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Application of fluorescent substrates to the *in situ* detection of prostate specific antigen

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ABSTRACT

The forensic identification of body fluids frequently presents an important source of genetic material and investigative interpretation. However, presumptive testing techniques presently employed in the discrimination of biological fluids are subject to criticism for poor specificity, lack of fluid localisation ability and detrimental effects on DNA recovery rates. The recognition of fluid-specific biomarkers by fluorogenic substrates may provide a novel resolution to these issues but research has yet to establish any pertinent *in situ* fluid detection applicability. This study therefore utilises a fluorogenic substrate (Mu-HSSKLQ-AFC) specific to the seminal protein prostate specific antigen in an effort to detect human semen deposited on a number of surfaces typical to criminal investigation. The ability of fluorescent fluorogenic substrates to simultaneously identify and visualise biological fluids *in situ* is demonstrated for the first time, whilst the production of complete STR profiles from fluid sources is also confirmed to be completely unaffected by substrate application.

1. Introduction

Locating and identifying body fluids such as semen, blood and saliva can often aid the progression of criminal investigation by providing intelligence on the nature and circumstance of an offence and may additionally associate or exonerate a suspect through the isolation of genetic material.

A number of 'presumptive' screening assays exist to rapidly exclude or indicate fluid presence, employing simple biochemical processes in order to generate colorimetric changes within a given substrate. Those indicating the presence of blood rely on the oxidation of haem to catalyse substrate-specific reactions [1–3], whilst intra-fluidic enzyme activity provides the basis for the testing of semen and saliva [4,5]. However, previous validation studies have established limitations in the usefulness and evidential strength of these assays. With the exception of the chemiluminescence phenomenon exploited in the detection of blood by Luminol, presumptive tests cannot be used to localise fluid depositions, thereby necessitating time-consuming visual searches prior to analysis. Furthermore, the molecular targets examined by these tests are not fluid-specific, often leading to false positives between different fluid types and other non-fluid substances [6–8]. Detrimental effects on the recovery of DNA from fluid depositions have also been demonstrated after some presumptive test applications [9,10].

Currently the most widely used presumptive test for semen identification is the Brentamine assay for the detection of acid phosphatase, an enzyme secreted into semen by the prostate gland [4]. However, the requirement of specialist knowledge and equipment often makes this test problematic. Results are subject to a high level of expert interpretation [11–13], whilst Brentamine toxicity also necessitates use of a fume hood.

Recent improvements in fluid assay specificity have utilised immunological testing strips for the detection of fluid-endogenous protein biomarkers [14–16]. However, these testing processes do not allow for the retention of fluids following application, potentially sacrificing a valuable source of material for genetic profiling [17].

Our research group has made initial efforts in the design of novel body fluid analysis techniques, developing a fluorescent biosensor complex specific to Glycophorin A, an erythrocyte membrane protein used in the identification of human blood [18]. However, whilst demonstrating effective glycophorin detection via decreases in fluorescence intensity, the 'turn-off' nature of signalling restricts the use of this sensor in visualising discrete fluid deposits *in situ*. A 'turn-on' fluorescence based assay is therefore preferable for simultaneous identification and localisation purposes.

The proteolytic digestion of peptide substrates to release fluorescent by-products within the same molecular unit may be considered an attractive signalling mechanism for *in situ* fluid detection. High specificities make enzyme recognition elements ideal candidates for fluid analysis, whilst 'turn-on' increases in fluorescence

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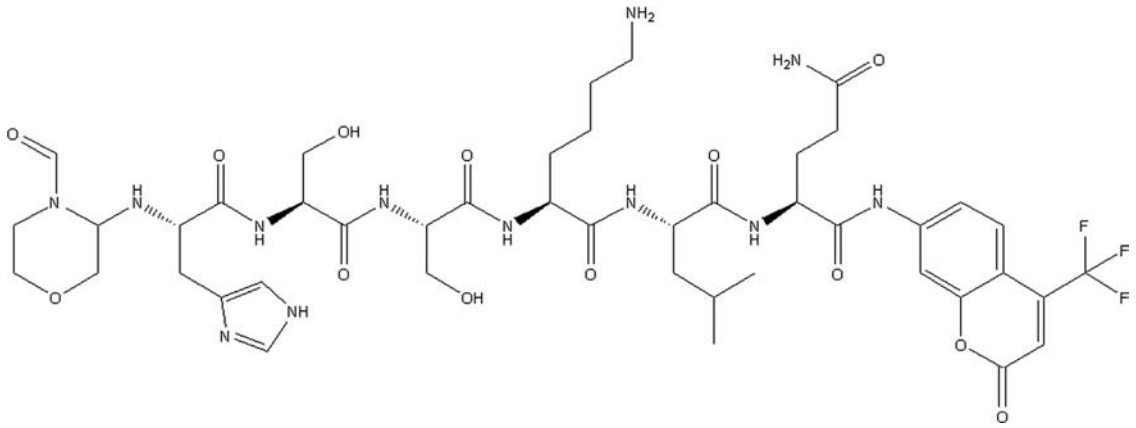

Fig. 1. PSA fluorogenic substrate – a hexapeptide consisting of amino acid sequence HSSKLQ, terminally labelled with a fluorescent coumarin derivative. Specific digestion of the peptide by PSA yields highly fluorescent 7-amino-4-trifluoromethyllcoumarin.

intensity upon target interaction may allow the visualisation of *in situ* fluid depositions. Furthermore, unlike irreversible antibody-based reactions, enzyme targets may interact with multiple substrate molecules to amplify signal production. Simultaneous detection of multiple fluid enzymes may also potentially be achieved by exploiting fluorophores of differing wavelengths in a single multiplex assay.

A central appeal of fluorogenic substrates as an alternative to current presumptive assays is that DNA-degrading oxidative processes, such as those exploited by Luminol and Leucomalachite green, are not required to generate a positive response [6]. With research yet to explore the effect of substrates on genetic material, investigation into the possible interference of reagents with DNA amplification, quantitation or profiling may be considered pertinent.

This study therefore explores the use of fluorogenic peptide substrates specific to prostate specific antigen (PSA) for the simultaneous visualisation and identification of human seminal fluid. PSA is a semen-endogenous protein responsible for proteolysis of gel-forming Semenogelin 1 and 2 [19]. The unique expression level of PSA within seminal fluid, often produced in milligram levels per millilitre [19], has established its wide acceptance as a forensic biomarker for semen identification.

Denmeade et al. [20] produced 12 peptide substrates for monitoring PSA activity based on amino acid sequences directly adjacent to mapped PSA cleavage sites of Semenogelin 1 and 2. These substrates utilise 7-amino-4-methylcoumarin fluorophores, which after amide bond conjugation to peptides undergo excitation and emission wavelength shifts, restricting fluorescence output. Subsequent separation of the fluorophore from the peptide by serine protease hydrolysis occurs in the presence of PSA and restores fluorescence.

The particular substrate MU-HSSKLQ-AFC (Fig. 1) displayed the highest specificity for PSA, arising from its resistance to similar proteolytic enzymes found within body fluids. Whilst this substrate has found routine use in the recognition of prostate cancer markers, it has yet to be applied towards the detection of human semen.

The fluorescence response of substrate MU-HSSKLQ-AFC to dilutions of semen within solution, as well as to whole semen extracted from *in situ* swabs, was measured via spectrofluorometry to determine the ability of the fluorogenic substrate to detect free PSA within seminal fluid. Further *in situ* detection ability was examined, testing substrate performance against semen deposits on glass slides and a number of surfaces typically encountered within forensic casework. Assay reagent was also applied to depositions of blood, saliva and urine to confirm substrate specificity. MU-HSSKLQ-AFC was lastly applied to semen samples for subsequent SGM plus profiling to assess reagent effect on each stage of the profiling process.

2. Materials and methods

2.1. Reagents

2.1.1. Fluorogenic PSA substrate

Lyophilised Prostate Specific Antigen Fluorogenic Substrate (Mu-HSSKLQ-AFC) was purchased from EMD Millipore (Massachusetts, USA) and dissolved in 109.5 μl DMSO to make an 8 mM stock solution before dilution in PBS to a working concentration of 400 μM.

2.1.2. Body fluid collection and storage

Blood, semen, saliva and urine samples were taken after informed consent. Blood samples were drawn by venipuncture and stored in a BD Vacutainer® Plus tube (Oxford, UK) containing 3.2% sodium citrate coagulation preservative. All tissue samples were stored at 4 °C until analysis.

2.2. Instrumentation and procedures

2.2.1. Spectrofluorometry

Fluorescence measurements were conducted on a BioTek Synergy HT spectrophotometer (Vermont, USA). Dilution curves were constructed through the addition of 100 μl of diluted semen (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) in a 96-well microplate to 100 μl of 400 μM PSA fluorogenic substrate and measured with appropriate blank (200 μl PBS) and negative controls (100 μl PBS, 100 μl assay reagent). Swabs taken from *in situ* semen depositions were extracted in 100 μl of PBS and added to 100 μl of working concentration substrate. All fluorescence emissions were recorded at room temperature in duplicate using Ex400/Em528 ± 20 nm wavelengths (for the measurement of emissions at 508 nm) immediately after mixing.

2.2.2. Slide microscopy

Fluorogenic PSA reagent was tested against seminal dilutions (1:25, 1:50, 1:100, 1:200, 1:500, 1:1000) deposited on glass slides as a demonstration of *in situ* substrate sensitivity. Semen volumes of 10 μl were applied to the centre of each slide before the direct 10 μl addition of substrate. Duplicates of each dilution were performed. Negative reagent-only controls were applied on the same slide as a measure of background reagent fluorescence, whilst blank controls consisting of semen-only applications were also used to monitor possible analyte auto-fluorescence. The simulation of dry depositions was achieved through the application of 10 μl of seminal fluid to glass slides, which were subsequently allowed to dry overnight. Reagent was then applied directly at the point of analysis.

Images were taken in the dark immediately after application on an Olympus SZX12 fluorescence microscope (Tokyo, Japan) and internal CCD camera. BV filtration (Ex 400–440 nm) was used for substrate excitation, whilst all additional microscopy parameters were kept constant (hue=359, saturation=255, white balance=64, contrast=0, brightness=1023, gamma=10, magnification= ×8.5) in order to restrict result variation.

Investigations into substrate specificity were also undertaken with application to depositions of whole blood, urine and saliva on slides in order to exclude the possibility of inter-fluidic cross-reaction.

2.2.3. Surface microscopy.

Eight different surfaces consisting of cotton, denim, felt, leather, paper, plastic, polyester and wood were chosen to reflect materials on which body fluids are commonly deposited within criminal investigations. All surfaces were cut to fit the size of a microscope slide. In a similar manner as commercial assay testing, 10 μl depositions of human semen were applied to the surfaces and allowed to dry overnight. PSA substrate was applied directly to depositions in 10 μl volumes with the same negative and blank controls previously described.

Images were once again recorded on an Olympus SZX12 fluorescence microscope utilising BV filtration (Ex 400–440 nm). All measurements were performed in duplicate.

200 μl of working concentration substrate was lastly dispersed directly over a semen deposition on leather using a common atomising spray bottle to reflect ideal future application methods.

2.3. DNA profiling

50 μl of PSA substrate at working concentration was added to 150 μl of human semen to observe effects on DNA recovery after application. DNA was extracted using the QIAmp® DNA Mini kit (Qiagen, Manchester, UK) according to the supplied protocol and quantified with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Paisley, UK). Samples were diluted to 0.1 ng/μl prior to amplification with the AmpFlSTR® SGM Plus® PCR Amplification Kit (Applied Biosystems, Paisley, UK) using a Perkin-Elmer 9700 thermal cycler (Cambridge, UK). STR amplicons were resolved on an ABI3130 genetic analyser and evaluated using GeneMapper® software. Generated profiles were compared to a semen reference profile to examine potential inhibition.

3. Results and discussion

3.1. Spectrofluorometry

Whilst regularly demonstrating detection of purified PSA protein within solution [20,21], this fluorogenic substrate has never been used to target the native PSA contained within seminal fluid. The potential for reagent inhibition or physical fluorescence screening by other biomolecules present in a complex matrix makes it pertinent to investigate whether a positive signal can be generated from whole semen.

Appropriate fluorescence intensity changes to varying dilutions of seminal fluid were observed through spectrofluorometry. Constructed calibration curves were found to be consistent with those demonstrated by Niemelä et al. [21] for the detection of PSA within solution and established a quantitative linear relationship between semen concentration and substrate fluorescence (Fig. 2).

A commonplace practice within forensic investigation is to swab potential fluid depositions that require analysis at a later point. It is consequently vital that substrates react to material extracted from these swabs in the same manner as whole fluid.

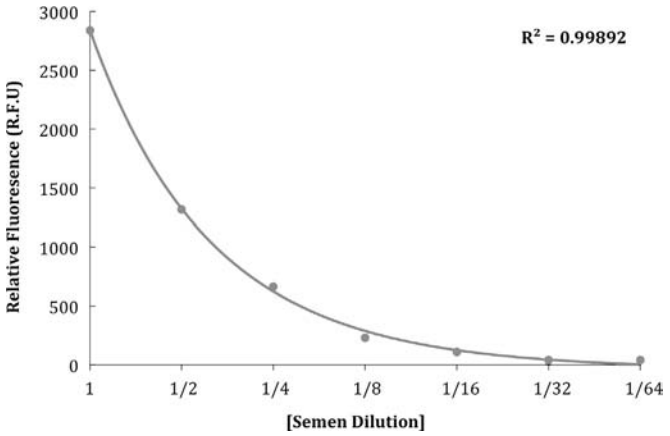

Fig. 2. Dilution curve demonstrating the relationship between seminal concentration and fluorogenic PSA substrate emission.

Swabs were therefore taken of semen deposited on a leather surface prior to extraction in PBS via brief vortexing. The collected material was analysed using the same procedure as whole fluid and did not display any differentiation in fluorescence response from its neat fluid counterpart (results not shown).

3.2. Slide microscopy

The main benefit of many presumptive tests is their ability to be performed at the point of fluid discovery. It is important that substrate reagents designed to detect biological fluids may do so *in situ* with only basic instrumental assistance for fluorescence observation, such as a portable alternative light source.

Human semen was deposited both on to glass slides prior to the direct application of PSA substrate. Any changes in fluorescence intensity were observed using standard fluorescence microscopy. Positive substrate reactions successfully identified all semen depositions, even at sensitive 1:1000 dilutions (Fig. 3). Similar results were obtained using simulated dry depositions.

Fluorescence emissions occurred immediately upon substrate application, promising potential for rapid fluid screening without extended incubation times. Background reagent fluorescence was not observed at any point during negative control testing, allowing for the visualisation of discrete fluid areas.

Additional studies of inter-fluidic substrate specificity were also undertaken with reagent applied to wet and dry depositions of whole blood, saliva and urine. In all cases, the PSA substrate did not generate a positive reaction, thereby demonstrating both high semen specificity, as well as resistance to proteases that may be present within other body fluids (results not shown).

3.3. Surface microscopy

The successful detection of semen by this substrate clearly confirms the high performance of fluorogenic substrates in the simultaneous identification and localisation of biological fluids *in situ*.

Nevertheless, clean glass slides are not representative of surfaces encountered within forensic casework. Surfaces on which fluids are typically deposited may potentially prevent the success of fluorescent substrates via absorption, movement restriction and physical screening effects. Furthermore, surfaces may contain a number of unknown substances that could inhibit substrate–target interaction. Fluorogenic PSA substrate was therefore applied, utilising the same slide testing procedure, to dry semen depositions on eight surfaces relevant to criminal investigation.

Despite the difficult nature of surface testing, positive results were generated on each of the eight surfaces, identifying and

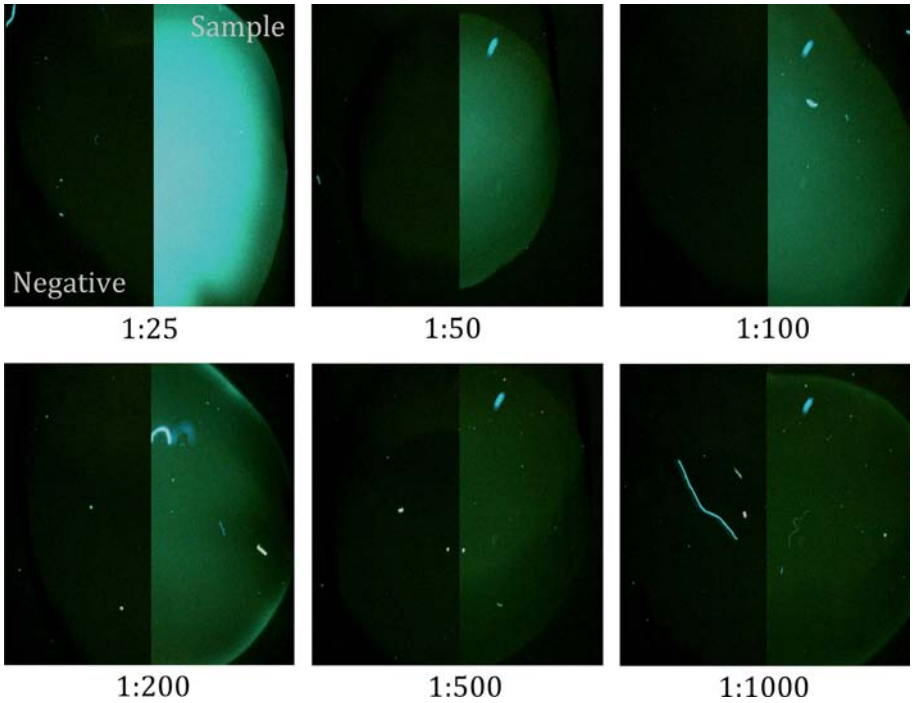

Fig. 3. Demonstration of *in situ* substrate testing of seminal depositions upon glass slides across a range of five dilutions. Duplicate measurements were taken and also displayed expected results.

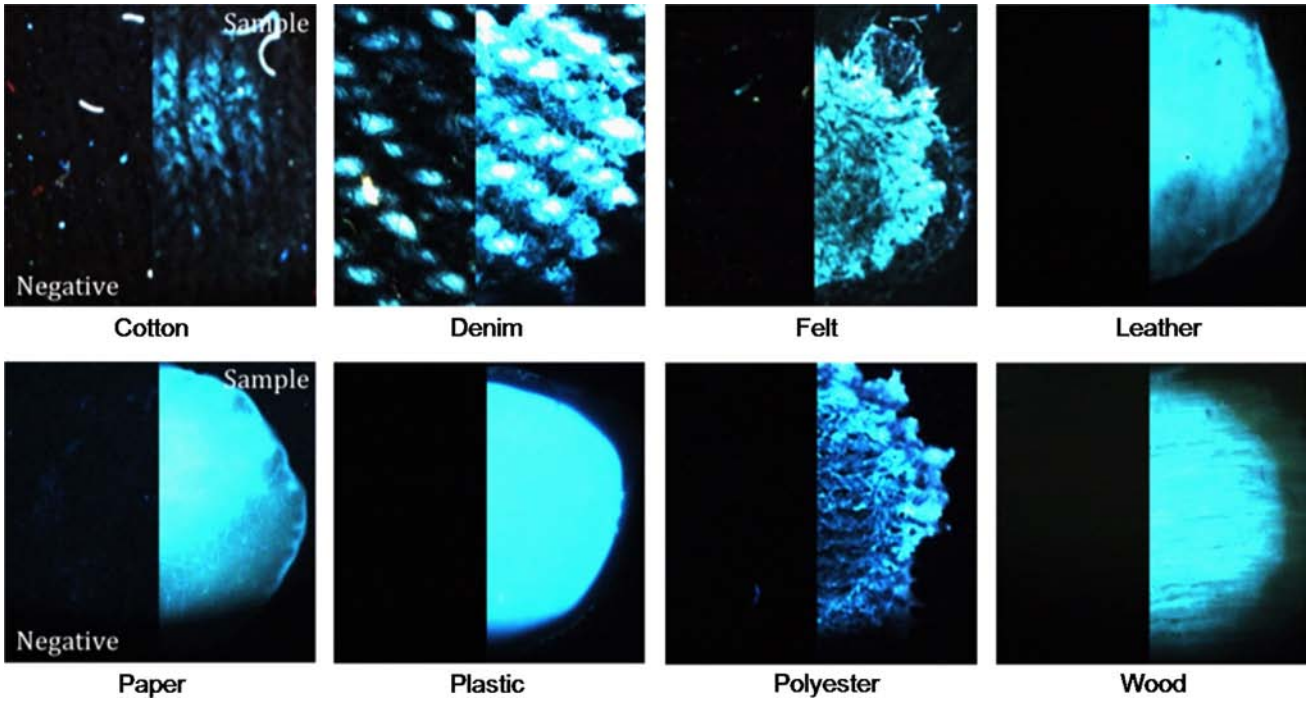

Fig. 4. Successful detection of human semen across eight forensically relevant surfaces by fluorogenic PSA substrate. Reagent-only negative controls are provided on the left side of each image.

visualising all semen deposits (Fig. 4). Once again, substrate-only negative controls did not exhibit any observable fluorescence upon application. Furthermore, surface material had little effect on the ability of the substrate to generate positive results, with no assay interference occurring during testing.

The direct spray dispersal of reagent over a large evidential surface may be considered the most efficient process of localising fluid deposits. 200 μl of substrate was applied to seminal depositions on leather using a common 1 ml atomising spray bottle in order to examine the viability of this method. Positive substrate emissions easily localised all semen depositions, elucidating discrete staining areas and confirming the validity of this technique (results not show).

3.4. DNA profiling

Validation studies by Tobe et al. [10] have previously demonstrated a significantly reduced recovery of high molecular weight

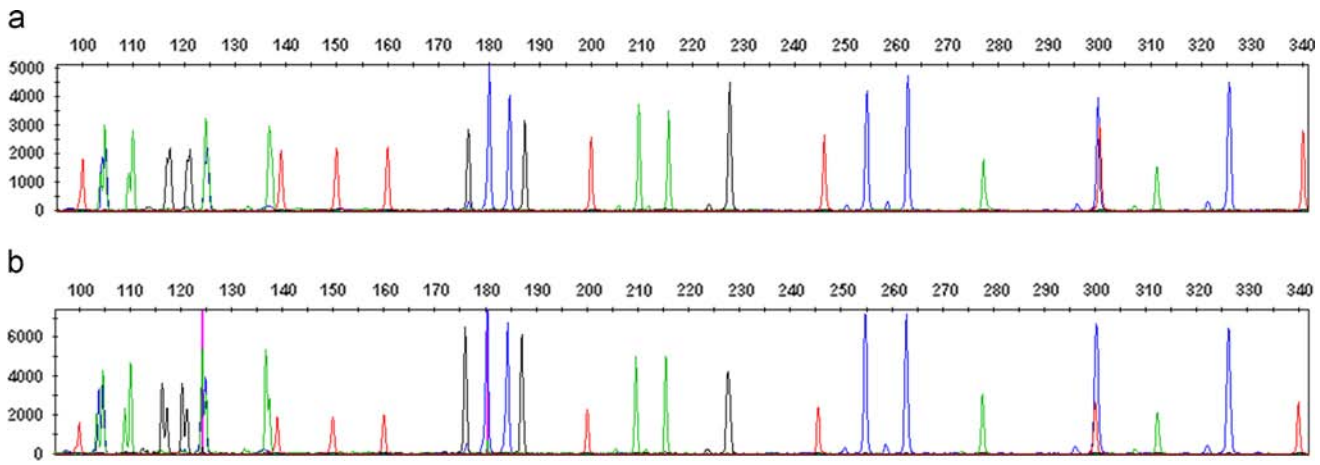

Fig. 5. Genetic profiles generated from (a) semen reference profile and (b) semen sample with applied PSA substrate. Profiles display no significant differences.

STR loci from biological fluids after some presumptive testing applications. Furthermore, the physical application process of the seminal acid phosphatase test has been shown to limit the amount of spermatozoa, and thus DNA, available for genetic profiling [13].

PSA substrate was applied to semen samples, which underwent extraction, quantification, amplification and profiling according to standard forensic protocols to examine the effects of assay application on the recovery of genetic material from biological fluids. Comparisons were then made to reagent-less semen standard (Fig. 5).

Successful genetic recovery from substrate-applied semen was established by Picogreen quantification, with an average concentration of 8.99 ng/μl DNA per sample extraction falling within expected values. Furthermore, full STR profiles were obtained from all assay applications, with no detrimental effects on high molecular weight loci. Differentiation of reagent-applied samples from their reference profiles was not observed at any stage of the profiling process outside the limits of normal experimental variation.

4. Conclusion

This study successfully demonstrates for the first time the ability of fluorescent substrates to identify biological fluid depositions *in situ*. Human semen was detected across a range of surfaces typical to forensic investigation with additional visualisation via a direct spraying application.

This particular substrate exhibited ideal increases in fluorescence intensity upon target interaction, even at sensitive 1:1000 seminal dilutions, giving opportunity for its use in contaminated fluid depositions or those washed in removal attempts.

Importantly, substrates were found to have no effect on DNA profiling processes after application to biological fluids and thereby negate the potential forfeit of fluid identification in order to maximise genetic material recovery.

An ideal application of fluid-specific substrates would likely exploit a number of different peptide sequences, each with separate emission wavelengths for simultaneous enzyme detection within several fluid types in a multiplex sensing system.

Displaying both an immediate and specific response to analyte presence, fluorogenic substrates have the potential to prevent month-long visual evidence searches by localising fluid depositions within a matter of seconds. Serious thought should therefore be given to the development of fluorogenic substrates as replacements to current presumptive testing techniques.

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