Article

Single-Metal Deposition for Fingermark Detection—A Simpler and More Efficient Protocol

Sébastien Moret [1,2]
Andy Bécue [1]

Abstract: This publication presents the latest optimization of the single-metal deposition technique (SMD II) and its comparison with the previous version (SMD I). In this study, endeavors were made to simplify and strengthen both the reagents and the detection procedure to obtain a technique that can be implemented in a standard operational laboratory. As a result, the proposed technique is simpler and faster because the monitoring of both temperature and pH is no longer required. Most importantly, the technique is (1) more efficient, with at least ca. 50% more marks detected with SMD II in comparison with SMD I (% obtained by using split marks) and (2) more robust regarding the processing of porous samples.

Introduction

Multi-metal deposition (MMD) and its evolution, the single-metal deposition (SMD), are two fingermark detection techniques that result in the selective deposition of gold nanoparticles onto fingermark ridges, followed by contrast enhancement through the *in situ* reduction of a metal: either silver for MMD [1] or gold for SMD [2]. Both techniques rely on similar detection protocols (Figure 1), and the choice for one or the other is only a matter of recipe and final contrast. MMD and SMD are very sensitive on fresh and old marks and can be applied on most types of surfaces (e.g., porous, nonporous, semiporous, adhesive tapes,

[1] École des Sciences Criminelles, University of Lausanne, Switzerland
[2] Centre for Forensic Science, University of Technology Sydney, Australia

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cartridge cases, wetted items). They are also recommended for the processing of unusual or problematic substrates, such as cling film wrapping material [3]. Regarding their potential advantages, MMD and SMD have been subject to extensive research and optimization processes throughout the last decades [1–9]. However, two commonly heard limitations of the technique are (1) a lack of robustness and (2) a labor-intensive procedure, preventing its application to case work [10].

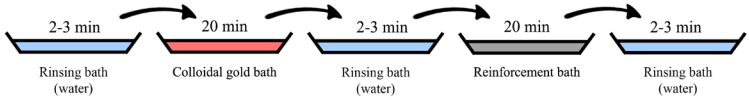

Figure 1

Illustration of the detection procedure (common to SMD I and SMD II)

Schnetz and Margot proposed an improvement of the original MMD [4, 5], named MMD II, in 2001 [1]. MMD II was characterized by a new gold synthesis procedure, improving the homogeneity of the nanoparticles, and a newly designed enhancement bath, giving more controllable and reproducible results. However, the colloidal gold synthetic procedure was tedious: to obtain 500 mL colloidal gold, it was necessary to bring two solutions to 60 °C before mixing them and heating the final solution to boil. The enhancement procedure required the use of an additional compulsory bath before the enhancement bath itself. Despite improved performances, MMD II was more complex to implement and more time consuming compared with MMD I. In 2007, single-metal deposition (SMD I) was proposed as an alternative to MMD II. The biggest difference was in the use of gold ions in the enhancement bath instead of silver [2]. When compared with MMD II, SMD I appeared to be simpler while keeping the same detection quality [8].

Despite these improvements, MMD and SMD still suffered from two major issues hampering their application in routine work: a strong pH-dependency and a labor-intensive procedure. Regarding the first limitation, the detection results are tightly related to the pH of the colloidal gold solution. According to Schnetz and Margot [1], the pH must be set between 2.5 and 3.0, with an optimum value of 2.65. This must be done by carefully adding citric acid in the colloidal gold solution while monitoring the pH value with a pH meter. To obtain optimal results, a precise adjustment must be performed before processing any

items. As a consequence, it was not recommended to process several porous items in the same colloidal gold bath or to re-use a colloidal gold solution to process successive samples. In a recent effort to reduce this dependency, it was observed that the addition of an amino acid (i.e., L-aspartic acid) during the colloidal gold synthesis resulted in good detection results at pH values outside the conventional range [9]. Regarding the labor-intensive aspect of the technique, the entire process (from the colloidal gold synthesis to the processing of items) may indeed take half a day. For people not accustomed to the technique, this time consumption may consequently deter someone from implementing this technique in his or her service.

This work aimed at addressing these limitations through three distinct studies: (1) the development of SMD II as an optimized and simplified evolution of SMD I, (2) the comparison of both techniques in terms of detection performances, and (3) an assessment of their robustness by processing a large amount of samples into the same colloidal gold solution.

Materials and Methods

Tetrachloroauric (III) acid trihydrate (Merck) and all other chemicals (Sigma–Aldrich) were of high purity grade and were used without further purification. Reverse osmosis deionized (RO/DI) water (18.2 Ω·cm) was used for the synthesis of colloidal gold, and deionized water was used for the enhancement baths. Bidistilled water (distilled twice) can be used in replacement of RO/DI water without any observed effect on the efficiency of the technique.

Fingermark Samples

All marks collected during this study were natural [11, 12]; no artificial enrichment was purposely incorporated (such as rubbing fingers on the forehead). Donors were asked to behave normally one hour prior to deposition, without washing their hands or applying cosmetics. Before leaving fingermarks, they were asked to rub their hands together to homogenize the secretions.

For the SMD II optimization study, two donors were asked to deposit natural fingermarks on four types of substrate (bleached and recycled papers, yellow paper envelopes, and transparent polypropylene sleeves) using printed templates, allowing the deposition of two depletive series of four marks. All marks were

cut in half to allow a valid comparison between the parameters under optimization (i.e., aspartic acid concentration, dilution factor, surfactant concentration, storage conditions, pH setting). The samples were left to age for 1 month in the dark, without any specific attention paid to the storage conditions (office drawer).

For the comparison study, the performances of SMD I and SMD II were assessed by using the following set of samples: 3 donors were asked to deposit 4 fingermarks on 14 different types of substrates (12 porous and 2 nonporous) (Table 1), following the deposition protocol described above. One-month and 2-year-old marks were considered for this study. A total of 336 marks were thus collected. For each donor and substrate, each mark was cut in half and one half was processed according to SMD II and the corresponding one according to SMD I. To prevent any bias because of a possible lateral difference of pressure of the fingertips during the deposition of the marks, right and left halves were equally distributed for each technique.

Surface Type	Substrate		Brand/Supplier
Porous	1	Recycled paper	Xerox
	2	Bleached paper	Xerox
	3	Recycled newspaper	unknown
	4	Paper envelope: grey	Elco
	5	Paper envelope: orange	Elco
	6	Paper envelope: brown	Elco
	7	Bleached paper	USSS
	8	Notepad	Elco
	9	Glossy newspaper	unknown
	10	Colored paper: blue	Elco
	11	Colored paper: yellow	Elco
	12	Thermal paper	Elco
Nonporous	13	Transparent PP	Coop (Switzerland)
	14	Mini-grip bag	Semadeni (Switzerland)

Table 1

Description of the samples used for this study.

For the robustness evaluation, an extended sampling composed of 9 sheets of paper (e.g., Xerox recycled paper, A4 format) each bearing 21 depletive marks was used (Figure 2). The marks were aged for 2 years and the series were cut in half so that each technique had the same amount of half marks and paper strips to process (Figure 2). The robustness test consisted in successively processing the strips by keeping the colloidal gold solution unchanged. A degradation of the detection efficiency was observed, because of the gradual increase in pH induced by the immersion of numerous paper strips in the solution. Because nonporous substrates, such as plastic, will not influence the initial pH value of the solution, they were not considered for this particular evaluation.

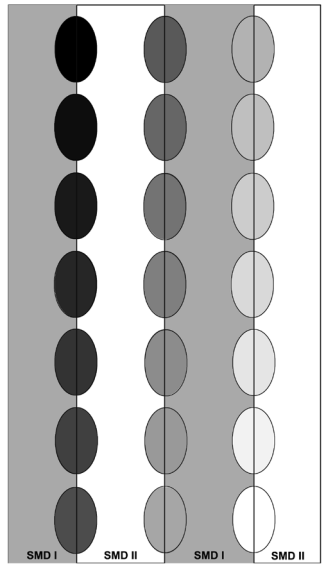

Figure 2

Template used for the robustness evaluation study. Nine sheets of paper (A4 format) like this one, each bearing a depletive series of 21 consecutive marks were considered in this test.

Sample Processing

SMD I was applied by following the procedure described by Durussel et al. [8] It consists of two main baths (colloidal gold deposition and contrast enhancement), preceded and followed by rinsing baths of deionized water (Figure 1). For SMD II, several parameters of the colloidal gold deposition step were optimized, which involved surfactant concentration in the colloidal gold solution, dilution of the working solution, pH adjustment, and influence of the working solution storage on the detection results. The contrast enhancement step remained unchanged from SMD I (this procedure is available elsewhere [8]). The complete SMD II protocol can be found in the Appendix.

With the exception of the robustness study, each paper sample was processed in its own volume of colloidal gold solution. This procedure aimed at preventing any detrimental effect that was due to a preceding immersion (e.g., pH modification). Moreover, to ensure a valid comparison between SMD I and SMD II, two corresponding half marks were processed in their respective colloidal gold bath, but were then immersed in the same enhancement bath.

To assess the robustness of the two techniques, it is important to know how many successive porous samples a given colloidal solution can process without observing an excessive drop in its ability to detect fingermarks. To do so, nine successive series of half marks were processed while keeping the colloidal gold bath unchanged. A volume of 750 mL was considered for this test, which corresponds to ca. 1.5 cm of liquid height in the dish. The samples were then processed altogether in the same enhancement bath.

Evaluation

After their detection, the marks were compared and ranked by following an existing procedure [13]. The marks were first scanned at a resolution of 1200 dpi using an Epson Perfection V330 Photo, without any digital enhancement. Each picture was stored in RGB and gray modes. A score was then associated with each half mark by three independent latent print comparison experts. The scoring scale ranged from 0 to 3 (Table 2), according to the clarity of Level 2 characteristics (i.e., minutiae). An automated procedure was set up so that an assessor faced one half mark at a time, randomly chosen among the pool of scanned marks. By doing so, each half mark was rated independently from its corresponding half. For each mark, the assessor could see both RGB and gray mode images of the mark. The average scores of each technique on each substrate were then calculated and compared.

Score	Description
0	No ridges are visible at all, no sign of fingermark.
1	Ridges are visible over a small area of the mark or over the whole mark, but it is extremely difficult to retrieve second-level characteristics (such as minutiae) due to extremely poor ridge details.
2	Ridges are visible on almost the whole area of the mark, and second-level characteristics can be retrieved. Nevertheless, the quality is not optimal due to a low contrast (strong background staining or faint ridges).
3	Ridges are very well defined on the whole mark. Second-level characteristics can easily be retrieved. The contrast is optimal with no (or extremely faint) background staining.

Table 2

Table used to assess the quality of the marks (reproduced from [13]).

Results

SMD II Optimization Study

The first part of the study consisted in the optimization and simplification of the overall SMD procedure, already initiated in a preliminary work [9]. The colloidal gold synthesis was simplified and the obtained volume increased to 2.5 L. The preparation and application of the working solution were also simplified (amount of surfactant, standardized pH setting without using a pH-meter), as summarized in Table 3. The successive evolutions of the technique (from MMD I to SMD II) are compared in Table 4, to emphasize each optimization step.

	Au-ASP [9]	Optimization Study						Final Value (SMD II)
L-aspartic Acid Concentration [% w/v]	1	0.5	1	2	3	10	25	2
Final Volume Through Dilution [L]	0.72	0.72		1.25		2.5	5	2.5
Surfactant Concentration [0/00] (Tween 20)	1	0.25	0.5		1		2.5	1
Surfactant Addition	before detection	before detection			right after synthesis			right after synthesis
pH Setting	citric acid (0.1 M)	citric acid (0.1 M)			citric acid (1 M)			citric acid (1 M)
	pH-meter monitoring	pH-meter monitoring			fixed volume of citric acid determined empirically			3 mL of 1 M citric acid for 100 mL of colloidal gold
pH Adjustment	before detection	right after synthesis			before detection			before detection

Table 3

Summary of the parameters tested for the SMD II optimization study.

	MMD I	MMD II	SMD I	SMD II
Colloidal Gold Volume Per Synthesis [L]	1	0.5	0.5	2.5
Temperature Monitoring During Colloidal Gold Synthesis	no	yes, 60 °C	yes, 60 °C	no
pH Adjustment	pH-meter (pH 3)	pH-meter (pH 2.65)	pH-meter (pH 2.65)	no pH-meter (recommended amount of citric acid)
Number of Baths	5 (including 3 rinsing)	6 (including 3 rinsing)	5 (including 3 rinsing)	5 (including 3 rinsing)
Enhancement Process	Silver on gold	Silver on gold	Gold on gold	Gold on gold

Table 4

Comparison between the various multi- or single-metal deposition (MMD and SMD) procedures.

Comparison Study Between SMD I and SMD II

Detection Efficiency

A total of 672 half marks (obtained from 336 marks) were processed and evaluated during the comparison study. The average scores for each substrate are illustrated in Figure 3. SMD II gave superior results for each substrate, with the exception of the gray envelope (substrate 4) for which SMD I gave slightly better results (an average score of 0.83 against 0.80 for SMD II). On recycled newspaper, orange envelopes, and brown envelopes (substrates 3, 5, and 6, respectively), both techniques performed similarly, with a slight advantage for SMD II. For all other substrates, there was a clear difference of quality in favor of SMD II.

More surprisingly, the age of the marks did not seem to influence the quality of the detection. There were indeed no significant differences between 1-month-old and 2-year-old marks (Figure 4). SMD II was superior to SMD I, independently of the age of the marks.

Similarly to most fingermark detection techniques, the results of both SMD I and II were donor dependent (Figure 5). Nevertheless, SMD II gave systematically superior results compared to SMD I for each donor. Throughout all substrates, SMD II was able to detect a total of 173 half marks (on a total of 336) of a score equal or superior to 1, compared to 117 half marks for SMD I. These numbers were comparable, because half marks were considered. It consequently means an increase of ca. 50% of detected marks when using SMD II.

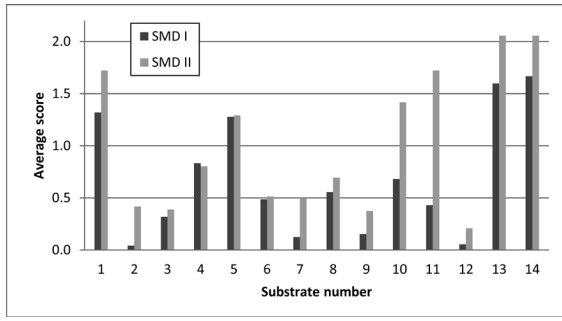

Figure 3

Average scores obtained by SMD I and SMD II when applied on each substrate type during the comparison study (see Table 1 for details about the substrate number).

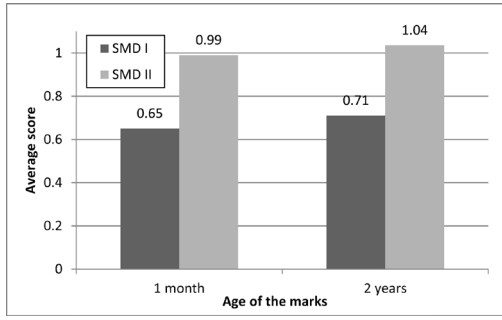

Figure 4

Average scores obtained by SMD I and SMD II when used to detect fingermarks of different ages during the comparison study.

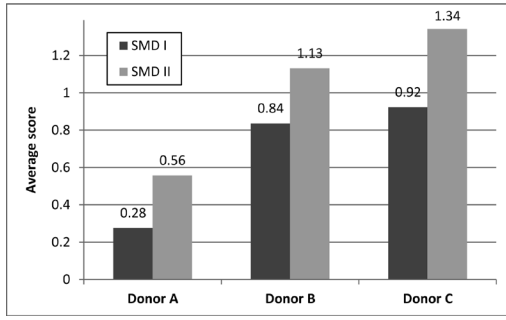

Figure 5

Average scores obtained by SMD I and SMD II when used to detect fingermarks from different donors during the comparison study.

Robustness Assessment

The detrimental effect of paper on the detection ability of MMD and SMD is not understood yet. However, it is hypothesized that some components of the porous samples may be released in the aqueous solution upon immersion, thereby increasing the pH of the working solution. As a result, the working solution loses its ability to detect fingermarks. The robustness test was thereby specifically designed to evaluate the influence of a large amount of samples processed successively in the same working solution to determine whether SMD II was more robust than SMD I. This test could also help determine the maximum amount of paper that can be processed with 750 mL of working solution. Figure 6 illustrates the evolution of the average scores (calculated from the 21 half marks present on the paper strips–Figure 2) because the nine sheets of paper were successively processed in the same working solution. Despite some variations that were due to the donor himself, it can be observed that the efficiency of SMD I tended to decrease with the number of immersed pages, especially after the fourth one. This effect was less pronounced for SMD II, which gave relatively consistent results throughout the experiment. The number of half marks detected on each page is reported in Figure 7. Similarly to Figure 6, it can be seen that the number of marks detected on each page by SMD I decreased with the number of immersed pages, whereas it remained quite constant for SMD II. On average, SMD I detected marks up to the 6th depletion on each sheet (on a total of 21 depletive marks per series), whereas SMD II gave successful results up to the 11th and 12th depletion on average. In total, SMD II detected 64 half marks (out of a total of 189) of a score equal or superior to 1, compared to 37 half marks for SMD I. It consequently means an increase of ca. 73% of detected marks when using SMD II on depletive series of marks.

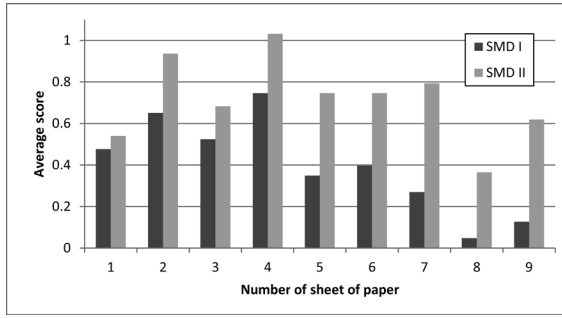

Figure 6

Average scores obtained for each sheet of paper, processed successively in 750 mL of working solution during the robustness study.

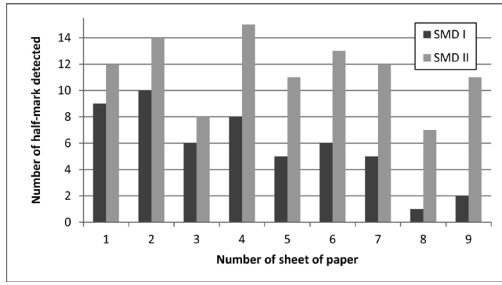

Figure 7

Number of half-marks detected by SMD I and II, when successively processing sheets of paper during the robustness study. Each sheet of paper bears 21 depletive marks.

Discussion

Colloidal Gold Synthesis

Contrary to the SMD I procedure, which heats two distinct solutions to 60 °C (i.e., gold precursor and sodium citrate, both in water) before mixing them and bringing them to boil, the SMD II procedure is done in one step. The gold chloride solution is directly brought to boil, thereby eliminating the tedious temperature monitoring. If all the required precursor solutions are already available (stored from a previous synthesis), the colloidal gold synthesis now takes about 20 minutes. The addition of Tween 20 just after the synthesis helps at stabilizing the solution for storage purposes. The new synthesis is quicker, simpler, and produces 2.5 liters of working solution in one process (compared to 500 mL for SMD I). The colloidal gold solution is very stable and can be stored under refrigeration a 4 °C for at least 6 months in polypropylene containers. After this limit, one should pay attention to its color (the solution should remain ruby red) and to the absence of any sediment on the bottom of the container. If the solution becomes purple, it should be discarded. All syntheses performed during this study were made using RO/DI water. Bi-distilled water can also be used without any noticeable differences on the final results. Regarding the use of deionized water, satisfactory results were obtained but no thorough study was performed on this kind of water.

The use of siliconized glassware [1] is no longer required in the SMD II protocol. This treatment was mandatory for MMD II or SMD I to avoid any detrimental effect (uneven gold deposition, background, and glass staining). Because of the high-quality glassware produced these days, this tedious pre-treatment now appears obsolete. No unwanted glass staining was noticed during the study.

Colloidal Gold Working Solution

Regarding the colloidal gold working solution, three main parameters differ from SMD I and SMD II: the presence of L-aspartic acid, the dilution factor, and the pH setting. The effect of L-aspartic acid has been discussed in detail elsewhere [9]. Regarding the dilution factor, the working solution in SMD II is more diluted compared to SMD I. As a consequence, a greater volume of working solution can be obtained from the same amount of gold precursors, the costs are reduced, and it also leads to better results in terms of background staining.

Finally, accurate pH-monitoring is no longer required for SMD II whereas the use of a pH-meter was mandatory for SMD I (as well as for MMD I and MMD II). Given that SMD II was proven to be less pH-sensitive than SMD I, a calculated amount of citric acid is now proposed in the recipe (i.e., 3 mL of 1 M citric acid for 100 mL of colloidal gold solution). This alteration results in a valuable amount of time saved.

Number of Donors and Choice of Substrates

In this study, two donors were chosen for the SMD II optimization study and three for the comparison with SMD I. This constitutes a slight deviation from the International Fingerprint Research Group (IFRG) recommendations [14]. However, it does not weaken the conclusions of this study because (1) the MMD and SMD are well-established techniques whose efficiency is mostly driven by the nature of the substrates, (2) the use of half marks allowed a reduction in donor dependency during the comparison between the two techniques, and (3) an extended number of substrates were considered, mainly porous ones (e.g., office paper, notepad, envelope, newspaper). Because MMD and SMD are known to perform well on nonporous substrates, there was no need to proceed to an extensive testing of this type of substrate.

SMD I Versus SMD II

The evaluation of SMD II was performed on a total of 336 natural fingermarks of 2 different age intervals, obtained from 3 donors and deposited on 14 different substrates. The results were assessed by three independent evaluators. Given that half fingermarks were considered in this study, it was possible to readily compare both techniques in terms of efficiency. When a difference in detection is observed between two corresponding halves, it can be associated to the detection technique rather than to the donor intra-variability.

The comparison of SMD I and SMD II has shown that on average, SMD II performed much better than SMD I on most substrates. It has also been shown that the SMD II solution was more robust regarding the successive processing of porous samples using the same working solution. As illustrated in Figure 6, SMD II was still able to detect 11 marks on the last of the 9-page series that were successively processed in 750 mL of working solution, whereas SMD I was only able to detect 2 on the last page. This means that by following the SMD II procedure, more porous samples could be processed in the same colloi-

dal gold solution, even though it is still recommended not to overload the detection bath. The authors recommend a minimum height of 1.5 cm of working solution in the chosen dish.

Finally, with ca. 50% and 73% more marks detected by SMD II in the comparison and robustness studies, respectively, the increased efficiency of SMD II over SMD I has been demonstrated.

Further Improvements

The critical step of MMD and SMD is clearly the first one, namely the colloidal gold deposition process (Figure 1). This step has been improved and strengthened to its maximum so far. The second step (i.e., the metal enhancement) is characterized by the deposition of silver on gold for MMD I and II, and of gold on gold for SMD I and II. The choice for gold on gold enhancement has been done for laboratory considerations (similar reagents and reduced costs). However, the contrast obtained with a gold enhancement has been recently considered as too weak by Fairley et al., who compared MMD I, II, and SMD I [10]. It is true that an SMD-processed mark will look grayish whereas an MMD-processed mark will appear darker. It is, however, important to recall that the colloidal gold deposition and the metal enhancement are two independent and interchangeable steps (Figure 1). Therefore, it is possible for someone to choose the SMD II procedure for the colloidal gold deposition, then to combine it with an MMD-like enhancement step using silver ions. However, it appears important to pursue research efforts to find more cost-effective and efficient ways to reinforce the contrast, perhaps using a metal that has not yet been considered.

Implementation in an Operational Laboratory

The various optimizations and simplifications presented in this study showed that SMD II can be effectively implemented in an operational laboratory. This technique should be considered on a case-by-case basis when unconventional items are encountered. They may be difficult surfaces or items that have been exposed to humidity, where standard techniques such as cyanoacrylate fuming or amino acid reagents would be inefficient. SMD II must not necessarily be applied in each case, but should be considered as a last chance technique, when the conventional ones have failed. The authors recommend placing SMD II at the end of the conventional sequences for nonporous and porous surfaces. Moreover, the technique can be implemented in a standard laboratory. It does not require any

specific glassware or complicated equipment. It is not necessary to undertake the work under a fume hood because all solutions are aqueous. Furthermore, the overall procedure involves only limited hazards. It is, however, recommended to manipulate gold chloride solution with care because it is corrosive. All solution waste must be disposed of in accordance with the local lab procedures. Because 2.5 L of colloidal gold solution can be synthesized at one time, processing large items is no longer problematic. Finally, if colloidal gold is available in the lab and stored in a refrigerator, the application of SMD II takes less than one hour for the item to be processed. This may result in the detection of new fingermarks and may bring valuable information for the investigation.

Conclusion

This study presents the optimization and the simplification of the single-metal deposition method, introducing SMD II as the best-so-far evolution of the technique (see Appendix for application protocol). SMD II is simpler, less expensive, and no longer mandates temperature and pH monitoring. When compared to SMD I, SMD II proved to be more efficient (increased number of marks detected) and more robust regarding the processing of porous substrates. In regards to these improvements, single-metal deposition (under its SMD II evolution) has reached a point where it could quite easily be implemented as a valid technique for fingermark detection in a standard police laboratory.

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For further information, please contact:

Sébastien Moret
Centre for Forensic Science, University of Technology Sydney
Broadway NSW 2007, Australia
sebastien.moret@uts.edu.au

Andy Bécue
École des Sciences Criminelles, University of Lausanne, Building Batochime
CH-1015 Lausanne, Switzerland
andy.becue@unil.ch

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Appendix

SMD II Protocol

Reagents

Solution A: 10% (w/v) tetrachloroauric acid trihydrate in RO/DI water.

This solution must be kept refrigerated and is stable for several months.

Solution B: 2% (w/v) trisodium citrate dihydrate in RO/DI water.

This solution is stable at room temperature for several months.

Solution C: 0.12 g sodium hydroxide and 0.38 g of L-aspartic acid in 25 mL RO/DI water.

L-aspartic acid is difficult to dissolve. The solution must be prepared under intense stirring. This solution is stable at room temperature for several months.

Solution D: 1 M citric acid monohydrate in RO/DI water.

This solution is stable at room temperature for several months.

Solution E: 1 g hydroxylamine hydrochloride in 50 mL RO/DI water.

This solution is stable at room temperature for several months.

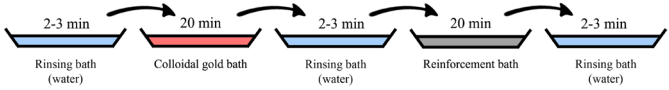
Colloidal Gold Stock Solution

Note: A volume of 2500 mL of gold colloid solution is prepared at once.

1. In an Erlenmeyer flask, mix 1 mL of solution A with 460 mL of RO/DI water and heat to boiling point under constant stirring.

2. In a beaker, mix 42 mL of solution B and 420 µL of solution C.
3. When solution 1 reaches its boiling point, quickly pour solution 2 into it all at once. Keep heating the solution under intense stirring until the solution turns deep ruby red.
4. Dilute with RO/DI water to reach the final volume of 2.5 L and add 2.5 mL of Tween 20 (preferentially using a positive displacement pipette) under stirring. The solution should be ruby red; purple tones at this stage may result from insufficient boiling time at step 3 and could seriously hamper the efficiency of the solution. Colloidal gold stock solution is stable for several months in the fridge, when stored in polypropylene containers.

Application Procedure



Note: Before processing samples, the pH of the colloidal gold working solution must first be adjusted using the recommended amount of citric acid (step 1 - below).

1. Remove from the refrigerator enough colloidal gold stock solution to process all the samples planned for the day (one batch of prepared solution will be stable for the day). Allow it to warm up to room temperature and adjust the pH by adding the proper amount of citric acid, calculated as follows: 3 mL of solution D per 100 mL of colloidal gold stock solution (added under constant stirring).
2. Rinse the sample with deionized water for 2 to 5 minutes (Bath #1).
3. Pour a sufficient amount of colloidal gold solution in a dish so that the liquid height is ca. 1.5 cm. Immerse the sample in the colloidal gold solution for 20 minutes under a gentle orbital shaking (Bath #2).

4. Intensively rinse the sample with deionized water for a few minutes (Bath #3).
5. Just before processing the sample, mix 200 µL of solution A with 200 mL of RO/DI water and 200 µL of solution E, under intense stirring. After a few seconds, transfer the solution to the dish dedicated to the enhancement and proceed immediately to step 6.
6. Immerse the sample for 20 minutes (recommended time) under a moderate orbital shaking speed (~70 rpm). Immersion time can be extended if necessary (Bath #4).
7. Rinse the sample with deionized water for a few minutes (Bath #5).
8. Hang the sample to dry at room temperature.

Remark: Step 4 aims at removing Tween 20 residues and unwanted adsorbed gold colloids from the sample surface. Insufficient rinsing at this step may result in background staining after the enhancement step. Step 4 also constitutes a break in the entire procedure. It is possible to let a sample remain in this rinsing bath until sufficient samples are to be enhanced.