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Research paper

Isolation and genetic analysis of pure cells from forensic biological mixtures: The precision of a digital approach

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ABSTRACT

Latest genotyping technologies allow to achieve a reliable genetic profile for the offender identification even from extremely minute biological evidence. The ultimate challenge occurs when genetic profiles need to be retrieved from a mixture, which is composed of biological material from two or more individuals. In this case, DNA profiling will often result in a complex genetic profile, which is then subject matter for statistical analysis.

In principle, when more individuals contribute to a mixture with different biological fluids, their single genetic profiles can be obtained by separating the distinct cell types (e.g. epithelial cells, blood cells, sperm), prior to genotyping.

Different approaches have been investigated for this purpose, such as fluorescent-activated cell sorting (FACS) or laser capture microdissection (LCM), but currently none of these methods can guarantee the complete separation of different type of cells present in a mixture.

In other fields of application, such as oncology, DEPArray™ technology, an image-based, microfluidic digital sorter, has been widely proven to enable the separation of pure cells, with single-cell precision.

This study investigates the applicability of DEPArray™ technology to forensic samples analysis, focusing on the resolution of the forensic mixture problem.

For the first time, we report here the development of an application-specific DEPArray™ workflow enabling the detection and recovery of pure homogeneous cell pools from simulated blood/saliva and semen/saliva mixtures, providing full genetic match with genetic profiles of corresponding donors. In addition, we assess the performance of standard forensic methods for DNA quantitation and genotyping on low-count, DEPArray™-isolated cells, showing that pure, almost complete profiles can be obtained from as few as ten haploid cells. Finally, we explore the applicability in real casework samples, demonstrating that the described approach provides complete separation of cells with outstanding precision.

In all examined cases, DEPArray™ technology proves to be a groundbreaking technology for the resolution of forensic biological mixtures, through the precise isolation of pure cells for an incontrovertible attribution of the obtained genetic profiles.

1. Introduction

Biological evidence collected as part of crime scene investigation (e.g. blood, body fluids, hair and other tissues) hold a potentially great deal of genetic and non-genetic information.

The analysis of collected biological evidence can provide information on the nature of biological fluids through standard biochemical and immunochromatographic assays. However, the final and most important goal of forensic analysis is to determine a genetic profile to identify the donor [1].

The standard forensic workflow for genotyping includes DNA extraction, quantitation, STR amplification and analysis using commercially available kits. This method is extremely efficient and today a full genetic profile can be obtained from as little as hundreds of picograms of DNA [2].

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Despite this high efficiency, the newest genotyping technologies can be inconclusive in case of biological mixtures, which are composed of biological fluids from two or more individuals. In most of these cases, a mixed genetic profile (*complex DNA profile*) will correspondently be produced. Nowadays, biological mixtures represent one of the major challenges in forensic genetics [2].

In a biological mixture, the genetic contribution of the individuals is generally unbalanced. Even when a mixture is composed of an identical volume of two or more different biological fluids (e.g. blood/saliva), the ratio of correspondent DNAs will still be different, because of the different cellularity present in the fluids. This will further impair the identification process through a series of stochastic effects, such as preferential amplification, which may affect PCR [2].

In many circumstances, in a biological mixture one contributor is represented at a minimal level. The genetic trace of this donor is likely not to be detected because of sensitivity limits or reaction saturation by the major component; typically, the minor contributor of a DNA mixture cannot be detected when ratios exceed 1:20 [3].

The challenge of biological mixture analysis is thus to correctly resolve the complex DNA profile into its components, regardless of the relative DNA quantity and its cellular source.

Today, the standard approach to a complex DNA profile is its statistical deconvolution and the method preferentially applied is Likelihood Ratio (LR) assessment. LR is defined as the ratio of two probabilities of the same evidence that are evaluated under two alternative hypotheses: the prosecution hypothesis (numerator) or the defense hypothesis (denominator) [4]. Elaborated statistical computation of LR for complex DNA profile is generally operated by specifically developed and validated software, based on probabilistic attribution of profile match. Despite the efforts of the community to optimize statistical analysis, genetic profiles deconvolution cannot always be fully achieved [5].

The need for robust solutions for the forensic mixtures problem has generated many attempts to develop alternative approaches. Among these methods, differential DNA extraction is largely applied to stains and vaginal swabs in sexual assault cases [6]. However, this method can only be applied to the separation of the DNA released from a mixture of sperm and epithelial cells and shows some further limitations: the sensitivity with few sperms is relatively low, it does not always guarantee the complete separation of DNA from different cell types, and does not provide the direct evidence of the cellular source.

Recently, next-generation sequencing (NGS) technology, with its high-throughput capacity of massive parallel sequencing (MPS), has also been applied to forensic genetics, increasing informative data by adding to STRs analysis, the SNPs dataset [7]. With NGS, the detection of all DNA contributors present in a mixture is attempted thanks to the deeper sensitivity and broader information reached. Although NGS represents a further analytical method to manage mixtures, it fails to provide a complete and clear resolution of the problem, as profiles attribution is still carried out by statistical analysis [7].

In both approaches, DNA is extracted directly from the biological mixture: cells are disrupted, the DNA is released in solution and remaining cell components are discarded through purification; in this way, the information on cell phenotype gets lost [7].

The most desirable solution for the biological mixture problem would be to physically separate the different type of cells *a priori*, and then perform the standard genetic analysis on a purified cellular source, providing the correspondent genetic profile along with morphological and phenotypical assessment. Many different approaches have been applied for this purpose, such as Laser Capture Microdissection (LCM) and Fluorescence Activated Cell Sorting (FACS). Although improved with time, both technologies never achieved the full endorsement of the forensic community, because they failed in reaching throughput, sensitivity or purity needed [8–10].

In recent years, DEPArray™ Technology (Menarini Silicon Biosystems, Italy, MSB), a highly-automated, image-based system that allows pure cells isolation with single-cell resolution, was successfully applied to different biological settings to purify cells from mixed samples, providing a 100% homogeneous sample for the subsequent genetic analysis [11–16].

A 13 μl suspension of fluorescently labelled cells is loaded into the single-use DEPArray™ Cartridge, which is then inserted into DEPArray™ System. Programmable electrodes are embedded in the microelectronic chip constituting the floor of the manipulation chamber within the cartridge. Activating the electrodes, the dielectrophoretic (DEP) force traps cells in field cages and keeps them in stable levitation, while the system automatically acquires cell images, measures fluorescence intensity and morphological parameters across different fluorescent channels. Collected data are displayed in the CellBrowser™ Software, which provides multiple analysis tools for cells identification and selection: the high level of technology and automation minimizes the operator interaction with the DEPArray™ system to the selection of the desired cells. Selected cells are collected in homogeneous groups, or as single cells, with clean drops of buffer into a 0.2 ml tube, and are then ready for further genetic analysis.

The capability of DEPArray™ technology to deliver pure cells from a mixed population has been widely described in oncology research [11,15,17–21] but it clearly holds a great potential also to relieve the problem of forensic mixture.

This work aimed first to develop a forensic workflow based on DEPArray™ technology and characterize its performance in resolving forensic mixtures. The workflow starts collecting intact cells from the evidence; it includes a step of cell preparation for DEPArray™ experiment and enables standard genetic analysis on the isolated pure cells. The first goal was to identify the different cell types present in simulated mixtures, and deliver pure cells for the genetic analysis. The second aim was to assess the applicability of the standard forensic methods for DNA genotyping and quantitation, developed for DNA extracted directly from biological evidence, to cells recovered by DEPArray™, meeting sensitivity and robustness criteria for human identification.

Finally, the defined workflow was applied to the resolution of some biological mixtures related to a sexual assault case, to explore the possibility to reliably address the forensic mixture dilemma.

2. Materials and methods

2.1. Simulated forensic mixtures analysis

A set of simulated biological mixtures was prepared using blood, saliva and semen, being the biological fluids most frequently found in crime scene investigations. Mixtures were processed to prepare an immunofluorescently labelled cell suspension suitable for DEPArray™ system; pure cells isolated by DEPArray™ technology were analyzed with standard forensic methods. The applied workflow is displayed in Supplemental Fig. S1.

2.1.1. Simulated mixture preparation

Blood, semen and saliva were collected by Carabinieri Scientific Investigation Department (in Italian: Reparto Investigazioni Scientifiche di Roma, Italy—R.I.S.), with written consent from donors. Blood collection (donors n = 5) was performed in EDTA tubes by antecubital venipuncture. Saliva (donors n = 3) and semen (donors n = 2) were collected in sterile tubes.