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A novel fluorescence-based method in forensic science for the detection of blood *in situ*

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Abstract

Full DNA profiles can be generated from just a few cells; however these profiles can be contaminated from other cell types present at the crime scene. We report here on the development of an immunofluorescent technique to spatially locate human-specific blood *in situ* and also on the ability of this technique to detect individual leukocytes and the DNA contained within them. Four monoclonal mouse anti-human antibodies were evaluated; anti-glycophorin A to detect erythrocytes and anti-CD45, anti-myeloperoxidase (MPO) and anti-histone H1 to detect the nucleated leukocytes. Each antibody was labeled with either Alexa Fluor 488 or 568 for direct application to blood smears which allowed the simultaneous detection of erythrocytes and leukocytes. Furthermore, because histones are DNA binding proteins, the application of anti-histone H1 allowed the detection of DNA within a blood smear. Importantly it was found that full DNA profiles could be achieved after using this method with similar peak area ratios compared to untreated cells. The fluorescent antibodies were found to be human-specific with the exception of anti-histone H1 due to its conserved sequence. However, used in combination with anti-CD45 or anti-MPO the location of DNA from human-specific leukocytes could be detected. The technique was also tested on older blood stains and was still found to be sensitive and cell-specific after 4 months. Following the optimization of the methodology, the fluorescent antibodies were applied to short lengths of black cotton fibres covered with human blood spots. Although the background fluorescence from the cotton was found to be high, erythrocytes and even individual leukocytes could easily be detected, indicating that this technique could be used to detect extremely minute amounts of blood. Used in combination with laser capture microdissection (LCM), this method could be used to pick off individual leukocytes for LCN DNA techniques.

Keywords: Antibody; Blood; Fixation; Fluorophores; Immunofluorescence; Microscopy

1. Introduction

In the last decade, there has been a significant improvement in the sensitivity of DNA profiling [1]. The limit of detection for a DNA profile with a standard procedure is approximately 100–500 pg of DNA. However, with less than 100 pg of DNA, low copy number (LCN) techniques are required, where the most popular method is to raise the number of cycles from 28 to 34 cycles [2,3]. However, recently it has been shown that full DNA profiles can be consistently obtained from the standard 28 cycles from as little as 20 pg of DNA using post-PCR purification [4]. This should allow DNA profiles to be obtained from just a few cells (single cell ~6 pg DNA). Therefore, the ability to detect trace amounts of sources of human DNA at crime scenes or on items submitted to a forensic laboratory is of paramount importance.

Blood is one of the most common sources of DNA found at a crime scene and is typically detected by presumptive tests such as Kastle Meyer (KM), luminol, leucomalachite green (LMG) and Hemastix®, which rely on the peroxidase-like activity of haemoglobin. However, these tests are not human-specific and are prone to providing false positives. For example, luminol, which allows the detection of very small droplets of blood down to 1 ng, has been found to give false positives with a wide variety of domestic and industrial substances that might be mistaken for haemoglobin [5]. However, there have been some improvements to luminol over the years to reduce interferences and to increase the chemiluminescence emission [6,7] and

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compared to the other presumptive tests is the most specific and sensitive. Furthermore, in a recent study DNA was not successfully recovered or amplified after the LMG and Hemident™ tests had been applied [8].

Testing kits based on immunological detection of blood have been developed, such as the SERATEC® HemDirect test which uses an immuno-chromatographic lateral flow test strip. This test is sensitive down to 40 ng/mL of haemoglobin and can be performed in approximately 5 min. However, it has been reported to cross-react with primate blood and is known to give false negatives due to a high hook effect [9]. A more recently developed immunological-based blood detection test, the Rapid Stain Identification-Blood (RSID™-Blood), is based on the detection of erythrocyte glycophorin A. This test is human-specific, has a limit of detection of 100 nL of blood and is reported to eliminate the problems of false negatives as well as false positives from certain food types [10]. However, the use of immuno-chromatographic cassettes either requires swabs to be taken from a sample area or where suspect stains are deposited on fabric, part of the area in question is dissected and the sample is then extracted.

We report here the development of a novel technique based on immunofluorescence to spatially locate human-specific blood *in situ* and the detection of nucleated leukocytes within that blood stain for the purpose of LCN DNA collection. We use a range of blood and human-specific antibodies to detect erythrocytes and leukocytes *in situ* and present evidence that the location of DNA can indirectly be visualized using this technique. Furthermore, we demonstrate that the technique can be used to detect blood spots on dark fabric and distinguish them from background fluorescence.

2. Materials and methods

2.1. Chemicals, equipment and sources

Human blood was collected by venipuncture from healthy volunteers after informed consent using BD Vacutainer® Safety-Lok™ Blood Collection Sets (Becton Dickinson, Oxford, UK) and 3 mL evacuated sterile blood vacutainers (Becton Dickinson, Oxford, UK) containing 7.5% EDTA. Mouse, guinea pig and rabbit blood was collected by venipuncture and stored in 1.5 mL eppendorf tubes containing 7.5% EDTA. Pig, cow and ferret blood was supplied in EDTA from B&K Universal Ltd. (Hull); rat, chicken and sheep blood was supplied in EDTA from Harlan UK Ltd. (Oxford). All blood tubes were stored at 4 °C until further analysis. Fluorescent antibodies were also tested against human blood which had not been exposed to EDTA. This blood was again collected by venipuncture and immediately used before coagulation. Saliva was collected from healthy male and female volunteers in 15 mL Falcon tubes (Becton Dickinson, Oxford, UK) and was used immediately. Semen was collected and stored at −20 °C until use.

The following reagents and materials were obtained for the study: adhesive, pre-cleaned microscope slides (26 mm × 76 mm × 1 mm, X-Tra™ Adhesive) (Surgipath, Peterborough, UK); pre-cleaned glass microscope slides (76 mm × 26 mm × 1 mm) for use as spreader slides and glass cover slips (22 mm × 22 mm, thickness No. 1) (VWR International, Lutterworth, UK). Cells were fixed with the following reagents: methanol (analytical grade) and dichloromethane (analytical grade) (Fisher Scientific, Loughborough, UK); acetone (HPLC grade) (Rathburn Chemicals Ltd., Walkerburn, UK); ethanol (AnalaR) and propan-2-ol (GPR) (VWR International). Other laboratory materials included: PBS, PAP pen, bovine serum albumin, casein and DMF (Sigma–Aldrich, Poole, UK); fluorescent anti-fade mounting medium (Dako, Ely, UK).

Antibodies and fluorophores purchased for the studies were as follows: mouse anti-human glycophorin A monoclonal antibody (HIR2), mouse anti-human CD45 monoclonal antibody (MEM-28) and mouse anti-human MPO monoclonal antibody (2C7) (Abcam plc, Cambridge, UK); mouse anti-human histone H1 monoclonal antibody (AE-4) (AbD Serotec, Oxford, UK); goat anti-mouse IgG Alexa Fluor 568 (anti-IgG_{568}) (Invitrogen, Paisley, UK); Alexa Fluor® 488 and 568 carboxylic acid, succinimidyl ester for antibody labelling (Invitrogen) and Zeba™ Desalt Spin Columns (0.5 mL) (Perbio Science UK Ltd., Cramlington, UK). Protein concentrations and fluorophore labeling ratios were measured on a ND-1000 spectrophotometer (LabTech International, Ringmer, UK).

For DNA profiling, the QIAamp® DNA mini kit was purchased from (QIAGEN®, UK); the Quantifiler® Human Quantification kit and the AmpFlSTR® SGM *plus*™ amplification kit were purchased from Applied Biosystems, USA. Plates were quantified on an ABI Prism 7000 and DNA profiling was performed on an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, USA).

Brightfield and fluorescent images were acquired on a Zeiss Axioskop 2 MOT plus stereomicroscope (Carl Zeiss Ltd. Welwyn Garden City, UK) or on a Leica DMIRE2 confocal microscope and imaged using a TCS SP2 system. On the Zeiss system, blood, saliva and semen smears were imaged using either a 20 × 0.5 or 40 × 0.75 dry lens or a 100 × 1.3 oil lens and illuminated with either a halogen lamp for brightfield, or a 100 W Hg arc lamp for fluorescence. Images were captured with an AxioCam HRC CCD camera and processed using Axiovision 3.1 software. On the Leica system blood smears were imaged using a 40 × 0.75 lens and illuminated using a 488 nm Argon laser to excite Alexa Fluor 488 and a 543 nm HeNe laser to excite Alexa Fluor 568.

2.2. Antibody labeling

Alexa Fluor (AF) dyes (AF488, AF568), carboxylic acid, succinimidyl ester were dissolved in DMF to a concentration of 1 mg/mL. Buffer exchange was performed by adding 70 μL of antibody solution to a 0.5 mL Zeba desalt spin column equilibrated with 100 mM $NaHCO_3$ (pH 8.5). Zeba columns, typically show 95% retention of salts and other molecules <1000 MW and high protein recovery (c. 90–95%). Dye was added at a ratio of 10:1 dye to protein and reacted for 1 h in the dark at room temperature. Unbound dye was then removed