International Journal of Legal Medicine (2019) 133:419–426
https://doi.org/10.1007/s00414-018-1912-7

METHOD PAPER

Whose blood is it? Application of DEPArray™ technology for the identification of individual/s who contributed blood to a mixed stain

K. Anslinger[1] · B. Bayer[1]

Received: 12 May 2018 / Accepted: 13 August 2018 / Published online: 18 August 2018

Abstract
The interpretation and statistical evaluation of mixed DNA profiles often presents a particular challenge in forensic DNA investigations. Only in specific combinations can single cellular components of a mixture be assigned to one contributor. In this study, the DEPArray™ technology, which enables image-assisted immunofluorescent-sorting of rare single cells using dielectrophoretic (DEP) forces, was applied together with different preliminary tests to identify the individual/s who contributed blood to a given mixture. The technique was successfully applied in two routine casework samples. In order to ascertain how old a stain can be and still be processed successfully, white blood cells from two 10- and one 27-year-old stains were investigated. Depending on the stain's age, the associated DNA degradation level and the number of target cells successfully isolated, the final profile reflects a compromise between the gain of information due to isolation of pure cells of a specific cell type from a single contributor and the loss of discriminatory power due to incomplete profiles caused by DNA degradation.

Keywords DEPArray™ technology · Mixed DNA profiles · Cell separation · White blood cells · Cold case

Introduction

Treatment of mixed traces

The investigation of mixed traces represents a particular challenge in forensic DNA investigations. Routine cases involve many mixtures consisting either of homogeneous components as in blood-/blood-mixtures or inhomogeneous components as in a vaginal swab with adhering sperm cells. Direct DNA analysis often leads to mixed DNA profiles, which can be more complicated to interpret and statistically evaluate than single source samples [1, 2]. Only in exceptional cases, for example, when the profile shows a major component, the alleles of a single contributor can be deduced. However, the mixture ratio sometimes does not allow the detection of a minor contributor's alleles at all, or at best, only partial profiles of the minor contributor can be derived. In particular, the statistical interpretation of these partial profiles is one of the main emphases of forensic research [3]. However advanced interpretation techniques may be, and even if pre-tests show the presence of blood and saliva in a given mixture and the STR analysis results in a corresponding mixed profile, one cannot determine from whom either component originates. Yet, exactly, this information may be of great interest for the burden of proof in cases where the persons involved live together or had legitimated contact before the crime.

Separation of mixed stains

In order to yield unmixed DNA profiles, to enable profiling of a minor mixture component or to assign a specific DNA profile to a cell population, several techniques for the separation of cell populations contained in mixtures were developed. In 1985, Gill and colleagues described a method for the separation of sperm and epithelial cells known as differential lysis [4]. Other approaches used laser microdissection or fluorescence-activated cell sorting (FACS) for the isolation of sperm or other cells that can be distinguished by means of histological or fluorescent in situ hybridization [5–8]. Yano and colleagues described the use of anti-human AB0- and

✉ K. Anslinger
katja.anslinger@med.uni-muenchen.de

[1] Institute of Legal Medicine, Ludwig-Maximilians-University, Nußbaumstr. 26, 80336 Munich, Germany

CD45 antibody-coated microbeads and centrifugal separation for the isolation of white blood cells originating from donors with different blood groups from mock-mixed blood stains [9]. In this study, the application of the DEPArray™ technology (Menarini, Silicon Biosystems, Bologna, Italy) for the isolation of white blood cells from mixtures containing blood from at least one contributor was investigated from the perspective of a feasibility study.

In general, single fluorescently labeled cells can be isolated into compartments (called "cages"), identified by image-assisted and moved independently in a controlled manner using the DEPArray™ technology. Thus, differently labeled single cells from different cell populations of epithelial, blood, or sperm origin can be separated and recovered from a mixture. In order to specifically stain and isolate epithelial cells, leucocytes and sperm cells, a corresponding forensic kit (DEPArray™ Forensic Sample Prep Kit) was developed by Menarini, Silicon Biosystems. As described elsewhere [10], using this kit, epithelial cells are stained with anti-cytokeratin (CK) antibody against a human epithelial antigen expressed in a vaginal tract epithelium and in a mouth epithelium, leucocytes are marked with an anti-CD45 antibody recognizing a human lymphocyte surface antigen, and for the sperm cells, a sperm head specific antibody is used. In a first validation study, proof of principle was demonstrated [10]. Moreover, in an earlier study, our group applied this technology in the context of chimerism determination after allogenic bone marrow or stem cell transplantation [11].

From our routine case work, five samples from five different cases were chosen for further investigations with the DEPArray™ technology. The first two samples were less than 3 months old. For these samples, the presence of blood was proven by a chemical as well as an immunohistochemical test, and mixed profiles were obtained via routine DNA profiling. In order to ascertain how old a stain could be and still be processed successfully with this technology, we simultaneously investigated two 10- and one 27-year-old stains, respectively. White blood cells were stained in all the samples, epithelial cells were co-stained only in one sample using the DEPArray™ Forensic Sample Prep Kit (Menarini, Silicon Biosystems, Bologna, Italy). Pure cell aliquots were isolated in all experiments, and DNA was analyzed using standard forensic kits.

Material and methods

Material

For this study, a total of five samples (named with sample A to E) from five different cases (in analogy also named with A to E), currently investigated in our lab, were chosen for processing with DEPArray™ technology (Table 1). Samples A and B were taken from flat (several centimeters in diameter) and maroon appearing stains on two different suspects' clothing's. Preliminary tests were carried out as a part of our routine forensic workflow. Weakly positive results for both stains were obtained in two preliminary serological screenings for the presence of blood and human blood, respectively (Combur3Test®, Roche, Rotkreuz, Schweiz and Seratec® HemDirectHemoglogin Test, Göttingen, Germany). One the other hand, a serological test for the presence of α-amylase (Seratec®) as well as for the presence of semen (PSA Semiquant, Seratec®), two body fluids, which possibly also can include a relevant number of white blood cells, showed negative results. Routine STR profiling of samples taken from the clothes mentioned above led to DNA-mixtures originating from at least two persons. From each of these stains, another sample was withdrawn for processing using the DEPArray technology. In case A, a cotton swab soak with PBS was used; in case B, it was a nylon-flocked PBS-soaked swab.

In order to determine the influence of the stain material's age on the staining and separation procedure using the DEPArray technology, blood samples of three different cold cases, recently analyzed in our lab, were examined in parallel. In detail, there was a 10-year-old bloodstain on a wooden stick (case C, Fig. 1a) and a 10-year-old bloody vaginal swab (case D), both from rape cases, as well as a 27-year-old blood stain taken from a victim's bra in a murder case (case E, Fig. 1b). Sampling was carried out using nylon-flocked swabs soaked in PBS. The original STR profiles from unsorted D and E showed distinct signs of degradation (Fig. 1c). Moreover, the bra showed some moldy spots. Based on the publication from Vernarecci et al. a degradation index ($DI = concentration_{small\ amplicon}/concentration_{large\ amplicon}$) of 2.75 and 3.7, respectively for D and E was determined via DNA quantification with real-time PCR, amplifying two amplicons of different size [12]. All cases and the corresponding samples are listed in Table 1.

Cell isolation using DEPArray™ technology and molecular genetic analysis

Cell identification on the DEPArray platform is performed by a combination of immunostaining and bright field imaging. Before loading on the device, cells resuspended from the sample are stained using antibodies specific for white blood cells, sperm cells, and/or epithelial cells using the corresponding Forensic Sample Prep Kit (Menarini Silicon Biosystems, Bologna, Italy) according to the manufacturer's instructions. In order to identify leukocytes in all samples mentioned above, white blood cells were stained with anti-human CD45 PE. For the bloody vaginal swab of case D, epithelial cells were additionally stained with FITC anti-cytokeratine. Furthermore, the nuclei in all five samples were

Table 1 Overview about the investigated samples: the cases and corresponding stains (A–E), age of the stains, the individual staining of each sample, the number of cells separated per cell population, and the results of STR profiling were listed

Case	Stain description	Age of the stain	Staining	Number of cells per aliquot and population	STR profile quality detected/expected alleles
A	Blood stain on suspects cloth	< 3 months	CD45 PE DAPI	12 and 13 white blood cells	Full profile 32/32
B	Blood stain on suspects cloth	< 3 months	CD45 PE DAPI	21 white blood cells	Full profile 30/30
C	Blood stain on a wooden stick	10 years old	CD45 PE DAPI	44 white blood cells	Partial profile 23/31
D	Bloody vaginal swab	10 years old	CD45 PE DAPI	54 white blood cells	Partial profile 26/30
D	Bloody vaginal swab	10 years old	FITC-cytokeratine DAPI	45 epithelial cells	Nearly complete profile 29/30
E	Blood stain on bra	27 years old	CD45 PE DAPI	40 white blood cells	Partial profile 9/29

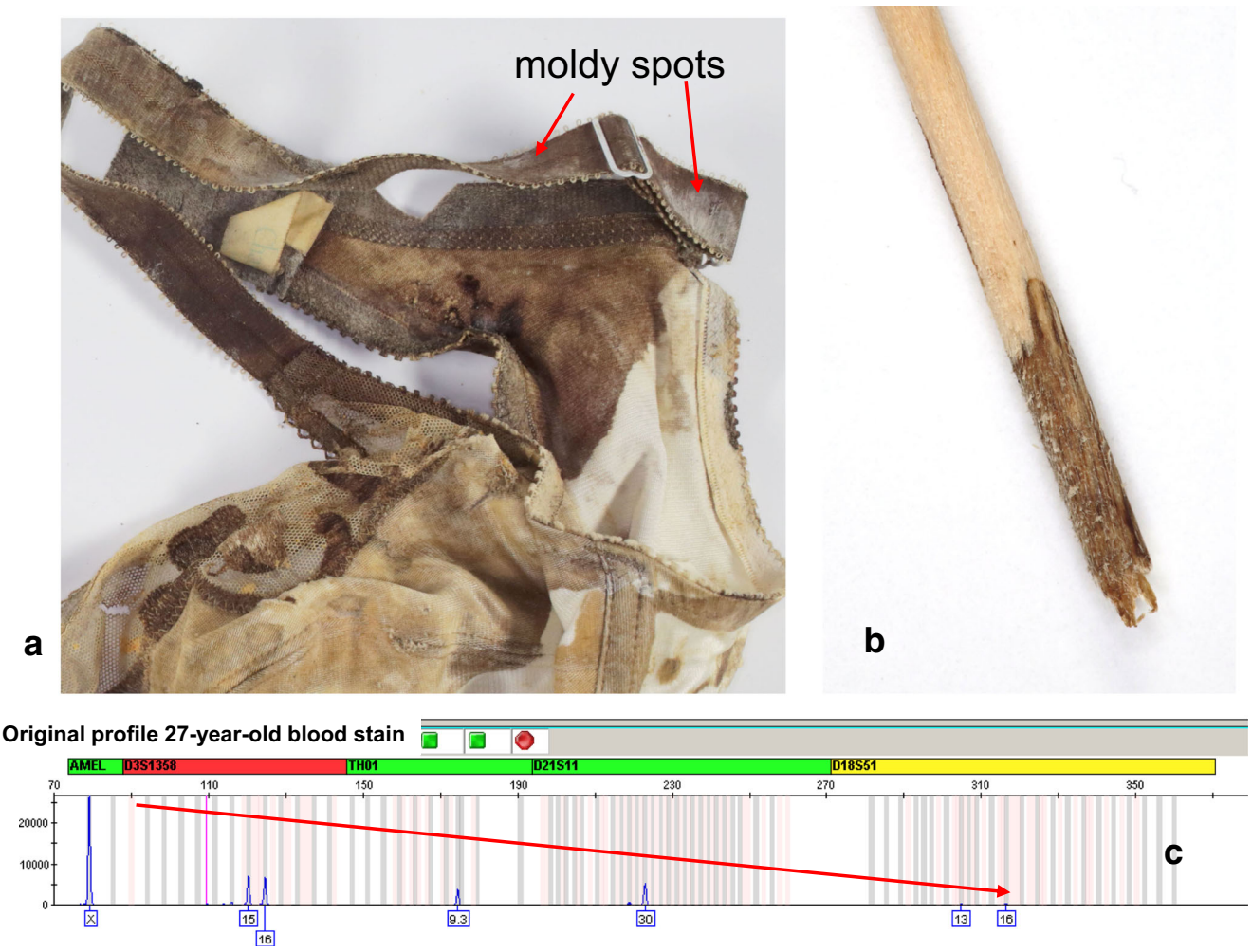

Fig. 1 **a** Photo of the 27-year-old blood stain on the bra (case E), also showing some moldy spots. **b** 10-year-old blood stain on the wooden stick. **c** Original STR profile from the 27-year-old blood stain (case E) showing distinct signs of degradation (declining peak highs of the larger PCR fragments)

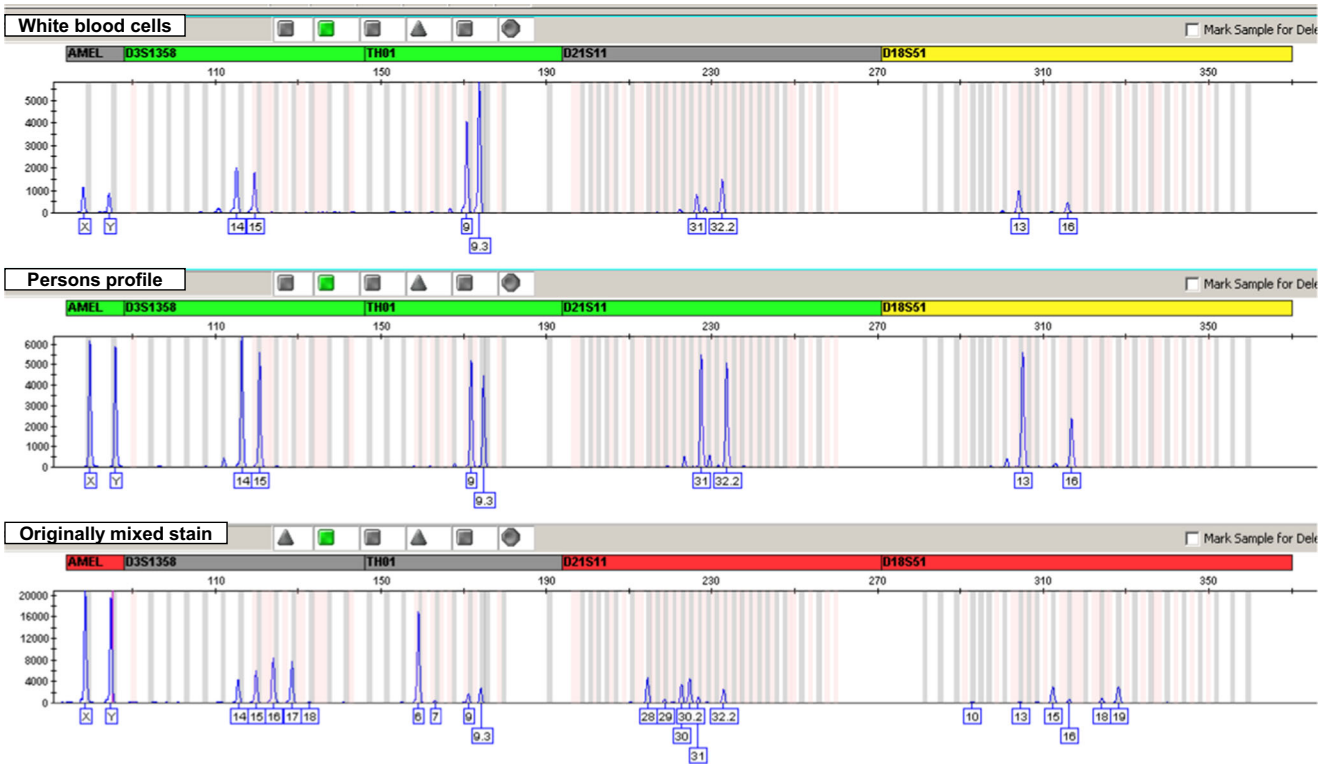

Fig. 2 From top to bottom: blue dye channel of the white blood cells, the persons profile and originally mixed profile, amplified with the PowerPlex® ESXfast System (Promega), including the peak heights in rfu (relative fluorescence units)

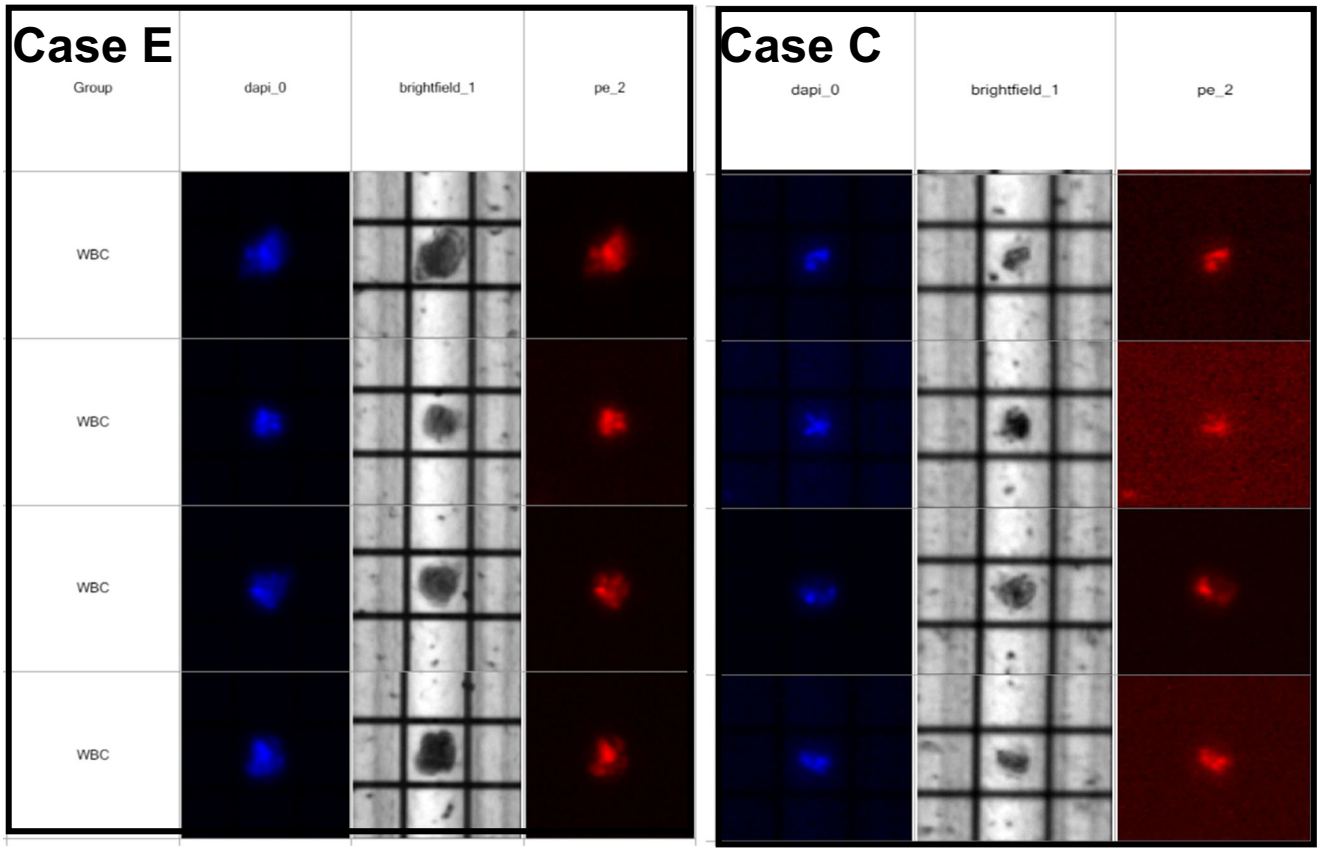

Fig. 3 Abstract of the stained and selected white blood cells from the 27- (case E) and 10-year-old blood stains (case C) (all cells satisfying the conditions "in cage," DAPI positive and PE positive). Additionally, the corresponding bright field and DAPI pictures were shown

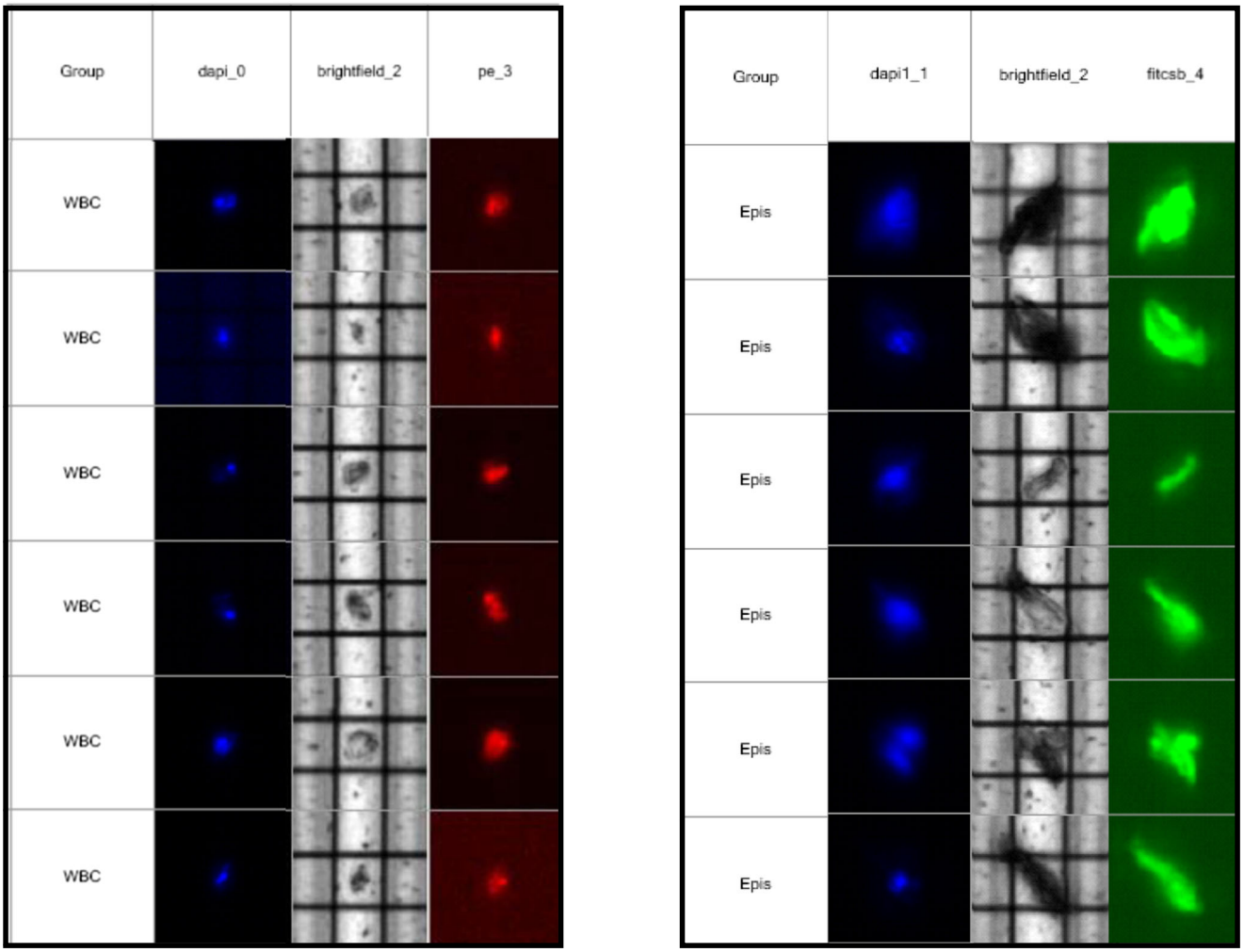

Fig. 4 Abstract of the selected PE-stained white blood- and FITC-stained epithelial cells from the 10-year-old bloody vaginal swab of case D. Additionally, the corresponding bright field and DAPI pictures were shown

stained with DAPI (Table 1). Samples were washed and subsequently loaded into the DEPArray™ cartridge. All DEPArray™experiments were conducted according to the manufacturer's specifications. Using the CellBrowser software (Menarini Silicon Biosystems, Bologna, Italy), the criteria "InCage" (position of the cell inside the electrode cage, such as it can be moved by the system), DAPI positive and PE positive were used for classification of white blood cells. "InCage," FITC positive, PE negative, and DAPI positive were the criteria used for classifying the epithelial cells from the vaginal swab. For sample A, two aliquots containing 12 and 13 leukocytes were recovered in separate tubes. For sample B, two aliquots containing 21 leukocytes each were recovered. To counteract known or possibly present degradation effects [12], several aliquots with higher cell numbers (40 to 54 leukocytes and 45 epithelial cells) were isolated from samples of the three cold cases C, D, and E (Table 1).

According to manufacturer's instructions, DNA was isolated with the DEPArray™ LysePrep Kit (Silicon Biosystems, Bologna, Italy). Using the Multiplex-PCR PowerPlex® ESXfast Systems (Promega, Madison, WI, USA), 16 autosomal loci (D3S1358, VWA, FIBRA, TH01, SE33, D8S1179, D21S11, D18S51, D16S539, D2S1338, D19S433, D22S1045, D1S1656, D10S1248, D2S441, and D12S39117) as well as the sex-determining amelogenin system were amplified on a Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Following our in-house-validated protocol, PCR was carried out in a reaction volume of 14 μl and a 30 (for the samples of case B) and 32 (for the samples of the cases A, C, D and E) cycle PCR program, respectively; apart from that, the manufacturer's instructions were followed. Separation of amplicons according to fragment length was performed on a 3500xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was carried out using the GeneMapper®ID-X Software v1.4 (Thermo Fisher Scientific, Waltham, MA, USA).

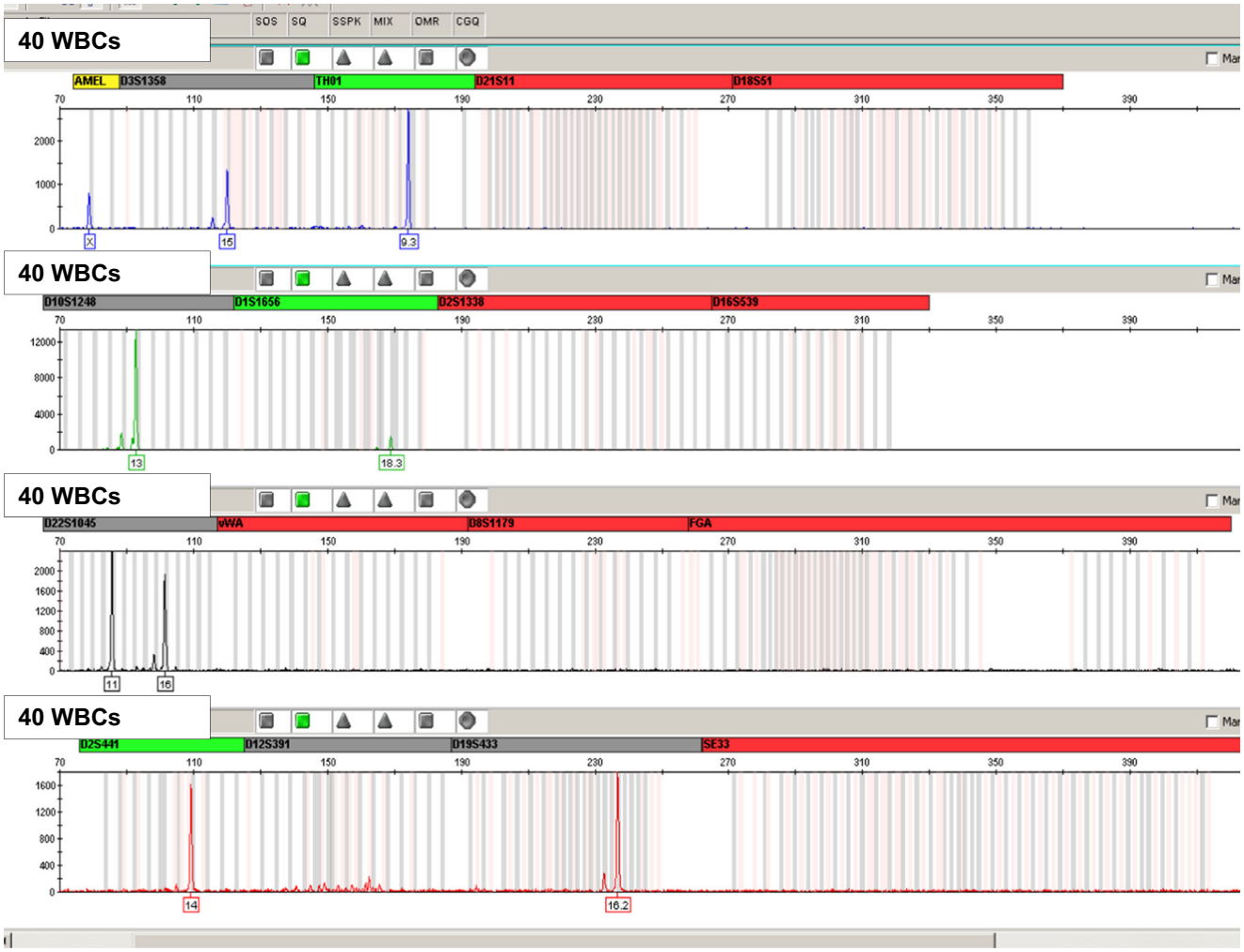

Fig. 5 Partial profile, obtained from 40 white blood cells (27-year-old blood stain, case E)

Results and discussion

In case A, two aliquots containing 12 and 13 white blood cells, respectively and in case B, two aliquots containing 21 white blood cells each, were separated via DEPArray™ technology. STR profiling resulted in corresponding single male profiles for both samples from each case (Table 1). These profiles completely matched with profiles of persons involved in each particular case. In Fig. 2, a comparison of the profiles of the pure white blood cells, the person involved in the case and the originally mixed stain from case A is shown (just the blue dye channel from each profile as an example). From the perspective of a feasibility study, we were able to show that this technology is quite suitable for determining which cell type (blood in this case) was contributed to a mixed stain of interest by a particular person. In combination with the look of the stains (flat and maroon appearing), the two positive preliminary serological screenings for the presence of blood (which proved the presence of at least one blood component in the mixture) and the negative test results for the presence of α-amylase and semen, we were able to determine who contributed the blood to each stain.

Cold cases (cases C, D, and E): For all samples of the three cold cases, a whole series of typically PE and DAPI positive cells could be detected. Examples of the picture gallery images of the stained and selected white blood cells obtained from case C and E are shown in Fig. 3. Moreover, numerous epithelial cells matching the criteria "InCage," FITC positive, PE negative, and DAPI positive could be detected in the sample from case D, the 10-year-old bloody vaginal swab. Example pictures of the selected PE-stained white blood as well as FITC-stained epithelial cells are shown in Fig. 4. Because of their variation in size and weight, white blood cells and epithelial cells have different "InCage" positions. In order to get clear DAPI signals for both cell populations, the focus must be adjusted for each cell type. The different settings were named with DAPI 0 and 1 (Fig. 4). Taking together the staining results, one can assume that there are enough cells showing intact epitopes for antibody binding and that the cells contain DNA packed in their nuclei. As recommended by

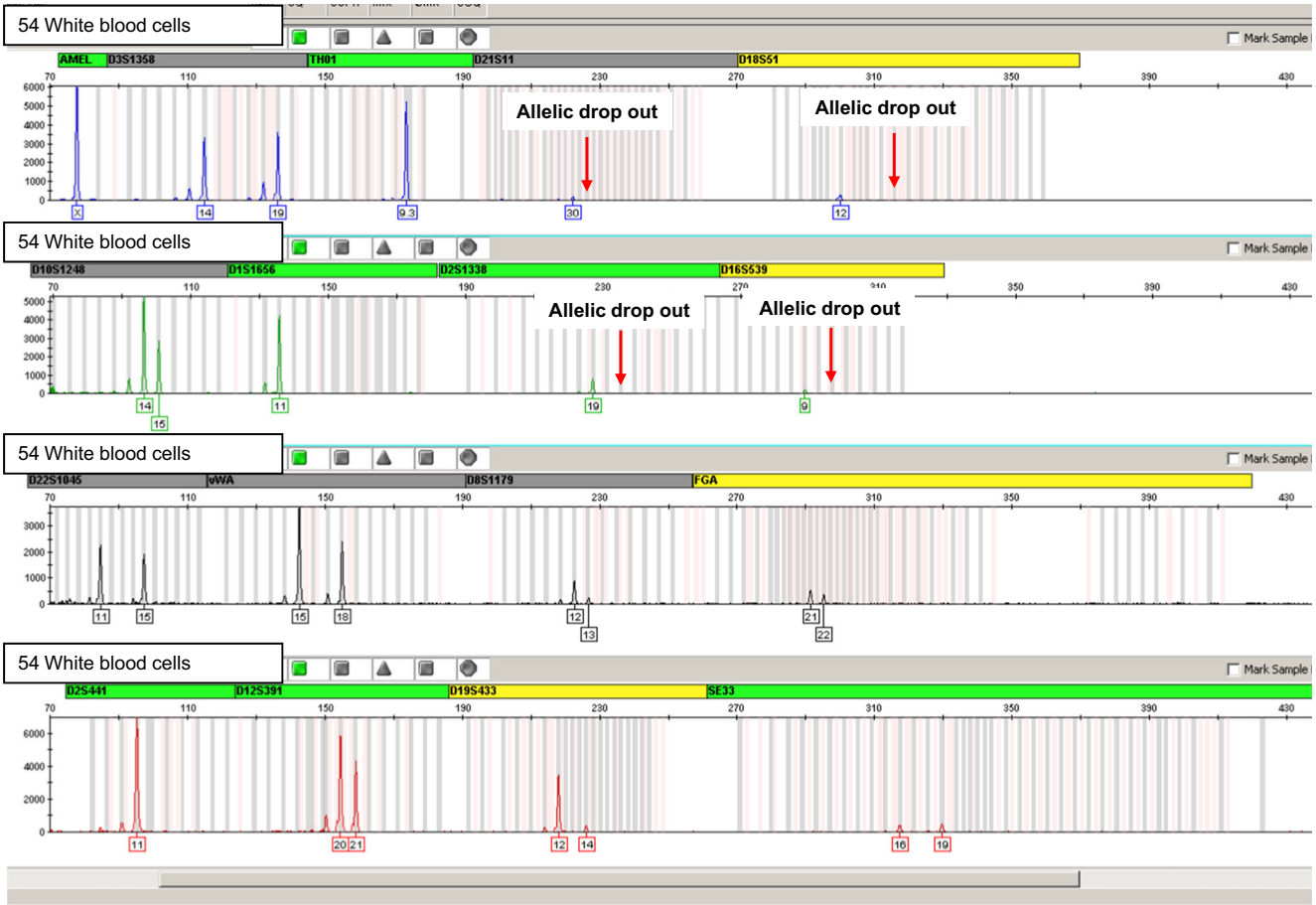

Fig. 6 Partial profile, obtained from the 54 white blood cells from the 10-year-old bloody vaginal swab (case D). Allelic drop outs are indicated with red arrows

Vernarecci et al., the number of selected cells per sample was increased (40 to 54, respectively), in order to compensate for the effect of DNA degradation on STR profiling. For the 27-year-old sample from case E, a partial profile consisting of 9 out of the expected 29 alleles (3 complete autosomal loci, 4 loci showing only 1 of 2 expected alleles, and amelogenin) could be obtained. In contrast, the profiles from case C and D, the two 10-year-old stains, showed much fewer drop outs. Twenty-three of 31 (case C) and 26 of 30 (case D) expected alleles for the white blood cell fractions and 29 of 30 expected alleles for the epithelial cell fraction from case D respectively could be detected (Table 1; Figs. 5 and 6).

All obtained alleles match with the corresponding persons' alleles and drop-ins could not be observed. A 100% concordance could be reached. Therefore, at least a "nonexclusion" hint on who could have contributed the blood could be obtained. However, compared with the original mixed profiles obtained from the stains, the discriminatory power (i.e., the calculated likelihood ratio for the obtained partial profile) was slightly increased. A possible explanation for the low number of alleles detected in sample E could be that the number of selected cells is not sufficient to fully compensate for the effect of degradation, which probably occurred before sample collection or during sample storage, but is also conceivable that a further damage and/or loss of DNA could happen throughout the staining procedure, especially during the permeabilization prior to DAPI staining. The reason could not be ultimately clarified throughout this study. As we know from the original investigations of the bloodstain on the 27-year-old bra (case E), enough nondegraded DNA for the generation of a full profile could be obtained from a 1-by-1-cm cutting of the blood stain. But, conclusions about the corresponding number of intact cells could not be deduced. Also, it cannot be excluded that parts of the DNA in the unsorted samples derived from "cell free" DNA, which no longer is surrounded by an intact DAPI and PE stainable cell [13]. Therefore, the results from the classical investigation and the DEPArray™ isolated cells could not be compared directly concerning profile completeness. Ways to improve the results of the DEPArray™ isolated cells may possibly be the optimization of cell number per aliquot in relation to DNA degradation (when possible) as well as the separation of numerous aliquots of one sample combined with STR profiling using different kits, especially kits with miniSTRs [12, 14]. For this reason, a systematic

study with stains of different storage times will follow. Nevertheless, from the perspective of a feasibility study, we could state that white blood cells from cold cases up to 27 years old could be stained and analyzed successfully but, depending on the stain's age, the associated DNA degradation level and the number of target cells successfully isolated, possibly the final profile reflects a compromise between the gain of information due to isolation of pure cells of a specific cell type from a single contributor and the loss of discriminatory power due to incomplete profiles caused by DNA degradation.

Acknowledgments We thank Menarini Silicon Biosystems for the provision of the DEPArray™ system, as well as for the valuable support and Eppendorf for providing necessary laboratory equipment.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

1. Gill P, Brenner CH, Buckleton JS, Carracedo A, Krawczak M, Mayr WR, Morling N, Prinz M, Schneider PM, Weir BS (2003) DNA commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures; DNA commission of the International Society of Forensic Genetics. Forensic SciInt 160:90–101
2. Ulbrich W, Anslinger K, Bäßler G, Eckert M, Fimmers R, Hohoff C, Kraft M, Leuker C, Molsberger G, Pich U, Razbin S, Schneider H, Templin M, Wächter A, Weirich V, Zierdt H, Schneider PM (2016) GemeinsameEmpfehlungen der Projektgruppe "Biostatistische DNA-Berechnungen" und der SpurenkommissionzurbiostatistischenBewertung von DNA-analytischenBefunden. Rechtsmedizin 26:291–298
3. Coble MD, Buckleton J, Butler JM, Egeland T, Fimmers R, Gill P, Gusmão L, Guttman B, Krawczak M, Morling N, Parson W, Pinto N, Schneider PM, Sherry ST, Willuweit S, Prinz M (2016) DNA Commission of the International Society for Forensic Genetics: recommendations on the validation of software programs performing biostatistical calculations for forensic genetics applications. Forensic SciInt Genet 25:191–197
4. Gill P, Jeffreys A, Werrett D (1985) Forensic application of DNA 'fingerprints'. Nature 318:577–579
5. Verdon TJ, Mitchell RJ, Chen W, Xiao K, Van Oorschot RAH (2015) FACS separation of non-compromised forensically relevant biological mixtures. Forensic SciInt Genet 14:194–200
6. Elliott K, Hill D, Lambert C, Burroughes T, Gill P (2003) Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic SciInt 137:28–36
7. Anslinger K, Mack B, Bayer B, Rolf B, Eisenmenger W (2005) Digoxigenin labelling and laser capture microdissection of male cells. Int J Legal Med 119:374–377
8. Anslinger K, Mack B, Bayer B, Eisenmenger W (2007) Sex-specific fluorescent labelling of cells for laser microdissection and DNA profiling. Int J Legal Med 121:54–56
9. Yano S, Honda K, Kaminiwa J, Nishi T, Iwabuchi Y, Sugano Y, Kurosu A, Suzuki Y (2014) DNA extraction for short tandem repeat typing from mixed samples using anti-human leukocyteCD45 and ABO blood group antibodies. Forensic SciInt Genet. 10:17-22
10. Fontana F, Rapone C, Bregola G, Aversa R, de Meo A, Signorini G, Sergio M, Ferrarini A, Lanzellotto R, Medoro G, Giorgini G, Manaresi N, Berti A (2017) Isolation and genetic analysis of pure cells from forensic biological mixtures: the precision of a digital approach. Forensic SciInt Genet 29:225–241
11. Anslinger K, Bayer B, von Máriássy D (2018) Application of DEPArray™ technology for the isolation of white blood cells from cell mixtures in chimerism analysis. Rechtsmedizin 28:134–137
12. Vernarecci S, Ottaviani E, Agostino A, Mei E, Calandro L, Montagna P (2015) Quantifiler® Trio Kit and forensic samples management: a matter of degradation. Forensic SciInt Genet 16: 77–85
13. Vandewoestyne M, Van Hoofstat D, Franssen A, Van Nieuwerburgh F, Deforce D (2013) Presence and potential of cell free DNA in different types of forensic samples. Forensic SciInt Genet 7:316–320
14. Butler JM, Shen Y, McCord BR (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 48:1054–1064