

**Investigation of infinite focus microscopy for the
determination of the association of blood with fingerprints**

FRANCESE, Simona <<http://orcid.org/0000-0002-1381-1262>>

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Investigation of Infinite Focus Microscopy for the determination of the deposition mechanisms of blood within fingerprints

Abstract

The determination of the type of deposition mechanism of blood within fingerprints at the scene of violent crimes is of great importance for the reconstruction of the bloodshed dynamics. However, to date, evaluation still relies on the subjective visual examination of experts. Practitioners encounter three types of scenarios in which blood may be found in fingerprints and they refer to the following three deposition mechanisms: *(i)* blood marks, originating from a bloodied fingertip; *(ii)* marks in blood, originating from a clean fingertip contacting a blood contaminated surface; *(iii)* coincidental deposition mechanisms, originating from a clean fingertip contacting a clean surface, leaving a latent fingerprint, and subsequent contamination with blood.. The authors hypothesised that, due to differences in distribution of blood in the furrows and on the ridges, the height of blood depositions on the ridges and furrows (and their relative proportions), will differ significantly across the three depositions mechanisms. A second hypothesis was made that the differences would be significant and consistent enough to exploit their measurement as a quantitative and objective way to differentiate the deposition mechanisms.

In recent years, infinite focus microscopy (IFM) has been developed, allowing for the computational generation of a 3D image of the topology of a sample via acquisition of images on multiple focal planes. On these bases, it was finally hypothesised that the application of this technique would allow the distinction of deposition mechanisms *(i)* to *(iii)*. A set of preliminary experiments were designed to test whether IFM was "fit for purpose" and, subsequently, to test if any of the three deposition mechanisms scenarios could be differentiated. Though IFM enabled the analysis of tape lifted samples with some success, for samples produced and analysed directly on the surface of deposition, the results show that the measurements from any scenario will be highly dependent on the original surface of deposition (both in terms of its nature and of the variable exposure to environment); as crime scenes exhibit a wide range of possible relevant surfaces of deposition, the technique showed to not have the desired wide appeal for inclusion into a standardised set of protocols within a routine crime scene workflow.

1.1 Introduction

Blood is the type of evidence frequently encountered at the scene of violent crimes. It can be found as a stain or in within fingermarks. Confirmation of the presence of blood within a fingermark can greatly inform investigations and judicial debates by corroborating or disproving a suspect's/defendant's statement regarding their presence at the scene or around the time of bloodshed.

However, still to this day, one challenge concerns the false positive- and false negative-free detection and confirmation of the blood presence. Several blood enhancement techniques (BET) are currently employed [1–4] by crime scene investigators and crime labs. However, these methods are prone to false positives as they lack specificity and are, therefore, called presumptive. Francese's group has previously developed a multi-informative, confirmatory approach for detecting blood and establishing its provenance in stains [5] as well as fingermarks [6,7] by employing Matrix-assisted laser desorption ionisation-Mass Spectrometry (MALDI-MS). Additionally, MALDI-MS imaging has allowed for the detection of multiple blood signatures in stains [5] and for their visualisation on fingermark ridges [6,7]. The enhanced specificity of the method is likely to reduce the rate of false positives/negatives although a quantitative measure of this occurrence is yet to be provided.

Another current challenge is to determine the type of deposition mechanisms by which blood is deposited within a fingermark. Although the literature does not explicitly report on the different mechanisms of deposition [8,9], blood pattern analysts are often confronted with three different types of deposition mechanisms (Dr G. Langenburg personal statement) : *i*) blood marks, which originate from a bloodied fingertip, where blood would, in principle, only be expected on the ridges; *ii*) marks in blood, which originate from a clean fingertip contacting a blood-containing surface, where blood would in principle be expected on ridges and in the valleys of the mark; *iii*) coincidental deposition mechanisms, which originates from a clean fingertip contacting a clean surface, leaving a latent fingermark, and subsequent contamination with blood. In these instances, interaction of the blood and the mark produce a visible blood contaminated mark (also called faux blood mark). In this case, blood might also be expected on ridges and in valleys of the mark.

When blood appears on the ridges of the fingermark and there is lack of blood in the furrows, this is considered the normal “signature” appearance of a blood mark. When blood appears in the furrows, but none appear on the ridges, this is called a “tonal reversal”. Tonal reversals and blood in furrows can complicate interpretation of the evidence. Tonal reversals can be observed in all three of the aforementioned deposition mechanisms.

These three types of deposition mechanisms indicate three forensic scenarios and three different crime scene dynamics, the distinction of which could greatly contribute to the reconstruction of the events around the bloodshed. MALDI-MS imaging could have theoretically enabled the differentiation between type *i*) deposition mechanism and the remaining two scenarios. A first insight into this capability was provided in previous work with regards to the order of deposition of fingerprints associated with condom lubricants [9], where it was possible to distinguish a mark left by a contaminated fingertip (type *i*) deposition mechanism) from a mark left by a clean finger on a contaminated surface (type *ii*) deposition mechanism). However, due to the nature of deposition mechanisms types *ii*) and *iii*), a two-dimensional MALDI-MS image does not allow for their differentiation, as the contaminant is expected to be present throughout the entire image in both cases.

To date, the type of deposition mechanisms is determined by the subjective visual evaluation of experts in the field including blood pattern analysts. The subjective nature of the examination may naturally lead to incorrect conclusions (and therefore to incorrect interpretation of the dynamics of the bloodshed), especially around marks that visually present the same characteristics, despite stemming from different types of deposition mechanisms. For instance, Praska and Langenburg [11] reported that type *iii*) latent fingerprints subsequently exposed to blood and treated with blood enhancement techniques could appear as genuine type *i*) blood marks, with their differentiation ranging from difficult to impossible. They also reported unpredictable interactions between the latent mark and blood and that blood dilutions can generate both faux blood marks and tonal reversals, depending on the age of the mark, angle and duration of contact with blood [11]. Furthermore it has been reported that the appearance and clarity or tonal reversal of ridge detail is affected by other conditions of deposition such as blood volume, drying time and pressure, as well as environmental factors including humidity, air flow and temperature of blood, body and air [12]. Additionally, the observation has been reported that, contrary to popular belief, tonal reversal is not produced by excessive pressure of deposition, but rather by longer drying time of blood on the fingertip prior to deposition; the previously accepted mechanism for tonal reversal creation in blood was that a bloody fingertip pushing blood on a surface would displace blood from the ridges into the furrows. With greater pressure, the effect was more likely to occur. This mechanism was shown to be incorrect [12].

All of these observations indicate an extremely challenging scenario for both visual inspection from experts and certainly for MALDI-MSI capabilities. Therefore an alternative different approach that is analytical and objective must be identified.

The use of various different microscopy techniques in forensic analysis has been extensively reviewed [13], outlining their applications in the identification of toolmarks, fracture patterns, fibres and ballistic evidence as well as biological specimens such as hair, pollen and insects, to name a few. Despite the array of techniques and set ups, most microscopes only generate two-dimensional data. They have to be focused on one feature of the sample, thereby not allowing for height or topology features to be measured without rotating or re-locating the sample, which often is impossible or impractical.

In recent years, with the advent of focus variation, newer techniques such as confocal microscopy and infinite focus microscopy (IFM, also referred to as focus variation microscopy or FVM) have been developed, allowing for the computational generation of a 3D image of the topology of a sample via acquisition of images on multiple focal planes. While confocal microscopy is based on transmitted light and routinely used for biological samples, IFM is based on reflected light. Background information about its development and principle of operation are described in the literature [14,15]; in brief, the software generates an optical image with a large depth of field by stacking images of each focal plane and producing an in-focus image based on the coordinate points that are best focussed. This then allows for the representation of the topology of a sample. As such, IFM allows for the combination of rapid, non-contact microscopic images with 3D topology data of complex geometric samples presenting an angle of up to 85° for highly reflective surfaces and high surface roughness. This can be achieved with a vertical and lateral resolution of down to 10 nm and 400 nm, respectively. Additionally, several analytical tools are available within the software, allowing for various measurements to be performed on the sample, ranging from profiles, heights and volumes to statistical surface parameters.

As the sample stage is considerably larger than a usual microscope stage, it can hold items weighing up to 20 kg [14].

IFM is frequently used in quality control and analysis of wear in material engineering and production processes, e.g. measuring metal parts and corrosion [14,16]. In addition, it has been applied to characterise biological samples that are not amenable to confocal microscopy due to their opacity, such as teeth and bones. This technology has been used in a medical context [15,17,18], as well as in the analysis of archaeological and anthropological samples [19–24] and even in the comparison of toolmarks in a forensic context [25–26].

Following this promising trend, the application of IFM to determine the type of deposition mechanisms of blood and fingerprints based on blood/ridges heights and IFM's topological capabilities, was deemed to be a reasonable research hypothesis in the present paper,

assuming validity of the hypothesis that ridge height differences consistently exist among the three deposition mechanisms. Specifically, the authors hypothesised that, through 3D measurements of representative samples, *significant* differences in ridge and valley heights may be observed. As an example of the rationale that followed, it was theorised that in scenario *iii*), blood covering a latent mark would fill the valleys and cover the ridges, presenting at large a relatively flat surface with ridge heights becoming undetectable. As opposed to scenario *iii*), in scenario *ii*), a mark deposited in blood might present significantly detectable ridge heights in comparison with the valleys. Furthermore, ridge heights observed in scenario *i*) marks might be different from *ii*) and *iii*) marks as there should be no blood present in the valleys. If the "ridge height determination concept" was known to be an effective method of method differentiation, this would allow for more facile and quantifiable method of assessing the deposition mechanism of blood marks and reduce the likelihood of erroneous or conflicting conclusions due to subjective analysis, especially of particularly challenging evidence. To date, no one has explored this approach to differentiation.

This work illustrates a range of measurements to test the overall hypothesis that IFM can distinguish between the three different types of blood-fingerprint deposition mechanisms. In order to establish the validity of any subsequent measurements on marks associated with blood, the error of measurement was determined by measuring the same feature on a stable, non-biological sample, a penny coin, five times a day for five days. Following this study, suitability of a range of surfaces and lifting tapes, allowing the generation of IFM images and subsequent ridge height measurements, was tested. Of particular interest was the surface roughness which could have interfered with height measurements. Suitability of IFM was also challenged through analysis of aging blood marks in a time course experiment over the course of 33 days. The composition of fingerprints is known to start changing immediately after deposition [27] and it has recently been reported that fingerprints migrate on non-porous surfaces, with the extent of migration depending on the surface [28]; these two occurrences may affect the ridge height. The time course aimed to test IFM robustness in meaningfully measuring ridge heights in the aging samples. Finally, a range of blood marks encompassing the three types of deposition mechanisms was analysed to attempt discrimination between deposition mechanisms *i*), *ii*) and *iii*).

However within this last experiment, height measurements differed drastically from the previous sample sets and largely fell within the standard error of measurement. It was determined that this was likely due to the surface properties of the substrate, such as the

wettability, which affects the spreading of blood and therefore the ridge height of any given sample.

Considering the variability of this type of evidence in real forensic cases with regards to the different surfaces of deposition, the range of forensically viable samples amenable to this analytical approach appears to be limited. Taking into account the large effect that the various surfaces of deposition may have on the ability of IFM to quantifiably differentiate between deposition scenarios, despite a reasonable hypothesis with respect to IFM capabilities in context, the technique was deemed to be unsuitable for the purpose intended by this study.

2.1 Material and Methods

2.1.1 Materials

CSI pre-cut lifting tape - fingerprint (#96113), Permacel J-Lar® Clear to the Core lifting tape 25mm (#96105), CSI Flexi tape (3M Polytape 1-1410; #96104) and CSI specialist tape - fingerprint (#96160) were purchased from CSI Equipment Ltd (Woburn Sands, UK). Cellulose Clear Tape ref. 3M 607 (3M Pressure Sensitive tape; #C32810), Sirchie fingerprint lifting clear tape (#S144L), Sirchie Search Polythene Lifting Tape Transparent (#S169PPA) and Serilux Style lifter (#B20653-100) were obtained from WA Products (Burnham on Crouch, UK). Clear, black and white gelatine lifters were supplied by BDA via WA Products Ltd (Burnham on Crouch, UK).

2.1.2 Methods

2.1.2.1 Determination of error of measurement - In order to establish the robustness of IFM measurements, a method was devised to determine the standard error of the instrument. In particular, a sample was selected with discernible features that the researchers were confident would not change during the duration of the measurement. To this end, a prominent feature, in this instance the profile of the Queen's nose, was measured on a one pence coin five times a day for five consecutive days and at different orientations of the coin. Unique features within the sample, such as dents or grooves, were used to reproducibly position the measurement line, in this case across the Queen's nose from one dent on the coin to another.

2.1.2.2 Sample preparation

2.1.2.2.1 IFM "fit for purpose" tests - Initially, blood marks were deposited directly on aluminium slides (prepared as previously described [29]) and glass slides; various lifting tapes and gel lifters were also trialled to reflect instances where it may be necessary to lift blood marks (for example from large or curved surfaces) in order to enable the acquisition of IFM images.

Blood fingermarks were prepared by pricking a clean fingertip with a lancet and rubbing it against another clean fingertip to achieve an even blood distribution. This second fingertip was then used to deposit blood marks onto the substrate and reloaded with blood for each further deposition (primary deposition). Depletion series were produced by loading the fingertip with blood and then 5 consecutive fingermarks were deposited, with no re-loading or replenishing blood in between the five depositions.. The volume of blood deposited could not be measured. This was due to the insurmountable difficulty in obtaining a clear mark depositing a controlled amount directly from the fingertip without it having dried prior to deposition.

In a further set of experiments, a white ceramic tile was cleaned with a window cleaning detergent and subsequently wiped with laboratory disinfectant prior to use as a sample deposition surface.

2.1.2.2.2. Preparation of marks reproducing the three deposition mechanisms. -

Whilst marks prepared as described in 2.1.2.2.1 were used in experiments testing the overall effectiveness of IFM use (evaluation of "fit for purpose"), a subsequent of experiments focussed on the three deposition mechanisms scenarios. For this set of experiments, samples deposited onto a tile were then enhanced using Acid Black 1 (AB1) as described in the Home Office edited *Fingermark Visualisation Manual* [30]. J-Lar® *clear to the core* tape was used to lift AB1-enhanced primary deposition blood marks by carefully positioning the tape over the mark, using sufficient force to ensure good contact, minimising air bubbles but making sure the mark was not modified or smeared by the pressure applied by the fingertip. For storage and analysis, samples were taped into Petri dishes with the adhesive side facing up and kept at ambient temperature.

Samples consisting of non-enhanced blood marks, marks in blood and marks with blood in coincidental deposition mechanism were prepared directly onto plasticised PVC cards. The chemical nature of the PVC card was confirmed through FTIR-ATR analysis.

Blood marks were prepared as depletion series using either 5 μL (2 consecutive depositions) or 20 μL (4 consecutive depositions) of blood. Blood of the specified volume was loaded onto the finger with a micropipette, and then a micropipette tip was used to quickly spread the blood drop into a thin, uniform layer of blood on the finger.

For the preparation of marks in blood, different volumes between 5-30 μL were spread out into ovals-shaped stains of 2-3 cm length on the PVC cards, and the stains were left to dry 1-2 minutes, depending on blood volume, before the deposition of a clean fingermark in the blood.

Lastly, faux blood marks were created by depositing a latent mark on the PVC cards, air drying for 72 hours and then dropping 15-20 μL of whole blood or diluted blood (50:50 H_2O :blood) on top of it. After 3 minutes of exposure to blood, the PVC card was tilted to drain the bio-fluid in order to allow for ridge detail to become visible, as it would otherwise be obscured by a near-opaque blood stain.

2.2 Data acquisition - All Images were obtained on an Alicona Infinite Focus Microscope (Alicona Imaging GmbH, Grambach, Austria) at a lens magnification of 5x. Brightness, contrast and image resolution were adjusted as necessary to ensure high quality images. Ridge height was analysed via profile form measurements of the acquired data using the instrument's software InfiniteFocus® (IFM Version 3.5.1.5).

For time course experiments, unique features within the sample were used to align them against the field of vision and for placement of the measurement line in order to ensure the same area of the sample was measured each time.

2.2.1 Statistical Analysis - Excel (version 14.0.7188.5002) and Prism (version 7.03) were used for calculation of the standard error and Dixon's Q outlier test ($Q = \frac{|\text{suspect-nearest}|}{[\text{largest-smallest}]}$), plotting of trend lines and generation of graphs of time course data.

2.2.2 Contact angle measurements - Contact angle measurements were performed using a Data Physics SCA202 Contact angle instrument. Data Physics OCA20 software was used to measure contact angle from the captured images using Ellipse Fitting as computation method. The collection parameters were as follows: Dosing liquid, volume and rate were 18M Ω , 0.1 μ L (water) and 0.5 μ L/s respectively. Contact angle measurements were made in duplicate, at randomly selected regions on each substrate.

3.0 Results and Discussion

A sample, stable over time with discernible features, in the form of a one pence coin, was used to establish the reproducibility of IFM measurements (through determination of the standard deviation) and evaluate any variation between measurements. Using this sample, it was anticipated that any differences in measured step height could be attributed solely to measurement variation. The findings were then used to inform the analysis of biological samples as to whether changes observed could be attributed to measurement variation or truly represented a change in the sample.

Five IFM images of the Queen's nose on the same one pence coin were acquired per day on five consecutive days, using the same instrument settings (resolution, z range, contrast, brightness), but using different orientations of the coin as shown in Fig 1.

Characteristic features surrounding the nose were identified and used as markers to set the measurement line (step height profile), which was placed as reproducibly as possible using the distance between characteristic dents for each measurement. Five measurements were obtained in order to determine if minor differences in the placement of the measurement line had an effect on the step height profile observed.

Some variations in the step height measurements obtained from 5 replicates, grouped by acquisition day, can be observed between multiple measurements of the same image (Fig 2). However, the majority of sample measurements appear to cluster well, indicating that minor differences in the placement of the measurement line do not have a drastic effect on the step height determination. This is also evidenced by 16 out of 25 sample images exhibiting a relative standard deviation (RSD) <1% across all five measurements, six with an RSD <2% and only three images showing an RSD between 2% and 2.8%, two of which can be corrected to <1% when performing the Dixon's Q outlier test and rejecting one measurement each accordingly.

The overall mean of the 123 measurements obtained (25 acquisitions with 5 measurements each, two measurements rejected) was calculated to be 103.7 μm with a minimum of 87.4 μm , a maximum of 111.6 μm and a standard deviation (σ) of 4.6. Based on this, the coefficient of variation was calculated to be 4.6% ($\sigma/\text{mean} \times 100$), meaning that there can be $\pm 4.6\%$ error of measurement in each measurement obtained.

As a range of sample surfaces can be expected to be present within crime scene scenarios, some consideration had to be given to the selection of such surfaces for this study as no standards protocols are available for the selection of representative sample surfaces. In general, enhancement techniques for fingerprints and blood are applied as suitable for porous, semi-porous or non-porous surfaces. It can be hypothesised that these surface properties also affect fingerprint ridge heights, e.g. by "absorbing" some of the deposit or affecting the deposit heights due to an uneven surface. Similarly, it can be expected that some substrates, such as smooth, non-porous surfaces, will be more suitable for this study than others. Uneven or porous surfaces can be expected to affect and distort ridge heights to a degree where the background surface roughness interferes with the target measurements. In contrast, it can be hypothesised that wet blood or fingerprint residue would spread further on smooth surfaces before it dries than it would on uneven or porous surfaces, resulting in smaller observable ridge heights. In addition, forensically relevant surfaces were selected with regards to the feasibility of their removal from the crime scene for analysis, or to the possibility to lift the fingerprints from them; the choice of surfaces also had to account for the IFM's stage size, operating mechanism and the preliminary application of BET likely to be carried out at the crime scene.

For these reasons, the suitability of a selection of different analysis surfaces was established in preliminary studies. Aluminium sheets have been selected as an example of ideal non-porous (but not completely smooth surface) for being flat and thin. Glass slides were also selected as representing smooth and non-porous surfaces. Additionally, various lifting tapes and gel lifters were selected to account for the fact that on occasion and when possible, the evidence may need to be lifted for further and remote analysis. The investigation of lifted marks could in fact be beneficial in those cases where photography of enhanced blood marks is not sufficient and lifting is required in order to facilitate removal of the mark for laboratory analysis. The selected surfaces were investigated with regards to their surface roughness, interactions with blood and the ability to yield good quality IFM images without considerable loss of data.

Unfortunately, as IFM image acquisition is based on the reflectance of light, glass slides and

gel lifters were found to be unsuitable because, although fingermark ridges were visible, no data could be acquired from the surrounding surface. This resulted in black regions in the images acquired representing loss of data. This occurrence made evaluation of the surface roughness impossible, as the clean surface could not be measured. Aluminium slides yielded suitable images. However, when larger volumes of blood, such as droplets or pools, were used to create marks in blood or coincidentally associated marks, the residue, once dried, did not adhere to the surface and started to flake off. Consequently, they had to be disregarded as a suitable substrate. Examples of IFM acquired images for each surface are shown in Figure 3.

Lifting tape was identified as a viable analysis surface for IFM measurements and several tapes were trialled for the lifting of blood fingermarks. Due to different compositions of the tape backing as well as the adhesives used, details of which were not disclosed in the product information for most tapes, not all lifting tapes provided equal results in lifting blood marks. In fact, a larger number of fingermark lifting tapes did not visibly lift any blood mark residue; additionally, due to the faint nature of some blood marks and more so their lifts, contrast was poor. For this reason and because crime scene marks would most likely be enhanced prior to collection, Acid Black 1 (AB1) enhancement was performed on the blood marks to increase contrast and allow for easier visual inspection of whether lifting was successful. Various tapes were trialled; Sirchie 144L and J-Lar® Clear to the Core tapes, in combination with AB1-enhancement of the blood mark, produced the highest quality lifts with regards to the largest portions or the entirety of the mark being lifted. Other tapes either did not lift at all or only lifted small, partial and incomplete areas of the mark and were therefore found unsuitable. Comparing the success rate and mark portion lifted between Sirchie 144L and J-Lar® Clear to the Core, J-Lar® was shown to be the most promising tape amongst those tested and hence used in a time course study to evaluate potential changes in ridge heights of the blood marks over time.

Ten primary deposition type *i*) blood fingermarks (hereafter M1-M10) were enhanced with AB1 and lifted with J-Lar® tape. Additionally, 2 depletion series of 5 marks each (M11-M20) were enhanced and lifted in the same manner. IFM measurements were acquired from the same area of a mark on the day of deposition (day 0), 5 days later and then at 7-day intervals up to 33 days in order to determine if potential changes in ridge height over time might affect discrimination between different deposition scenarios. It was noted that some marks did not produce images of sufficient quality on some days (and therefore those images had to be excluded from the dataset) and not all deposited

impressions could be lifted successfully. Characteristic features in each mark, such as ending ridges or islands, were then used as reference points to place a measurement line in the image. These reference points were used to determine the ridge height of the sample post-acquisition. Using the protocol described in the Methods section, five measurements were obtained per image. At each time point, an image of the same region of the mark was collected (aligned to the field of view using characteristic features in each mark) and the same reference points used for placement of the measurement line. Figure 4 shows time course plots for each fingerprint including the 5 repeat measurements for each acquisition. As observed previously in the coin sample, multiple measurements on the same image exhibit low amounts of scattering, and for the majority of samples measurements appear consistent. It was however observed that the measurements fluctuated between days without a clear trend amongst all samples, therefore the Dixon's Q test ($Q = \frac{|\text{suspect-nearest}|}{|\text{largest-smallest}|}$) for outliers was performed on the mean ridge height ($n=5$) of the most suspect samples (M1, 2, 3, 5 and 7), assuming normal distribution of the data. From this analysis, day 33 of mark 3 represented an outlier that was rejected ($Q = 0.711$ for a sample size of the 6 time points obtained, with $Q \geq 0.621$ critical for $P=0.05$).

In an attempt to detect potential trends in the data, lines of best fit were calculated and plotted for the time course data. Comparing the panels in Figure 4 A-C, however, it was evident that different trends for different marks could be observed. Marks 1, 7, 8, 9, 10 and 12 appear to exhibit a decrease in ridge height, possibly suggesting evaporation of water, marks 3, 4 and 5 demonstrate a trend towards increased ridge height, potentially indicating collection of dust/debris, and marks 2, 6 and 13 show little change in ridge height. The decrease in ridge height for marks 7, 8 and 12 fits better to an exponential function as they decrease (Figure 4D), which would support the hypothesis of exponential loss of water over time due to evaporation. It should be noted, however, that all marks had been stored together in the same way and such a scatter of results was therefore unexpected.

Comparison of these data with the previously established standard error of measurement (4.6%) shows that the minimum and maximum ridge height values for each mark across all time points show a much greater variation than 4.6%. This is therefore likely to show an actual change in the sample over time rather than an error in measurement, although a consistent trend with regards to increasing or decreasing ridge height was not observed. It was unclear if the scatter of results was due to the samples or the measurement strategy. Therefore additional experiments were conducted using samples from different types of blood-fingerprint deposition mechanisms; it was hypothesised that measurements of blood

ridges outside the fluctuating 25 μm and 84 μm range might be observed for other scenarios, therefore still permitting their differentiation within the 4.6% RSD measurement error.

Samples of all three deposition scenarios were deposited on PVC plastic cards and step heights measured using the strategies outlined previously. On this surface, across the different deposition types, volumes and drying times, periodically, but unusually, samples exhibited ridge heights of a maximum of 12.4 μm , with the majority ranging around 2-3 μm and a few being as low as 400 nm.

Although the absolute ridge height values are not of primary importance, it is important to note that they did not separate into clear groups according to the different deposition scenarios. Not only did the measurements mostly fall within the range of the instrument's error of measurement of 4.6% (σ), but they also failed to exhibit any obvious differences between deposition scenarios, making their differentiation impossible in this case. It should be noted that for dark samples it was difficult to obtain measurements due to the lack of contrast and reflectivity, and even where acquisition was possible, ridges were not measurable within the blood. The results were further investigated as to why the ridge heights, even of the same deposition scenario, were so much lower on PVC (Figure 5 A-D) than those analysed in previous experiments (Figure 5E) and also why ridge height measurements often did not match up the with observed position of the ridge (i.e. the valley was measured to be higher than the ridge (Figure 5F)).

Clearly, understanding the different surface chemistries and topographies should facilitate the elucidation of the nature of these differences. Therefore, contact angle measurements were performed. Although blood marks were initially deposited and left to dry on a ceramic tile before lifting, the determination of the contact angle indicated a much larger contact angle (128-128.2°) for the lifting tape than the PVC plastic (64-64.4°), meaning that any liquids or deposits exhibit reduced spreading across the surface in the case of the lifting tape, due to the nature of the adhesive coating. Any substance, in this case blood marks, deposited on PVC, on the other hand, could spread out much more before drying, thereby reducing the final ridge height. This finding implies that measurements of any deposition scenario will be highly dependent on the original surface, which cannot be controlled in a forensic case scenario, therefore greatly limiting the forensic applicability and feasibility of the method despite the ability to analyse lifted samples. For this reason, it would appear that the scope and applicability of IFM for the determination of the order of deposition of blood marks is somewhat limited. It may be feasible to outline idealised scenarios where the methodology is

applicable, but it does not have the desired wide appeal for inclusion into a standardised set of protocols within a routine crime scene workflow.

Following on these results, the group has diverted their interest towards MALDI MS Imaging, albeit exploiting instrumentation innovation enabling 3D topographical molecular maps as recently reported by the Spengler's group [31]. Despite the spatial resolution offered by this technology may not presently be suitable for determining the mechanism of deposition of blood and fingermarks, in the authors' opinion, this technological innovation appears to be the most suitable analytical tool to investigate to date.

4.0 Conclusions

This study aimed to determine the feasibility to use IFM for the differentiation of different types of blood deposition mechanisms within fingermarks based on the hypothesis that ridge height differences exist and on their measurement. This knowledge would inform investigations and provide more objective conclusions than those currently relying on the expert visual observation. The scenarios in question include blood marks, marks left in blood and clean, latent marks subsequently contaminated with blood (coincidental deposition mechanism), e.g. large volumes of blood shed at the scene, or contamination of blood during the attempted clean-up of a crime scene.

The IFM instrument's relative standard error of measurement was established to be 4.6% based on measurements of a stable sample coin. The changes in ridge height of a time course experiment conducted on lifted, pre-enhanced marks left by a bloodied finger were observed to have an RSD larger than 4.6% but the findings were inconclusive. Some marks showed increased ridge heights while others exhibited a decrease or reasonably stable ridge heights over time, despite all marks being stored under the same conditions.

The range of ridge height values determined on marks with blood pertaining to each of the three different deposition scenarios was found to be highly dependent on the deposition surface, pre-enhancement or lack thereof and chemistry of the lifting tape. As a range of different surfaces can be expected to be encountered at crime scenes and it is impossible to provide appropriate control measurements for each such surface, it was determined that the forensic applicability of the method would be very limited. Despite a promising hypothesis and the potential of success for some selected surfaces, IFM was therefore deemed unsuitable for the reliable, quantifiable differentiation of the three types of blood-fingermark deposition mechanisms as per initial research hypothesis based on the capabilities of the technique.

Based on results obtained, the authors believe to have confirmed the hypothesis on the existence of ridge height differences. However, the surface of deposition has a major impact on these differences. Confirmation of the hypothesis that the ridge height differences are consistent across the three scenarios (on "ideal" surfaces) and significant enough to enable discrimination, can only be achieved, in the authors' view, when and if an appropriate techniques with a lower measurement RSD can be tested.

5.0 References

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Legends

Figure 1. IFM images of the same coin in different orientations (A and B) with the measurement line placed in the same position, aligned to 2 dents in the coin.

Figure 2. Step height IFM measurements replicates of 5 image acquisitions (each shown by a different shape) per day (colour coded) of the same penny coin in different orientations and their median.

Figure 3. IFM images obtained from A: a blood drop on a latent mark on aluminium (type *iii*) deposition mechanism scenario); B: a blood drop on a latent mark on glass (type *iii*) deposition mechanism scenario); C: an AB1-enhanced bloodied mark lifted with J-Lar® tape (type *i*) deposition mechanism scenario); D: a blood drop on a latent mark on a clear gelatine lifter (type *iii*) deposition mechanism scenario).

Figure 4. IFM time course of AB1-enhanced blood fingerprints (type *i*) deposition mechanism scenario) lifted with J-Lar® tape with A-C: linear regression and D: exponential regression where the latter appeared to fit best. Mark 3, day 33, highlighted in red, is likely an outlier as calculated by Dixon's Q and has therefore been rejected.

Figure 5. IFM images and measurements obtained on a bloodied mark (A; type i) association scenario; 3rd depletion in 20 μ L depletion series), a mark in 10 μ L blood (B, type ii) association scenario), a faux blood mark (C; 15-20 μ L blood, type iii) and a faux blood mark (D; 50-50 dilution, type iii) in comparison to an AB1-enhanced bloodied mark lifted with J-Lar® tape (E, type i) and an example for the measurement mismatch observed in some images, in this case on a mark in blood (F, type ii)), where the green measurement point on the ridge is lower than the red point in the valley. The red line shows the measurement line and the red and green + signs correspond to the measurement points marked on the graph.