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"MALDI-CSI": A proposed method for the tandem detection of human blood and DNA typing from enhanced fingermarks

Abstract

Matrix Assisted Laser Desorption Ionization Mass Spectrometry Profiling and Imaging (MALDI MSP and MALDI MSI), in combination with bottom up proteomics, have proven to successfully detect and map blood-derived peptide signatures in blood fingermarks, with high specificity and compatibility with a number of blood enhancement techniques (BET). In the present study, the application of MALDI MSP and MSI to blood marks has been investigated further. In particular, the MALDI based detection and visualisation of blood has been explored in tandem with DNA typing. This investigation has been undertaken in a scenario simulating blood fingermarks on painted walls. In the present study, two sets of marks were analysed with each set comprising of a depletion series of four marks deposited on a surface treated to simulate painted walls: Set I - developed with Ninhydrin (NIN) and Set II- developed with Acid Black-1 (AB-1). For both sets, the application of MALDI MSP was successful in detecting haem and human specific haemoglobin peptide markers. MALDI MSI also provided molecular images by visualising haem on the ridge pattern enhanced by BET. The feasibility of successful and subsequent DNA profiling from the recovered fingermarks was also assessed for marks that had undergone enzymatic *in situ* digestion and MALDI MSI; it was observed that in 73% of the samples analysed, a DNA profile suitable for comparison was obtained. Based on these results, a possible operational workflow has been proposed incorporating the use of a MALDI MS based approach as a confirmatory test for human blood enabling subsequent DNA typing.

Keywords: MALDI, blood enhancement techniques, fingermarks, STR, DNA typing, crime scene.

Introduction

In violent crimes, such as murder, blood-contaminated fingermarks may be key evidence for crime scene investigators (CSI), as they can provide associative evidence by directly linking the suspect to the crime. These marks, which are commonly found on the walls of crime scenes, may be visible, partially latent or latent, with the latter requiring the application of enhancement techniques to visualise the ridge detail. Ninhydrin (NIN) and Acid Black 1 (AB-1) react with either the amino acids or the proteins in the blood, respectively [1] and are amongst the most commonly used enhancement agents for operational application on surfaces such as painted walls.

A fingermark suspected of being contaminated with blood may provide extra evidential weight if it meets three essential requirements: 1) determination of the presence of human blood in the mark to connect it to the crime; 2) sufficient ridge detail to provide the identity of the suspect by fingermark comparison; 3) full DNA profiling from the blood mark for confirmation of the victim's (most likely) identity.

The forensic determination of blood in marks or stains is generally conducted by using two main presumptive field tests: 1) Kastle-Meyer test to detect haem groups; and 2) Immunological tests, such as the Hexagon OBTI test for the identification of human blood. The former is not specific for human blood and prone to false-positives; the latter is considered more specific but may lead to false-negative results, especially after certain enhancement techniques are applied such as AB-1 and NIN. In addition, it has been reported that the sensitivity of the Hexagon OBTI test decreases with the ageing of the sample and when stored at temperatures above 4ºC [2], which may also affect the result of the test. Additionally, due to the presumptive nature of other tests employed for blood enhancement, a reliable, sensitive, robust and legally admissible method is necessary to confirm the presence of human blood in order for such evidence to be confidently presented in court.

One of the most published techniques for the analysis of latent fingermarks in recent years is Matrix Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry, in both profiling and imaging modes (MALDI MSP and MALDI MSI) [3]. Since 2014, these techniques have been adapted and extended as confirmatory tests for the detection of blood in stains and fingermarks in laboratory settings. Initial work enabled confirmation of blood presence through the detection of haemoglobin and haem [4] at concentrations of 1000 and 250,000,000 times lower than the physiological value, respectively. In one example reported by Francese [5], it was shown that, where NIN failed to enhance blood in the third mark of a depletion series, MALDI MSI yielded an image of the ridge pattern through the visualisation of haem. Compatibility with the prior application of AB-1 and NIN was demonstrated in fresh as well as aged specimens, such as a 9 year old blood palm print [6] and a 37 year old NIN enhanced mark on fabric [5]; there, a bottom up proteomic approach was employed, yielding the detection of multiple blood specific proteins thus increasing the specificity of the method. These proteins were visualised onto the ridge detail of blood marks in both fresh and 4 year old fingermarks [7,8]. Kamanna *et al* [9] adapted the approach to generate MALDI MS images of haemoglobin tryptic peptides discriminating between human blood and blood from a range of Australian mammals. Recent work by Kennedy *et al* [10] demonstrated extended compatibility with blood enhancement techniques (BET) and, in a blind study, confirmed the ability to discriminate blood from other biofluids and its provenance, down to animal species, within the system investigated. Importantly, it was also demonstrated that these MALDI

based methods correctly reported on the presence/absence of blood when the presumptive tests exhibited false positive/negative results. Given MALDI MSP and MSI capabilities as confirmatory tests for the detection of human blood from fingermarks, it is important to fully elucidate its potential use in a comprehensive forensic workflow where BET application is followed by DNA typing for downstream analysis.

Whilst MALDI MSP has shown, in a rare application of this technique, an ability to detect DNA fragments [11,12], it failed to provide full DNA profiles from blood or any other source [11]. In another report, Kamanna *et al* [12] showed that MALDI MS was unsuccessful when applied to blood detection following classic DNA profiling, due to the latter procedure removing trace proteins. Therefore, from an operational perspective, the application of MALDI MS based analyses downstream has been dismissed here and the hypothesis has been investigated that the application of this technique to blood marks does not prevent successful retrieval of DNA for further short tandem repeat (STR) analysis. It is known that short UV light wavelengths induce DNA degradation and should be avoided when subsequent DNA profiling is required [14]. However, for UV MALDI it is not known whether the irradiation at 337 or 355 nm (typical MALDI UV laser wavelengths used) is detrimental to DNA typing.

In this work, the first of its kind, MALDI MSP and MSI were evaluated for their compatibility and effectiveness in a multi-sequence processing of blood fingermark evidence, including DNA recovery and typing. At real crime scenes, the blood and the mark may come from different individuals. In the case of blood marks, a connection of the suspect to the crime is made by matching the mark to a fingerprint of perpetrator and the blood to that of the victim (which is generally present in much larger amounts). In this study the same donor was used to produce the blood and the mark as the sole purpose was to assess whether DNA profiling is possible at all after the application of MALDI MSP and MSI, employed to determine the presence of blood and its human origin. This work has been conducted in a simulation of a "real crime-scene" scenario in which blood marks are present over a simulated painted wall, and have been subjected to a workflow where they have been: 1) enhanced with either NIN or AB-1, 2) analysed by MALDI MSP and/or MSI and 3) finally subjected to DNA extraction, quantification, amplification and profiling.

Methods and Materials

1.0 Materials

Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich (Poole, UK). Acetonitrile was purchased from Fisher Scientific (Loughborough, UK). Trypsin Gold was obtained from Promega (Southampton, UK). RapiGest^{TM} was obtained in 1 mg vials from Waters (Wilmslow, UK). Aluminium plates were purchased from Sigma Aldrich (Israel) and acrylic matte white-wash water-based paint was purchased from Nirlat (Israel). Ninhydrin (NIN), ethyl acetate, glacial acetic acid and ethanol were purchased from Bio-Lab (Israel) and HFE-7100 was purchased from 3M (Israel). 5-sulfosalycilic acid was purchased from Sigma-Aldrich, (Israel) and Acid Black-1 (AB-1) was purchased from Spectrum (Israel). VACUETTE (R) Tubes containing EDTA (K3EDTA) were purchased from Greiner Bio-One and sterile gauze pads 100% cotton were obtained from NISSAN Medical Industries, (Israel).

2.0 Instrumentation and Instrumental Conditions

2.1 MALDI MSP and MSI Analysis

Two MALDI quadrupole time-of-flight (QTOF) systems were employed in this study; the Synapt G2 HDMS mass spectrometer (Waters Corp. Manchester, UK) and the QStar Elite mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). The former is equipped with a Nd:YAG laser set at repetition rate 1 kHz at a wavelength of 355 nm and the latter equipped with an SPOT 10 kHz Nd:YVO4 solid state laser (Elforlight Ltd, Daventry, UK), operating at 1 kHz at a wavelength of 355 nm, with an elliptical spot size of 100 x 150 µm and a pulse duration of 1.5 ns. Prior to any analysis, 0.5 µL of phosphorus red saturated solution in acetonitrile was used as calibrant for each instrument. MALDI QTOF instruments were calibrated in the range *m/z* 600 - 2500 (Synapt G2) and 100 - 1000 (QStar Elite). *MALDI MSP*- when using the Synapt G2 instrument, MALDI MS spectra were acquired in positive sensitivity mode in the range *m/z* 600 - 2500; positive mode was also selected on the QStar Elite although the aquisitionrange was set between *m/z* 100 - 1000; on the QStar Elite, the declustering potential was set at 15 and the focussing potential at 20 a.u, respectively. *MALDI MSI* - When using the MALDI Synapt G2, MALDI MSI data were acquired in positive sensitivity mode at a mass resolution of 10,000 FWHM with ion mobility separation enabled over the calibrated mass range. MALDI MS images were acquired in the *m/z* range 600 - 2500, at 100 x 100 µm spatial resolution, using variable IMS wave velocity, with the initial wave velocity and an end velocity (m/s) set at 900 and 100, respectively. When the MALDI QStar Elite was employed, all images were acquired in the *m/z* range 100-1000, in 'slowest' raster mode at a 100 x 150 µm spatial resolution.

3.0 Data Processing

Mass spectra were viewed both in MassLynx™, (Waters Corp., Manchester, UK) and in mMass, an open source multifunctional mass spectrometry software [17,18], upon conversion of the spectra into .txt files. Peak assignments were performed using an excel macro to aid manual assignments of all the peaks characterised by S/N>3:1. Ion signal annotations in mMass were made using an in-house database consisting of all theoretical *m/z* values for the blood specific peptides for human blood.

4.0 Methods

4.1 Preparation of deposition surface

Aluminium plates were coated with two layers of an acrylic matte white-wash water-based paint with each layer left to dry for at least 24 hrs prior to applying the second coat of paint. The plates thus prepared simulated a "painted wall" and were used to deposit blood fingermark depletion series. For blood marks deposited underneath the paint, a third coat of paint was applied over the blood fingermark (FM) 24 hrs after deposition. The blood fingermarks were generated by the same donor providing the blood and the "fingertip stamp" to produce this evidence.

4.2 Deposition of blood-contaminated fingermarks

Blood was collected from one donor in 4 mL sterile test tubes (BD Vacutainer) containing EDTA to prevent blood-clotting. A sterile medical pad was soaked with blood. Subsequently a fingertip contacted the pad prior to touching the painted surface in a depletion series of 4

fingermarks (FM). The samples were left to dry for over 24 hrs prior to development. Two sets of blood fingermarks were analysed two weeks after preparation: Set I - developed with NIN; Set II- developed with AB-1. In addition, two undeveloped control blood fingermarks were made over the paint.

4.3 Fingermark development

Development by NIN was performed by spraying a solution containing 5 g of NIN in 2 mL of ethyl acetate, 5 mL glacial acetic acid, 45 mL ethanol and 1 L HFE-7100. Development by AB-1 was performed in three stages: 1) spraying the samples with a fixing solution containing 20% aqueous 5-sulfosalycilic acid and waiting 15 min for denaturation to occur; 2) spraying with the development solution comprising of 1 g AB-1, 50 mL glacial acetic acid, 250 mL ethanol and 700 mL water; 3) spraying a washing solution containing 50 mL glacial acetic acid, 250 mL ethanol and 700 mL water to reduce the background staining. The developed blood fingermarks were allowed to dry overnight in a closed dark box at room temperature of 23.5°C and 42% relative humidity and then visualised under white light and photographed using the DCS5 system (Foster & Freeman). The images were saved in a 1:1 scale and 1000 dpi greyscale mode as per the standard requirements for operational work.

4.4 Extraction and enzymatic digestion of enhanced blood fingermark depletions.

Trypsin Gold and RapiGest™ were prepared immediately prior to proteolysis as at 20 µg/mL in 50 mM ammonium bicarbonate, containing 0.1% RapiGest (v/v). RapiGest™ (0.1% v/v in 50 mM ammonium bicarbonate solution) was added to Trypsin Gold (150 µg/mL in 50 mM ammonium bicarbonate solution) for *in situ* proteolysis and subsequent MALDI MSP analysis. For MALDI MSI analyses, 9 layers of the enzyme and surfactant solution were sprayed using the SunCollect automatic sprayer at a flow rate of 2 µL/min for each layer (SunChrom, Germany). The samples were then incubated for 3 hrs at 37ºC and the proteolytic digestion was stopped with the spray-coating of α-CHCA matrix. For *in-solution* digests, Eeach blood fingermark sample was swabbed with 70/30 ACN:H_2O. The swab head was removed using scissors and transferred to a 1.5 mL eppendorf containing 1 mL of 70:30 ACN:H_2O prior to sonication for 10 minutes. Subsequently, 10 µL of the extract was added to 40 µL of 40 mM Ammonium Bicarbonate and to 9 µL of trypsin. The *in-solution* extract was incubated for 1 hr at 37ºC and the proteolytic digestion was stopped with the addition of 2 µL of 5% TFA. Digests were stored at -80ºC until analysis.

4.5 Matrix application

α-CHCA matrix at a concentration of 10 mg/mL in 70:30 ACN:0.5% TFA was deposited by spotting 0.5 µL on top of the sample for MALDI MSP experiments. For MALDI MSI experiments, 5 mg/mL α-CHCA was sprayed post-enzymatic digestion using the SunCollect automatic sprayer, depositing 4 layers at a flow rate of 4 µL/min, at a nitrogen pressure of 3 bar.

4.6 DNA extraction, quantification, amplification and data analysis

DNA extraction from blood fingermarks and the positive control DNA (007) was carried out using the PrepFiler BTA™ Forensic DNA Extraction Kits (Life Technologies, Foster City, CA) [19] according to sample type, as determined by the operational protocols. Quantification was carried out using the Investigator Quantiplex Pro Kit® (QIAGEN®) on a 7500 Real-Time PCR System™ device (Applied Biosystems®, Israel) according to the manufacturer's

protocol. Quantification results were processed in Microsoft Excel. Amplification of extracted and quantitated DNA was performed using the VeriFiler™ Plus PCR Amplification 24 Kit (Applied Biosystems®, Israel) on the GeneAmp® PCR System 9700 cycler (Applied Biosystems®,Israel) [20]. Electrophoresis was performed using Performance Optimized Polymer (POP-4™) on a 3500xL Genetic Analyzer™ (Applied Biosystems®, Israel) Fragment analysis was conducted using GeneMapper@ ID-X software (Applied Biosystems®,Israel).

Results and Discussion

The present study initially assessed MALDI MSP and MSI based approaches for the detection/mapping of blood in marks 1) over a simulated painted wall, enhanced by either NIN or AB-1 and 2) underneath the paint. Subsequently, MALDI MSP and MSI analyses were followed by DNA extraction, quantification and profiling. This further investigation gained insights into whether or not MALDI MSP/MSI interfered with a subsequent DNA STR analysis. Two sample sets were prepared as depletions series of four blood marks (Fig. 1) each as follows: Set I - blood marks over the paint developed with NIN; Set II- blood marks over the paint developed with AB-1 (Fig. 1A). The first blood mark of each depletion series was split into 3 sections to carry out a number of different analyses (Fig 1B); generally, the bottom half was reserved for extraction and MALDI MSP analysis. The extract was either directly analysed in the low mass range to detect haem (MALDI QTOF QStar Elite), or proteolysed and analysed in the peptide mass range (to detect blood specific protein-deriving peptides (MALDI QTOF Synapt G2). The upper left and right quarters were reserved to direct haem imaging (QStar Elite) or digested *in situ* and then imaged in the peptide range (Synapt G2), respectively (Fig 2C). Each 2nd mark of the depletion series was split into two halves and reserved either to haem imaging (QStar Elite) or peptide imaging (Synapt G2); the 3rd and 4th marks of each depletion series were submitted to MALDI MSP only (on both MALDI QTOF instruments).

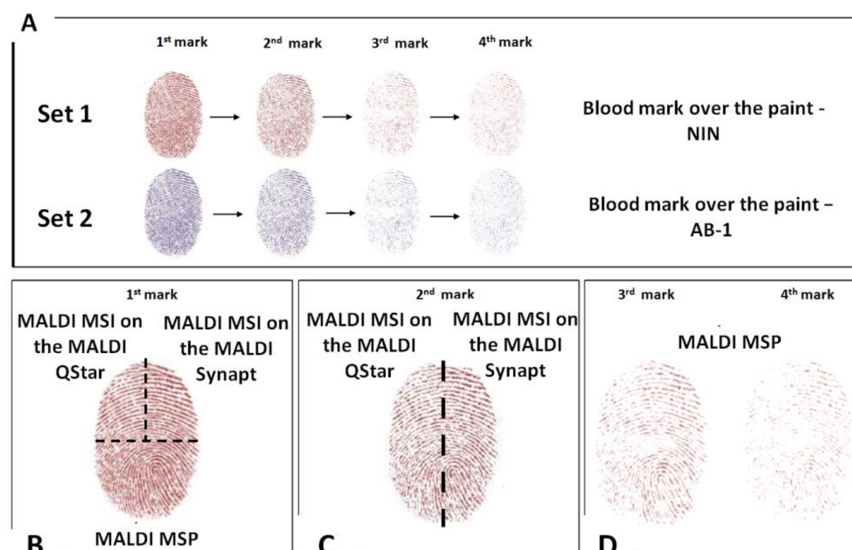


Fig. 1 Preparation of blood marks and analyses performed. (A) Schematic of the blood fingermarks depletion series (sets 1 and 2) prepared for the study. (B) Schematic of the splitting of the 1st blood mark of each depletion series for: MALDI MSP analysis on both

MALDI QStar Elite and Synapt G2 instruments, MALDI MSI of upper left quarter on MALDI QTOF instrument and MALDI MSI of upper right quarter, after enzymatic digestion on MALDI Synapt G2 *Si.* (C) Schematic of the splitting of the 2nd blood mark of each depletion series into two halves for: MALDI MSI on the QStar Elite and Synapt G2 instruments. (D) The 3rd and 4th mark of each depletion series were reserved for MALDI MSP of haem (on the QStar Elite) and blood derived peptides (on the Synapt G2) after enzymatic digestion.

MALDI MSP and MSI of untreated marks - Initial profiling analysis of the paint yielded a densely ion populated spectrum in the *m/z* range 600-2000 exhibiting the expected α-CHCA matrix clusters. However, in addition, a PEG based polymer was also detected (Fig. S1) across the entire mass range acquired, indicating a potential interference or ion suppression of blood molecules of interest, especially peptides, when analysing blood mixed with paint. However, the presence of this polymer was variable across the three replicates of the paint analysed, likely as a result of differential co-crystallisation with the matrix due to varying levels of "polymer segregation".
This is a known phenomenon in MALDI and was reported in previous work on polymeric condom lubricants [21]. It is possible that PEG based polymers could be used as additives and paint preservatives and contribute to the water-based nature of some paints. If that is the case, the detection of PEG was not surprising.
Two untreated (control) blood marks over the paint were analysed by either MALDI MSP or MSI. Prior to the MALDI MSI analysis, the mark was enzymatically digested. The recent study by Kennedy *et al.* [10] has determined that, within the system investigated, if both signals at *m/z* 1274.726 and 1529.734 were present (β and α haemoglobin, respectively) not only can blood presence be claimed but also human provenance can be confirmed. Therefore, particular focus was given to the detection of the two peptides at *m/z* 1274.726 and 1529.734 for detection and confirmation of human blood in enzymatically digested samples.

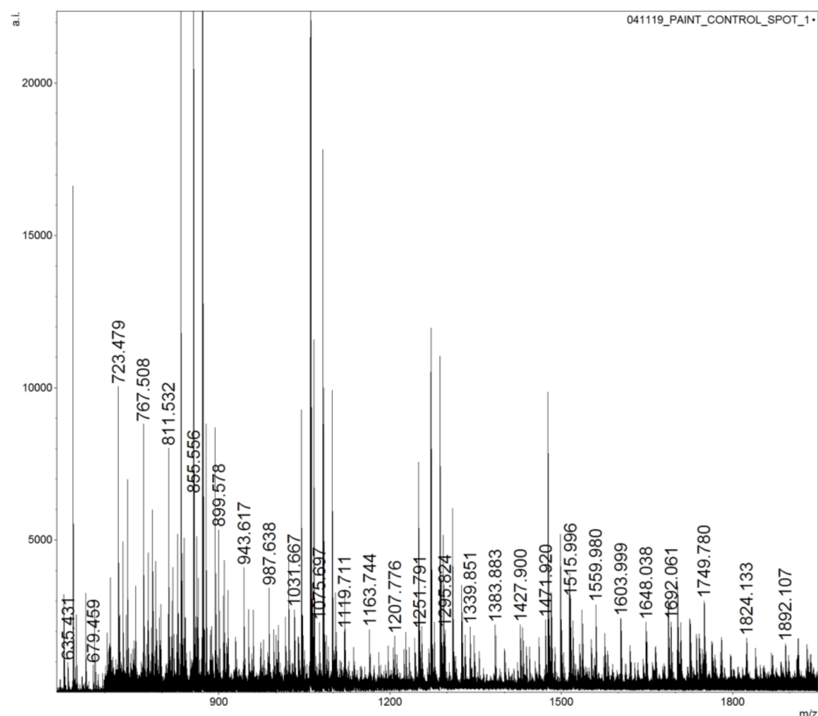


Fig. S1 MALDI MSP spectrum of acrylic paint used to simulate blood marks on walls in this study. The spectrum shows the presence of PEG polymers through ion signals distanced 44 units apart.

In profiling mode and on the Synapt G2, it was possible to detect both haem at *m/z* 616.094 and haemoglobin peptides, including those at *m/z* 1274.736 and *m/z* 1529.727 (Fig. 2A). Another blood mark (half) was subjected to MALDI MSI in the range *m/z* 100 - 1000 and a molecular image of haem at *m/z* 616.2 was obtained and superimposed onto the optical image of the developed blood mark (Fig. 2B). Haem co-localised with the areas where blood presence was revealed by NIN. However, the molecular image of haem, obtained (on the Qstar Elite) by normalising against on ubiquitous signal at *m/z* 855.6 did not permit visualisation of the ridges that were observable in the optical image. An analysis of the matrix deposition showed that the MALDI matrix was not deposited homogeneously across the mark and whilst molecular information is still detectable, images, in this circumstance, are generally compromised.
The non-homogeneity of the matrix could be due to the presence of the PEG based polymer, disrupting crystallisation. Another hypothesis is a potentially undetected discontinuous matrix spray of the automatic sprayer employed.

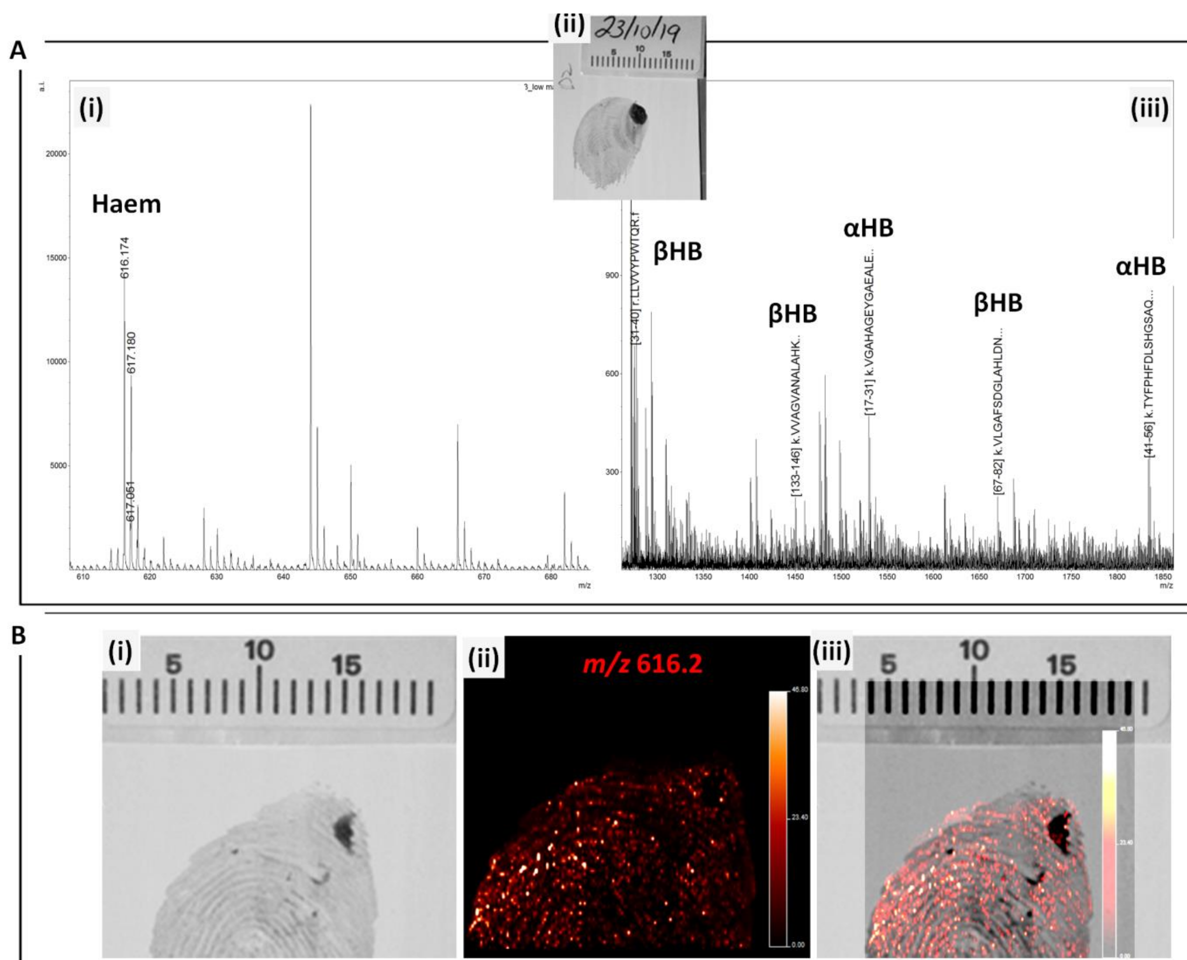


Fig. 2 MALDI MSP and MSI of two control blood marks (undeveloped) over the paint. A: MALDI MS spectra of haem (i) and haemoglobin peptides (iii) from an untreated control

blood mark (iii). B: Images of a second undeveloped blood mark over paint: (i) optical image, (ii) MALDI MS image of haem at *m/z* 616.2 acquired on the MALDI QStar Elite and (iii) the overlay of the MS image of haem with the optical image of the NIN developed blood mark.

MALDI MSP and MSI of enhanced marks over the paint - The application of MALDI MSP aimed to assess whether blood could be detected from enhanced blood marks over paint and, specifically, from which of the marks within the two depletion series investigated. All MALDI MSP analyses were run in triplicates. However, in this paper, only one replicate is shown. MALDI MSI was employed to additionally assess whether blood specific protein deriving-peptides could be visualised on the ridges of the mark, compatibly with the application of either NIN or AB-1, as observed in previous work [5-6,10], as well as "filling in the ridge pattern gaps" left by the enhancement through these presumptive tests.

<u>Set I</u>: *Blood fingermarks over paint enhanced with NIN (FMO-NIN)* - Haem was detected both at nominal *m/z* 616 (expected) and 617 across FMO-NIN-1, FMO-NIN-3 and FMO-NIN-4 marks (Fig. 3). The difference of 1 mass unit is due to the reduction of the haem iron from Fe^{3+} to Fe^{2+} and this has been observed previously [22]. The HB peptides at *m/z* 1071.557, 1529.749, 1833.898 (αHB, 2.8, 9.7 and 3.1 ppm mass accuracy respectively), 1274.729, 1314.669, 1449.794 (βHB, 2.7, -3.3 and -1.5 ppm mass accuracy respectively) and one Complement C3 protein deriving peptide at *m/z* 1930.079 (-1.9 ppm mass accuracy) have been detected in the 1st mark of the NIN depletion series. The 3rd and the 4th marks of the depletion series showed increasing PEG based polymer presence to the extent in which the 3rd mark (FMO-NIN-3) only exhibited 3 HB peptides, just above the S/N 3:1, at *m/z* 1529.730 and 1833.895 (αHB, -3.1 and 1.9 ppm mass accuracy, respectively) and 1274.716 (αHB, -7.3 ppm mass accuracy) and no peptides were detected in the 4th mark (FMO-NIN-4) (Fig. 3).
Overall, MALDI MSP indicated the presence of blood in the three marks of the NIN depletion set examined. For FMO-NIN-4, the presence of blood (through detection of haem) could be established but not its provenance.

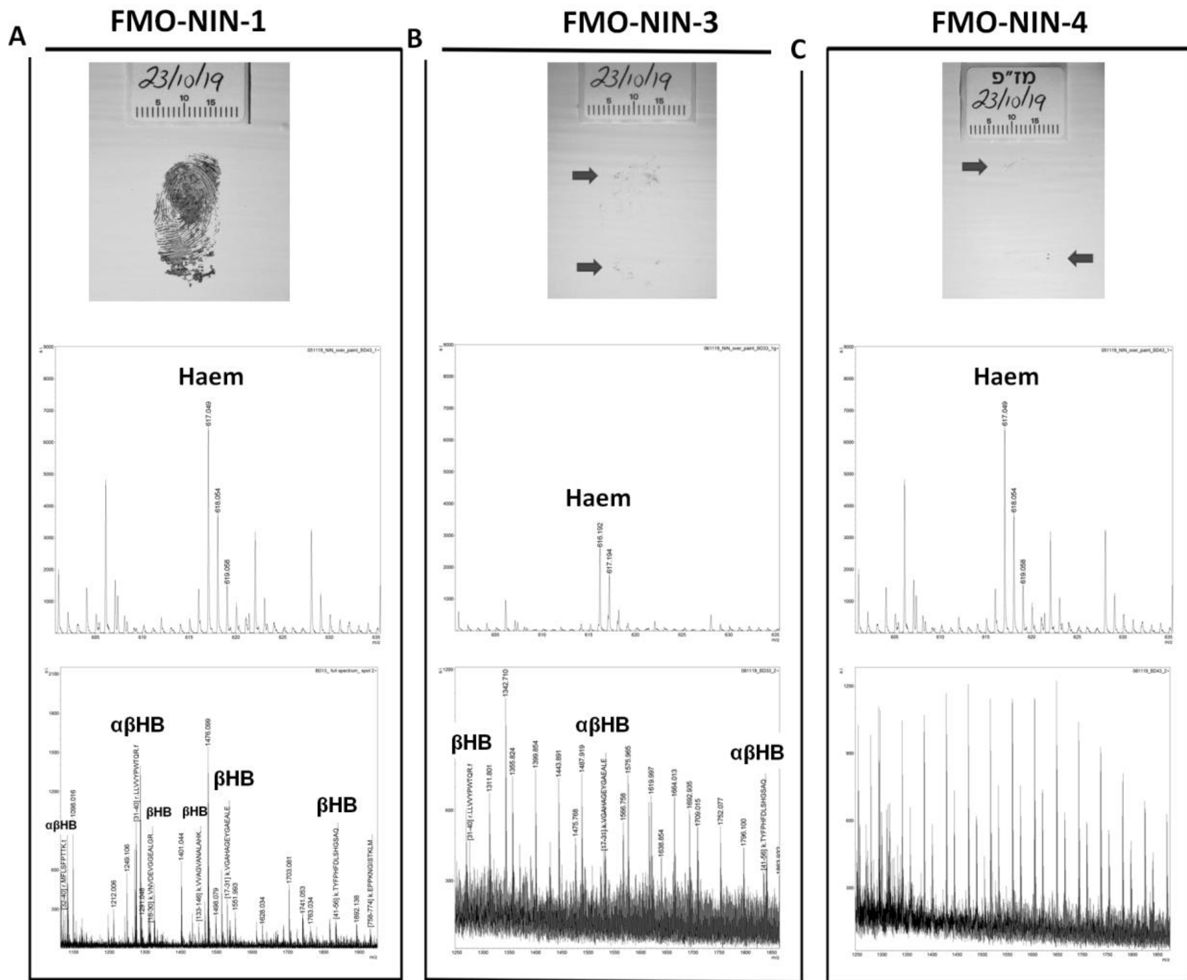


Fig. 3 MALDI MSP of 1st (FMO-NIN-1), 3rd (FMO-NIN-3) and 4th (FMO-NIN-4) mark of the Set I of blood fingermark depletion series deposited over the paint and enhanced with NIN. MALDI MS spectra of haem and HB peptides are shown for each mark.

MALDI MSI was applied to the 1st (FMO-NIN-1) and 2nd mark (FMO-NIN-2) of Set I depletion series. Imaging capabilities were confirmed for NIN enhanced blood marks "over paint" where molecular images of haem at *m/z* 616.2 could be generated albeit with no additional ridge detail (Fig. 4).

The marks were deposited without ensuring clarity of the ridge detail; therefore originally smudged areas could not be resolved by MALDI MSI. The overlay of the haem images with the optical images of the NIN enhanced blood marks shows haem localisation where NIN revealed the presence of blood, thus acting as a confirmatory test. Haem images were generated with and without normalisation against the total ion current (TIC). Normalisation yields a true reflection of the presence/location of the species that is being normalised. However, occasionally, depending on the distribution of the species against which normalisation occurs, this process may compromise clarity of some areas of the ridge pattern. The provision of both normalised and non-normalised images yields complementary ridge flow/details. Haem was confirmed by the presence of its in-source ion fragment at nominal *m/z* 557 which was also previously observed by Bradshaw *et al.* [4].

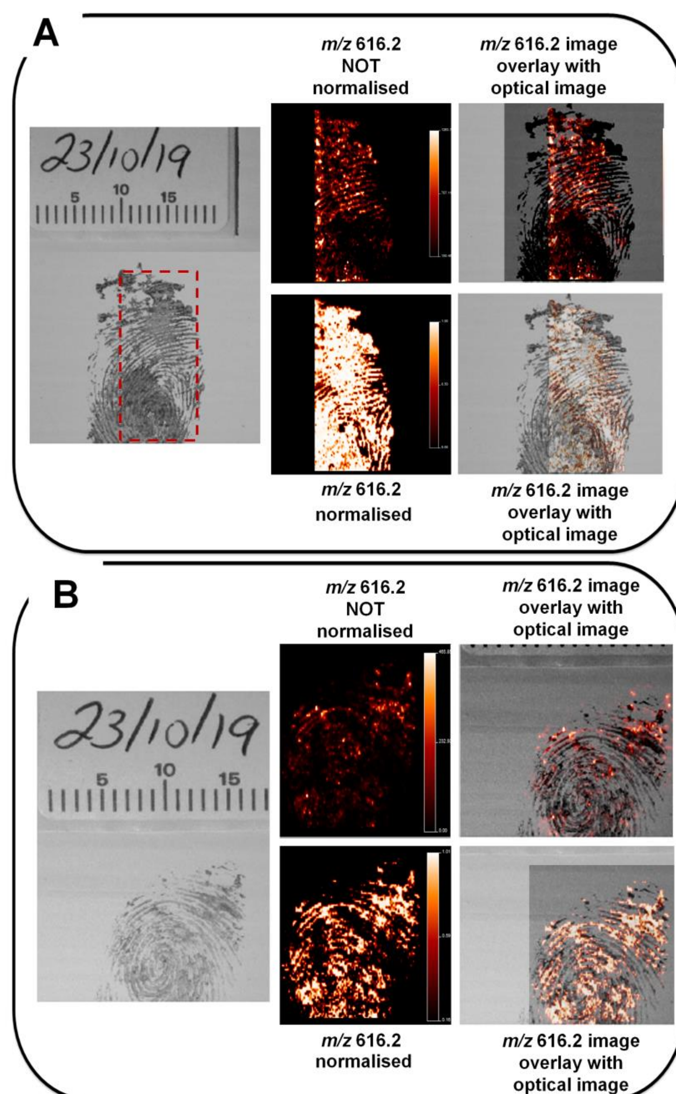


Fig. 4. MALDI MSI of NIN treated depletion series marks (SET I) over the paint. **(A)** 1st mark of the depletion series (FMO-NIN-1), submitted to MALDI MSI. The red dashed frame on the optical images of the NIN developed marks indicate the areas that have been imaged by MALDI MSI. Two molecular images of haem at *m/z* 616.2, both non-normalised and normalised against the Total Ion Current (TIC), are shown followed by the overlay of the MALDI MS image with the optical image. **(B)** 2nd mark of the depletion series (FMO-NIN-2), submitted to MALDI MSI. Two images of haem at *m/z* 616.2, both non-normalised and normalised against the TIC, are shown followed by the overlay of the MALDI MS image and the optical image.

On this occasion, the imaging data, acquired for the other half of the mark that underwent *in situ* enzymatic digestion prior to MALDI MSI on the Synapt G2 instrument, were poor and did not yield relevant blood specific protein-deriving peptides. A sensitivity issue was generally observed, with the instrument only yielding a reasonable molecular image for haem at *m/z* 616.17 (Fig. S2).

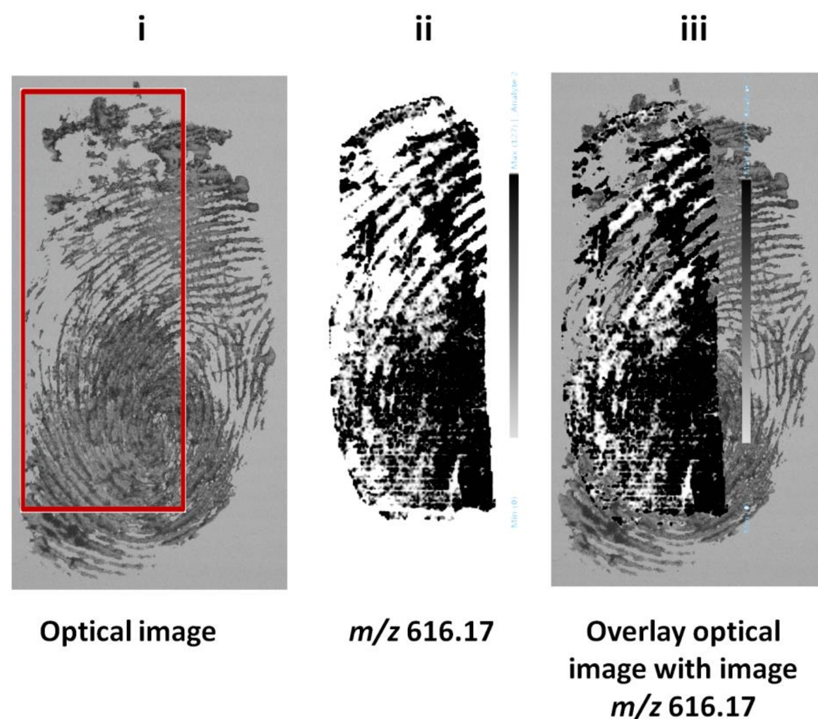


Fig. S2. MALDI MSI of FMO-NIN-1 on the Synapt G2 instrument. (i) Optical image of the mark showing the area imaged by MALDI MSI in the red frame; (ii) Molecular image of haem at *m/z* 616.7 superimposed with the optical image (iii)

<u>*Set II*</u> - Similarly for Set I, within the AB-1 enhanced blood mark depletion series deposited over the paint (FMO-AB-1), MALDI MSP was able to detect haem in all of the three marks of the depletion series, namely the 1st, the 3rd and the 4th mark of the series (FMO-AB1-1, FMO-AB1-3 and FMO-AB1-4). α and β HB peptides at nominal *m/z* 1275 and 1530 were detected in the 1st and 3rd mark for this depletion series (FMO-AB1-1 and FMO-AB1-3), together with additional HB peptides and other blood specific proteins although to a much lesser extent in FMO-AB1-3 (Table S1). The MALDI MS spectrum of the 4th mark of this depletion series (FMO-AB1-4) is heavily dominated by the PEG based polymer (in 2/3 replicates) and only exhibits the putative presence of Complement C3 protein (Table S1).
As for Set I, these MALDI MSP analyses enabled the confirmation of blood presence across the whole depletion series although it was, again, not possible to establish the presence of human blood in the 4th mark of the depletion series.

Protein	FMO-AB1-1		FMO-AB1-3		FMO-AB1-4	
	m/z	Mass accuracy (ppm)	*m/z*	Mass accuracy (ppm)	*m/z*	Mass accuracy (ppm)
αHB (P69905)	1071.555	0.5				
	1087.621	4.6				
			1129.741	4.6		
	1171.741	-8.0				
	1529.741	4.6	1529.741	-9.1		
	1833.899	3.7				
βHB (P68871)	952.508	-1.8				
	1274.835	7.6	1274.74	1.7		
	1314.667	2.0				
	1378.702	1.1				
	1449.805	6.4				
	1797.973	-7.2				
EMBP 4.2 (P16452)	1161.578	8.1				
Complement C3 (P01024)	960.556	9.3				
			1638.858	-1.3	1638.871	6.7
	1690.892	-1.7			1690.905	-2.7

Table S1. Putative blood protein assignments following MALDI MSP of digested bloodmarks on paint pre-enhanced with Acid Black 1. The 1st, 3rd and 4th marks of the depletion series (FMO-AB1-1, FMO-AB1-3, FMO-AB1-4 respectively) were analysed. Protein names are accompanied by Uniprot accessions numbers (https://www.uniprot.org/).

Figure 5 shows the optical images of the three AB-1 enhanced blood marks and representative mass spectra of haem and haemoglobin peptides indicating the presence of blood.

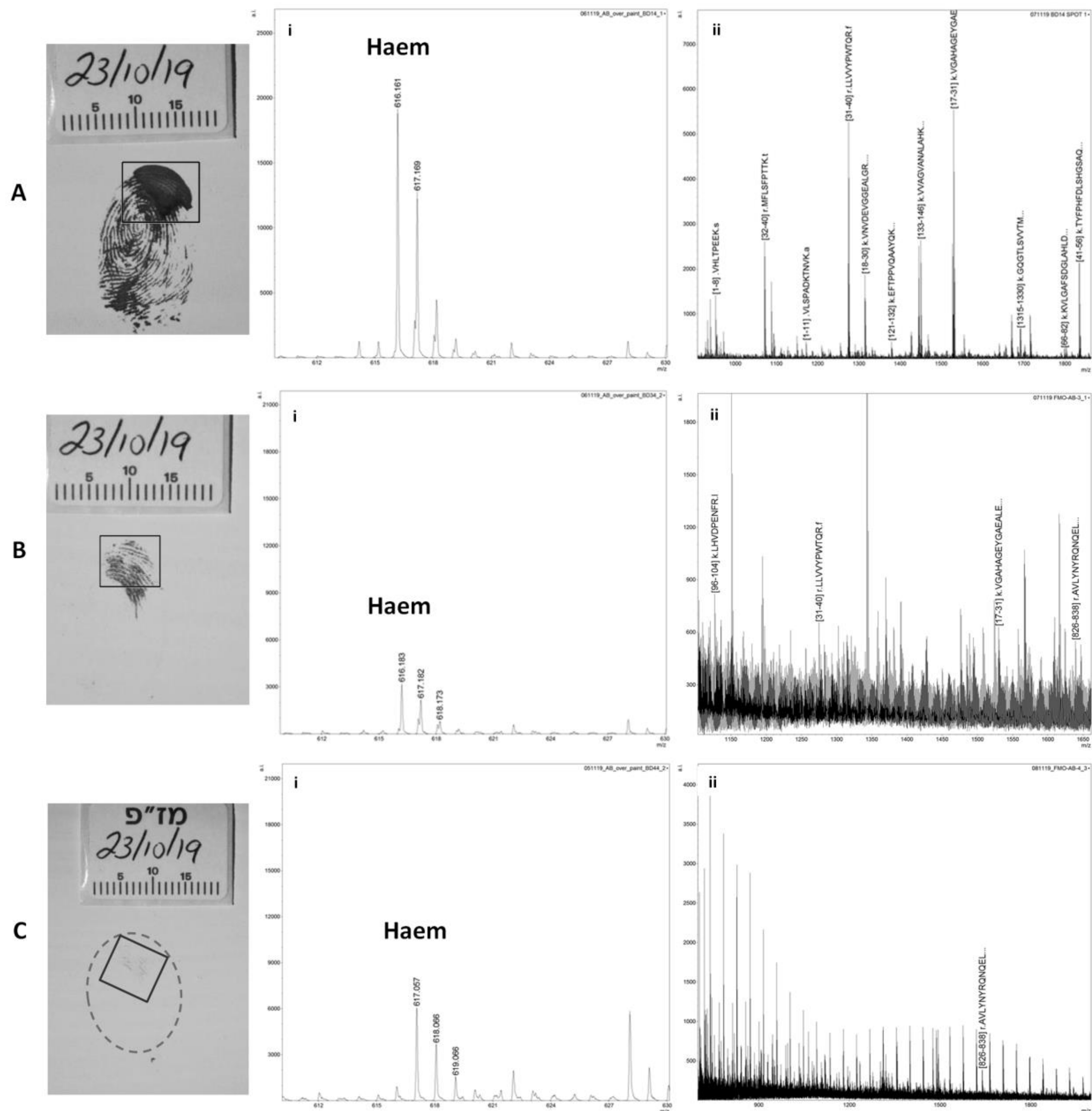


Fig. 5 MALDI MSP of 1st (FMO-AB1-1), 3rd (FMO-AB1-3) and 4th (FMO-AB1-4) marks of Set II (A, B and C respectively) deposited over the paint and enhanced with Acid Black 1 (AB-1). The squares in the optical images indicated the areas submitted to MALDI MSP. MALDI MS spectra of haem (i) and blood protein-deriving peptides (ii) assignments are shown for each mark.

MALDI MSI experiments were subsequently carried out. As for Set I, imaging analyses were performed on the 1st and 2nd mark of the AB-1 depletion series, namely FMO-AB1-1 and FMO-AB1-2 (Fig. 6).

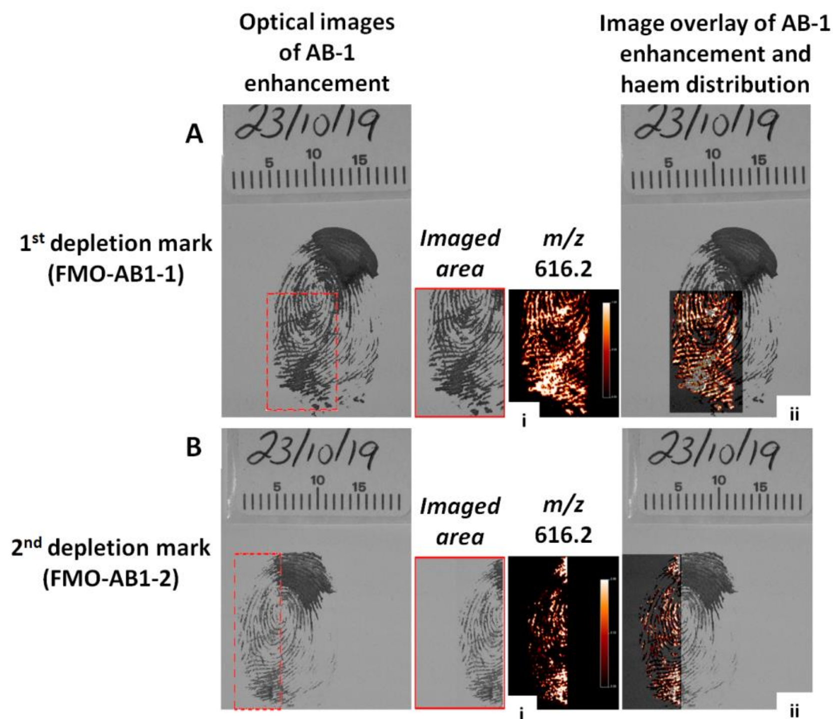


Fig. 6 MALDI MSI (QStar Elite) of AB-1 treated 1st and 2nd blood marks of a depletion series (Set II) over the paint (FMO-AB1-1 and FMO-AB2-2). (A) Optical image of FMO-AB1-1 showing imaged area highlighted with a dashed red frame, accompanied by (i) the MALDI MS image of haem at *m/z* 616.2 and (ii) and subsequent overlay with the optical image and.(B) The optical image of the FMO-AB1-2 mark is accompanied by the MALDI MS image of haem at *m/z* 616.2 (i) and (ii) the overlay between the two images

As it can be seen for FMO-AB1-1, haem was successfully and compatibly mapped on the ridge pattern in the areas where AB-1 presumptively indicated the presence of blood, with the MS analysis confirming the presence of this biofluid. Similarly to Set I, the Synapt G2 instrument underperformed and did not yield suitable images of the blood mark through the relevant blood specific protein-deriving peptides.

Insights into detection of blood under the paint

MALDI MSP analysis was also performed to obtain an initial evaluation of the feasibility of detecting blood after paint had been applied over it. This experiment simulated the scenario in which the perpetrator is attempting to conceal the blood on walls. MALDI MSP spectra show that it was possible to detect blood under three coats of paint through the detection of both haem (*m/z* 616.183, with a mass accuracy of 9.7 ppm) and several haemoglobin peptides (αHB at *m/z* 1071.554, 1529.734 and 1833.892, mass accuracy 1.1 and 0.5 and 1.2 ppm respectively; βHB 1126.564, 1274.726, 1314.665, 1378.700, 1449.789, 1669.876

and 1797.986, mass accuracy, 1.0, -0.5, 1.9, 2.9, 1.8, 1.5 and 7.3 ppm respectively) despite the presence of a PEG based polymer (in 2/3 replicates) (Fig. S3).

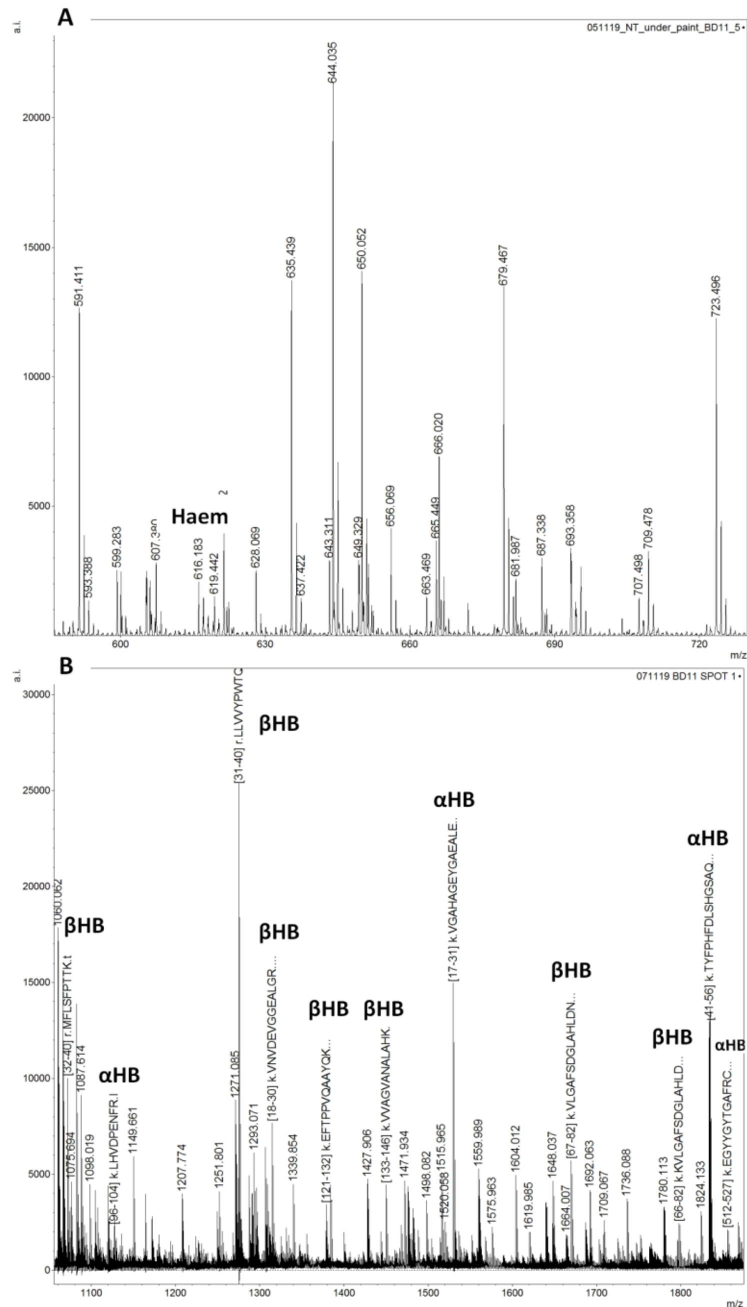


Fig. S3. Synapt G2 MALDI MSP spectra of blood "under the paint". Both haem (A) and haemoglobin peptides (B) could be detected.

PEG based polymers were detected throughout this study except within blanks and other control samples. As PEG-based polymers are known to ionise particularly well in MALDI, the detection of blood despite the PEG polymer was reassuring.

However, in future work, sample purification would be desirable to ensure maximum sensitivity for blood detection in depleted marks.

DNA Profiling from imaged marks

The blood fingermark extracts/in solution digests from Sets I and II that underwent MALDI MSP along with the 1st and 2nd depletion intact marks from the same sets that were imaged, were taken to the Israeli Police Biology & DNA lab for DNA profiling. DNA extraction, quantification and profiling were carried out on these samples. This sequential analysis was important to assess the feasibility of DNA testing *after* MALDI MS based analyses. All original extracted samples (portion of 1st depletion mark, entire 3rd depletion mark and 4th depletion mark in both Sets II and III), showed that the DNA concentration obtained was just below the recommended value required for subsequent PCR amplification (>0.0147 ng/µL) in operational work. This is a recommended DNA concentration for each commercial STR multiplex kit that has been employed in the study. Lower concentrations may still provide DNA profiles, but may result in partial profiles. It is possible that the extensive MALDI analysis performed on the digests has negatively impacted on the amount of DNA available for DNA testing.
DNA amplification was still attempted from the intact marks, even if its concentration was lower than the recommended value, to assess whether a compatible DNA profile for comparison purposes could be achieved. The profiles obtained were compared to the reference blood used to generate blood marks. Indeed, in 73% of these amplifications from the intact fingermark samples that underwent MALDI MSI, comparable profiles originating from the donor were yielded. This result means that a profile of at least 2/3 of the loci tested needs to be achieved, and in the case of the intact samples (FMO-NIN-1, FMO-NIN-2, FMO-AB1-1, FMO-AB1-2) complete profiles were achieved. From the half marks analysed for haem imaging, only FMO-NIN-1 and FMO-AB-1 had a DNA concentration above the recommended value, at 0.35 ng/µL and 0.25 ng/µL respectively. With reference to the half marks analysed for peptide imaging, only FMO-AB-2 had a DNA concentration above the recommended value, at 0.25 ng/µL. The remaining samples did not have DNA concentration above the recommended value but still provided full profiles (Supplementary Table S2).

Instrument	Sample	DNA Concentration (ng/µL)	
		Short	Long
QStar	FMO-D1	0.80	0.58
QStar	FMO-NIN-1	0.35	0.10
QStar	FMO-NIN-2	0.06	0.02
QStar	FMO-AB-1	0.25	0.11
QStar	FMO-AB-2	0.14	0.07
Synapt	FMO-NIN-1	0.12	0.03
Synapt	FMO-NIN-2	0.07	0.01
Synapt	FMO-AB-1	0.09	0.03
Synapt	FMO-AB-2	0.48	0.21

Table S2. DNA extraction quantification following MALDI MS based analyses. Blue rows indicate all the DNA concentrations that are suitable for DNA amplification. These findings are the first of their kind indicating a reasonable compatibility of DNA profiling following the application of MALDI MSI on enhanced blood marks.

The data confirm that exposure for a period between 1-3 hours (QStar Elite) and 10-20 hours (Waters Synapt G2) to the laser wavelength employed (355 nm) enables quantities of DNA that are suitable for further STR testing following MALDI analyses (as opposed to known detrimental exposure of short UV wavelengths commonly employedin some forensic analyses).
However, advanced MALDI instrumentation is capable to image a mark in around ten minutes at the same spatial resolution used here; therefore it is reasonable to speculate that it may be possible to obtain and amplify even higher quantities of DNA.
The DNA profiles were evaluated with respect to peak height and allele balance per locus, and compared to that of the donor, as per accepted analysis methods in the DNA lab within the Israeli Police. The limit of detection (LOD) threshold for minimum peak height of heterozygous alleles was set at 280 RFU, and the stochastic threshold for homozygous allele calls was made at 900 RFU (Internal Validation for VeriFiler™ Plus).
Figure 7 shows the histograms of parts of the recovered DNA profiles from the control (unenhanced) blood marks over paint (Fig. 7a) and blood marks developed with AB-1 imaged using the QStar Elite (Fig. 7b) and Synapt G2 (after proteolysis) (Fig. 7c). There was no difference in the quality of the profiles achieved after AB-1 enhancement.

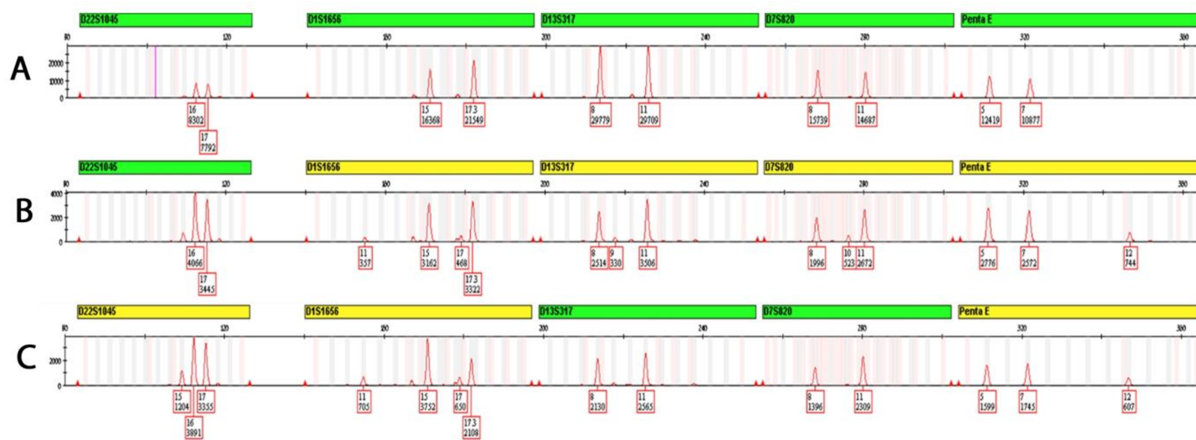


Fig. 7: Part of the DNA profiles recovered from 1st depletions of blood-contaminated fingermarks over paint.
A: no mark development; after ~1 h MSI (QStar Elite) a complete DNA profile of a single origin (23 full STR loci and XX Amel alleles) was retrieved; B: mark development by AB-1; after ~1 h MSI (QStar Elite) a complete mixed-dominant DNA; C: development by AB-1; after MSI acquisitions of up to 30 h (Synapt G2) following *in situ* proteolysis shows a complete mixed-dominant DNA.

The results from DNA recovery and STR amplification of blood fingermarks are summarised in Table 1 and indicate that, whilst a full single source profile was achieved from an undeveloped mark, the developed blood marks provided mixed DNA profiles. However, crucially, comparable profiles were obtained and whether the profile obtained was single source or a mixture is unimportant here as no precautions were taken to operate in a sterile environment. *Set I*, developed using NIN, yielded mixed partial profiles that were not suitable for comparison purposes. Although it is known in the literature that DNA testing can be

Depletion[a]	Development of blood-contaminated fingermarks								
	No development			Ninhydrin			Acid Black-1		
	DNA amount[b]	Number of STR loci[c]	DNA profiles suitable for comparison	DNA amount	Number of STR loci	DNA profiles suitable for comparison	DNA amount	Number of STR loci	DNA profiles suitable for comparison
1-Q	+	24	+	+	24	+	+	24	+
1-S	N/A	N/A	N/A	-	24	+	+	24	+
2-Q	N/A	N/A	N/A	-	21	+	-	24	+
2-S	N/A	N/A	N/A	-	20	+	+	24	+

Table 1: DNA recovery and STR amplification from the fingermarks after MALDI-MSI analysis. [a] 1-Q refers to 1st depletion imaged on QStar Elite, 2-S refers to 2nd depletion imaged on Synapt G2. [b] **+** indicates the DNA quantity found was above the lowest range required for PCR amplification (minimum of 0.25 ng/µL), **-** indicates the quantity below lowest range required for PCR amplification (less than 0.25 ng/µL). [c] The number of STR loci refers to the number of STR loci amplified.

carried out following NIN treatment [23], the Israeli Police reports of previous casework in which NIN has occasionally interfered with subsequent DNA profiling (Dr. Ravell Bengiat *personal communication*) thus reinforcing the hypothesis that it was this enhancement technique and not the MALDI MS based approach that prevented a DNA match with the reference profile. The DNA profiles obtained from the AB-1 samples (Set II) exhibited a dominant profile originating from the donor from both a simple extract and in the proteolytic digest following the extraction. Based on the results obtained, the Israeli Police has suggested a possible simplification to their current sequential processing protocol used when marks are suspected to be contaminated with blood (Fig. S4).

In this suggested alternative workflow: (i) the routinely used non-destructive process is applied first; (i) NIN or AB-1 are used if there is a positive indication for the presence of blood; (iii) if ridge detail is required, the sample is partially scraped/swapped, split and submitted to DNA profiling (victim/perpetrator blood match) and MALDI MSI; (iv) if ridge detail is not needed, sample is entirely scraped/swabbed, split and separately submitted to DNA profiling (victim/perpetrator blood match) and MALDI MSP of haem; (iv) if haem is present, full proteomic analysis by MALDI MSP is undertaken as a confirmatory test of human blood. Further studies including the investigation of sterile conditions and use of faster imaging mass spectrometers might lead to revision of this suggested protocol and implementation of MALDI prior to DNA testing where necessary.

The integration of MALDI MSP/MSI removes extensive cross examination during court proceedings with respect to the validity of presumptive tests as they would be followed by confirmatory mass spectrometric analyses whilst still allowing DNA testing.

Conclusions

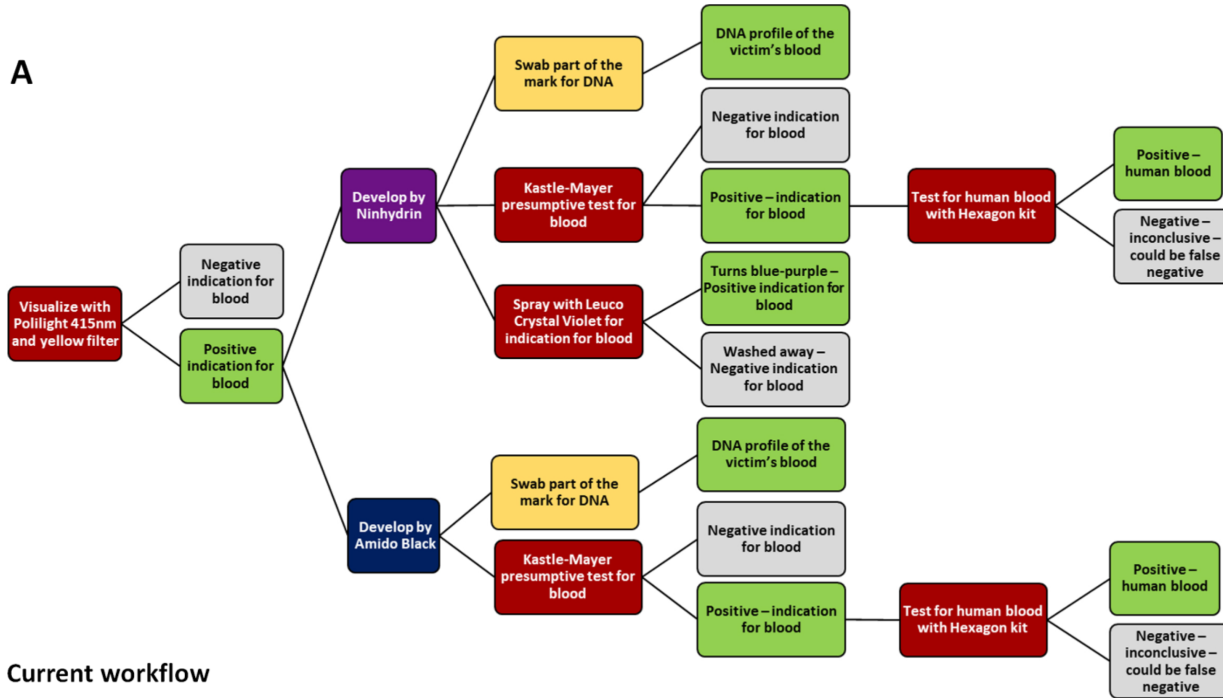
MALDI MS Profiling and Imaging have confirmed their capability to detect and image blood in fingermarks after the prior application of Ninhydrin (NIN) or Acid Black 1 (AB-1), this time, on a surface simulating a painted wall. The data demonstrate that the common acrylic paint employed to simulate blood marks on walls(despite containing PEG based polymer) does not hinder the detection of blood. However, as these conclusions are specific to the type of paint used, further work should include investigation of other types of paint.

Blood marks were examined in depletion series and this blood was reliably detected in all the 1st, 3rd and 4th marks of the depletion series "over the paint".

Additionally, for the first time, it was demonstrated that DNA recovery and profiling are possible after the application of MALDI MSI following both AB-1 and NIN development. While DNA profiles were obtained after the application of both AB-1 and NIN development, the data suggest that AB-1 is less destructive to DNA than NIN enhanced marks in which only partial profiles, not suitable for comparison purposes were obtained.

These results are very promising as they show that the short UV wavelengths of the MALDI laser, which until now were assumed to be detrimental to DNA, may still allow a successful recovery and profiling of DNA. Moreover, should MALDI MSP and MSI be recommended for operational work in Biology laboratories, protocols using sterile conditions should be adopted. However, whilst it will be possible to prepare samples for MALDI MS based analyses in sterile conditions, if contamination occurs inside the mass spectrometer, it will not be not possible to remove it.

On the basis of the results obtained, the authors suggest an alternative forensic workflow for suspected blood marks on painted walls, integrating for the first time the use of DNA profiling and MALDI MSP/MSI. Initial results also indicated the possibility to detect blood in marks under the paint (with reference to concealment attempts) and these promising results will be investigated further in future studies.


A
Visualize with Polilight 415nm and yellow filter
Negative indication for blood
Positive indication for blood
Develop by Ninhydrin
Swab part of the mark for DNA
DNA profile of the victim's blood
Kastle-Mayer presumptive test for blood
Negative indication for blood
Positive – indication for blood
Test for human blood with Hexagon kit
Positive – human blood
Negative – inconclusive – could be false negative
Spray with Leuco Crystal Violet for indication for blood
Turns blue-purple – Positive indication for blood
Washed away – Negative indication for blood
Develop by Amido Black
Swab part of the mark for DNA
DNA profile of the victim's blood
Kastle-Mayer presumptive test for blood
Negative indication for blood
Positive – indication for blood
Test for human blood with Hexagon kit
Positive – human blood
Negative – inconclusive – could be false negative
Current workflow

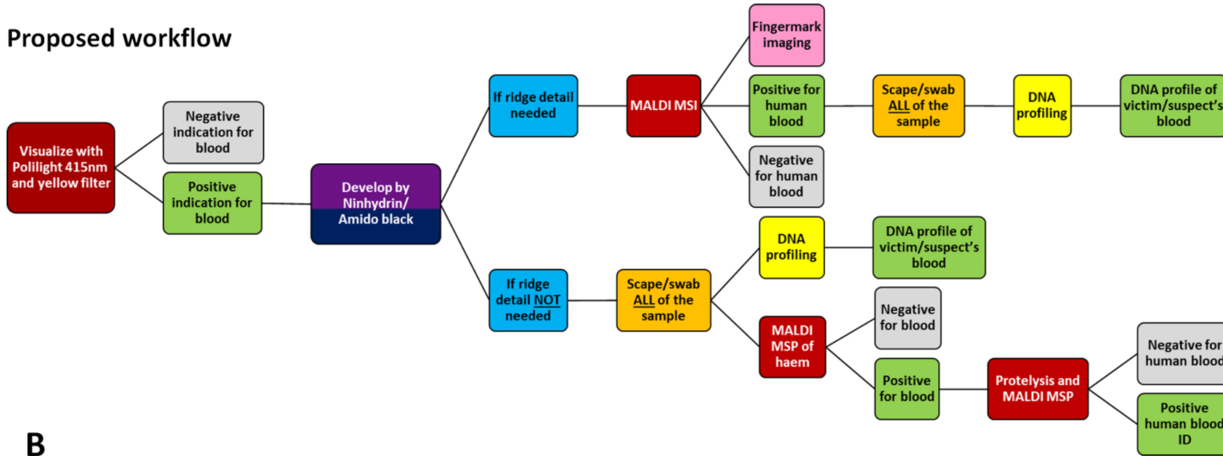

Proposed workflow
Visualize with Polilight 415nm and yellow filter
Negative indication for blood
Positive indication for blood
Develop by Ninhydrin/ Amido black
If ridge detail needed
MALDI MSI
Fingermark imaging
Positive for human blood
Scape/swab ALL of the sample
DNA profiling
DNA profile of victim/suspect's blood
Negative for human blood
If ridge detail NOT needed
Scape/swab ALL of the sample
DNA profiling
DNA profile of victim/suspect's blood
MALDI MSP of haem
Negative for blood
Positive for blood
Protelysis and MALDI MSP
Negative for human blood
Positive human blood ID
B

Fig S4. Current and suggested operational sequence of methods for development and testing of suspected blood-contaminated fingermarks in Israel. **A**: workflow includes non-destructive methods for blood visualisation, presumptive tests for blood of increasing specificity and DNA profiling. **B**: application of non-destructive methods first followed by blood enhancement techniques, DNA profiling and MALDI MSP and MSI of human blood.

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