selecting multiple antibodies that detect specific biomarkers in fingermarks [11, 14-16]. Thus, multiple immunolabeling significantly increases the information that can be acquired about the donor within one labeling session. This method is not restricted to two antigens. The two components described in this paper were chosen to prove the principle. In future research, interesting constituents to target can be (a) drug metabolites, like benzoylecgonine and 3,4-methylenedioxy-N-methylamphetamine, (b) food metabolites, like pheophorbide and (c) specific targets for gender determination including hormones [6, 11, 14-16]. Information about the donor of the fingermark may include or exclude potential donors, leading to the reduction of possible donors. Multiple immunolabeling is not limited to synthetic fluorophores either and upconversion nanoparticles can also be used as conjugates and thus for detection of antigens of interest. Furthermore the ability to apply immunolabeling to lifted fingermarks needs to be addressed to further increase the forensic value of this technique. We acknowledge that Hazarika et al. showed immunolabeling of two different drug metabolites in a single fingermark by physically dividing the mark into two different parts and subsequently performing single labeling on each part [15]. Our attempt to
reproduce Hazarika’s protocol, including the use of protein A/G coated magnetic particles, resulted in non-specific binding of the functionalized and non-functionalized magnetic particles to the fingermark and/or the carrier surface, as shown in figure 6. Because non-specific immunolabeling has little value for donor profiling purposes, we have sought to develop a method that is reproducible and has specific binding (avoiding non-specific binding as far as possible). Below we describe the steps that must be taken to overcome most of the experimental pitfalls in immunodetection, including the use of blocking steps, washing steps and proper control experiments.

Figure 6. Non-specific binding of magnetic particles to the fingermark residue. A: Fingermark incubated with non-functionalized protein A/G coated magnetic particles, magnetic particles bind non-specific to the ridges of the fingermark residue. B: Fingermark incubated with anti-glutamate functionalized protein A/G coated magnetic particles. In this example magnetic particles bind to the ridges of the fingermark residue.

In the first place, blocking the sites that have the ability to induce non-specific binding and that can be found on carrier surface, fingermarks and ABs, is an important step towards the specific detection of antigens. To prevent non-specific adsorption of the antibodies, it is necessary to block the membrane and fingermark with a surplus of proteins, such as bovine serum albumin (BSA) or skimmed milk powder (SMP) [21-22]. Blocking the free sites is possible, because the affinity of ABs to bind to their antigen is much higher than the affinity of the blocking agents to the unoccupied binding sites.

Next, the use of washing steps is important to remove unbound ABs from the sample, and thus to minimize unwanted staining. No washing or minimal washing steps will lead to more background staining and can affect the accuracy of the results. An alter-
native strategy to the conventional washing step suggested in literature is the use of magnetic particles and a magnet. However, we found it difficult to obtain homogenous washing steps with this method and circular contact patterns and incomplete washing can be observed, as is shown in figure 6-B. With larger volume traces, this might not be a problem but with low volume traces such as fingermarks this leads to false positive results [23].

Thirdly, control experiments are necessary to investigate the specificity of the used primary ABs. Several controls are required, including an isotype control, negative controls and a positive control. An isotype control consists of a non-immunized or irrelevant immunoglobulin of the same species and subtype as the primary antibody used during the experiments. Applying this control, non-specific binding and background staining of the primary antibody can be identified [21, 24].

We also investigated other antibodies (data not shown) that were able to detect a variety of keratins and cathepsin D in fingermarks. However, the anti-cathepsin D we used according to that described by Drapel et al. had the isotype polyclonal rabbit IgG, which appears to bind not only to its antigen, but also non-specifically to components in the fingermark [13]. Nevertheless, both antigens can be detected in fingermarks, using the right controls and choosing an isotype that does not bind non-specifically. Both antigens can therefore be selected to use for multiple immunolabeling of general components in fingermarks.

Interestingly, dermcidin was mainly found at the pore-sites, whereas albumin was found at the pore-sites, and more along the ridges. In a previous study, in which we investigated the compatibility of immunolabeling with a powdering method, we also found that dermcidin is excreted via the pores and consequently expressed at the pore-sites in the fingermarks [10]. These results were in correspondence with the results obtained by Drapel et al. [13]. The knowledge about the distribution of general components in the fingermark deposition might be useful in developing new fingermark enhancement techniques or to choose between the best suitable fingermark developing reagents. Antigens that are distributed more along the ridges can be used to develop continuous ridges, whereas antigens that are known to be excreted via the pores in the friction ridge can be used to develop pores. Finally, we also observed relative differences in distribution of constituents between different pores, information that can be of interest for the forensic researcher.

In this study, we observed a more diffuse detection of the antigens along the ridges on the non-porous surface, the glass slide, compared to the non-porous surface. This observation can be explained by characteristics of the surface. While placing the fingermark deposition on a porous surface the components have the ability to penetrate through the surface and/or are directly absorbed by the material. However, in the case
of a non-porous surface, the constituents can only move in a horizontal direction along the surface plane, leading to a more diffused fingermark deposition.

In this study, nitrocellulose membranes were used as surface to prove the principle. Another option could have been the use of PVDF membranes [13]. We preferred the use of nitrocellulose membranes, because our preliminary results showed lower background fluorescence and a smoother surface than the PVDF membranes that we had investigated.

5. CONCLUSION
In this study, we have described a reproducible immunolabeling protocol that allows the simultaneous detection of multiple components fingermarks. This technique can be used to obtain additional donor information from fingermarks, including gender, lifestyle and drug consumption. Additionally, specific detection of antigens present along the ridges or otherwise, present at the pore-sites, can also be used to enhance poor developed fingermarks. In conclusion, multiple immunolabeling can provide more information within one labeling session than included in the ridge pattern alone, and is therefore likely to become a promising method to be used in the field of forensic science.

REFERENCES