TECHNICAL NOTE

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Sex-specific fluorescent labelling of cells for laser microdissection and DNA profiling

Abstract Sex-specific isolation of cells from mixtures would greatly facilitate forensic casework. Thus, male and female cell mixtures were marked with a fluorescent X/Y-probe CEP X SpectrumOrange/Y SpectrumGreen DNA probe kit for fluorescence in situ hybridization, and single cells were isolated via laser microdissection (LMD). DNA profiling of LMD isolated, hybridized cells showed usable short tandem repeat profiles for at least 20 cells, which are comparable with results from other studies. To simulate casework samples, the method was also optimized for air-dried samples.

Keywords Laser microdissection (LMD) · Hybridization · Fluorescent labelling

Introduction

Laser microdissection (LMD) is currently the method of choice for single cell isolation [3], and its use in research has become widespread. For example, Budimlija et al. [4] used LMD for the isolation of chorionic villi from admixed maternal tissues for parentage testing. Di Martino et al. [5] demonstrated that the isolation of the lower part of a hair follicle via LMD reduces keratin contamination and thus favours successful DNA profiling. LMD was also used for the isolation of spermatozoa from stained slides containing sperm and vaginal cells [6, 8] and for the isolation of

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digoxigenin labelled, diploid male cells in a male/female epithelial cell mixture [2]. It was shown that LMD could greatly improve the recovery of male DNA, especially in cases with unfavourable mixtures of male and female cells. Both methods allow the detection and isolation of single male cells from a mixture with large numbers of female cells. Conversely, isolation of single female cells from mixtures containing many male cells may also be relevant. However, in such cases, the use of a Y-chromosome-specific probe is not the method of choice, as shown by Anslinger et al. [2], since false negatives can be caused by incomplete hybridization.

In order to obtain different signals for both male and female cells, we used the fluorescent X/Y-probe CEP X SpectrumOrange/Y SpectrumGreen DNA probe kit for fluorescence in situ hybridization (Vysis, Downers Grove, IL) [1, 9]. One SpectrumGreen labelled, Y-specific probe in this cocktail hybridizes to satellite region located Yq12; a second, SpectrumOrange labelled probe hybridizes to an X-specific alphoid satellite DNA located at the centromere Xp11.1-q11.1. After successful hybridization, female cells show two red signals, whereas male cells are recognized by one green and one red signal.

In this current study, mixtures of male and female blood cell samples were stained, and the labelled cells were isolated by means of the SL μCut LMD system from MMI (Glattburg, Zurich, Switzerland). To simulate casework samples, the method was also optimized for air-dried samples. To determine whether the fluorescent hybridization procedure or any of the reagents have an influence on DNA profiling, dilution series of 10, 20, 30 and 40 cells were excised via LMD, and DNA profiling was performed and compared with data published previously [2].
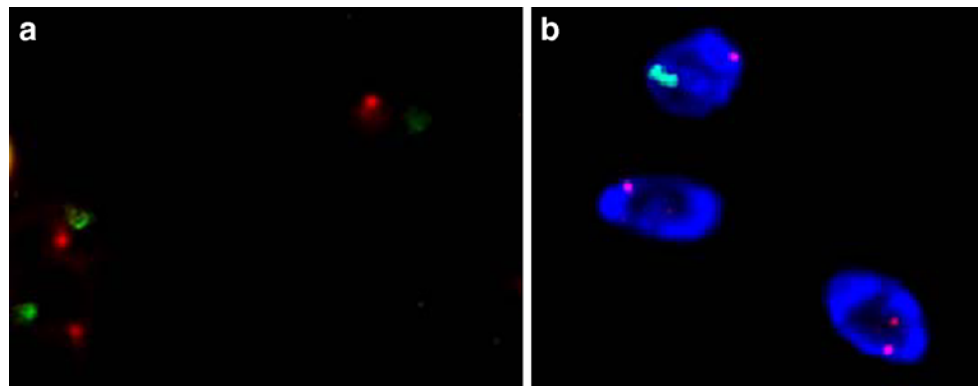
Materials and methods

Preparation of the slides To improve fixation of the cells on the membrane surface, the slides were incubated with poly-L-lysine solution P 8920 (Sigma, Deisenhofen, Germany) for 60 min at 37°C.

Fig. 1 Hybridized nuclei showing **a** three male cells viewed with a double filter and **b** one male (*upper one*) and two female cells (*lower ones*) viewed with a triple filter, which additionally shows the reciprocal staining of the nuclei with Hoechst 33258

Sampling Blood samples were taken from a male and a female individual, and 5 µl from each sample was added to the same slide. The solutions were spread out over an area of 1 cm². Some of the prepared slides were used directly for further treatment, others were air-dried for 1 week, and the cells were then transferred to another slide to simulate handling of dried bloodstains. In order to find a gentle method for the resuspension and transfer of cells from dried bloodstains to slides without disturbing cell structure, different solutions (0.8% Na-citrate buffer, 0.1% SSC, aqua bidest and 1× PBS) and transfer techniques were tested in parallel as follows:

1. Cells were collected with a pre-soaked swab, the swab was washed in 400 µl of each solution, and the cell density was increased by a gentle spinning step.
2. Dried blood samples were overlaid with different solutions, and the cells were collected by gentle pipetting. After drying all slides at 37°C, they were incubated in 0.8% Na-citrate buffer for 30 min at 37°C, fixed in glacial acetic acid/methanol 3:1 for 10 min at −20°C and air-dried.

Enzymatic digestion For enzymatic digestion, both air-dried and native blood samples were treated with pepsin solution (0.1% in 0.1 N HCL) at 37°C. To optimize the digestion of the cells, the enzymatic reaction was stopped at intervals from 20 min up to 2 h. The slides were incubated in PBS buffer including 30 mM $MgCl_2$ for

5 min, followed by 1-min incubation steps in 70, 80 and 100% ethanol. Negative controls, without pepsin digestion, were carried out in parallel.

Hybridization For hybridization, 8 µl of the fluorescence labelled X/Y-probe CEP X SpectrumOrange/Y Spectrum-Green DNA probe (Vysis, Downers Grove, IL, USA) was applied on the sample area of the slide, covered with a glass cover-slip, and hybridization was carried out in a Hybrite/2 J 1131 system (Abbott, Wiesbaden, Germany) using a 3-min denaturation step at 75°C, followed by an overnight hybridization step at 37°C. The post-wash procedure included several washing steps with 0.4× SSC (73°C) and 2× SSC (up to 5 min). The nuclei were stained with Hoechst 33258 (Serva, Munich, Germany).

LMD and DNA extraction From the stained slides, 10, 20, 30 and 40 cells were isolated using the SL µCut LMD system (MMI). These steps were repeated twice. DNA was extracted using the QIAamp DNA Micro kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions.

Short tandem repeat (STR) profiling and electrophoresis conditions A multiplex polymerase chain reaction (PCR) was performed using the AmpFlSTR SGM Plus Kit (Applied Biosystems, PE Corporation, Foster City, CA). From the LMD dissected samples, the whole DNA extract was added to the PCR reaction (at a volume of 50 µl), and
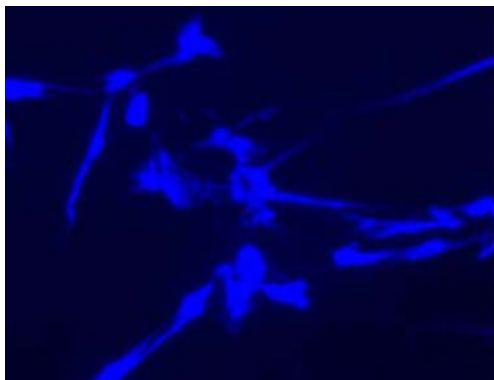

Fig. 2 Cells from a dried bloodstain transferred to the slide with a Na-citrate pre-soaked swab. Nuclei stained with Hoechst 33258
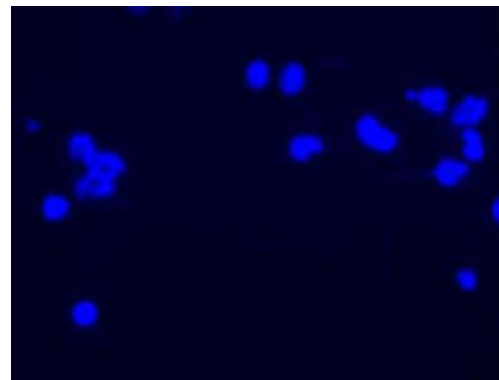

Fig. 3 Cells from a dried bloodstain transferred to the slide with aqua bidest by gentle pipetting. Nuclei stained with Hoechst 33258

34 cycles were carried out. The PCR products were analysed on an ABI PRISM 3100 Avant capillary electrophoresis system (Applied Biosystems).

Results and discussion

For native blood samples, pepsin digestion was unnecessary. With the described hybridization technique and without any further enzymatic reaction, the labelling of almost all male and female cells was possible. The male cells showed a red and a green signal and could be clearly distinguished from the female cells, showing two red signals. Stringent post-wash steps led to clear signals (Fig. 1). A distinct advantage over the digoxigenin method [2] was shown because at any time, female cells can be distinguished from false negatives caused by incomplete hybridization.

In contrast to native blood samples, dried bloodstains have to be treated with pepsin. Without enzymatic digestion, signals were undetectable. After 20 min, almost all cells showed fluorescent signals. With increased incubation times, the number of successfully hybridized cells decreases. Within 20 min of pre-digestion, the probe was able to reach the nuclear DNA, and further destruction of cell structure did not occur.

The most critical point for successful hybridization of dried bloodstains was the transfer of cells from the carrier material to the slide. After the transfer with a swab, the cells showed different degrees of degradation (Fig. 2) and corresponding lower numbers of fluorescent signals. It could be shown that the condition of the cells, especially the nuclei, influences the strength of the fluorescence signal. This is possibly based on the degree of condensation of the probe binding sites, a phenomenon which is comparable to the results of the pepsin tests mentioned above. Moreover, cotton fibres from the swabs were responsible for high background fluorescence. From the various solutions tested in this study, aqua bidest demonstrated the best results (Fig. 3). In summary, overlaying dried blood cells with aqua bidest and resuspension by gentle pipetting yielded superior results, which means the structure of nearly all blood cells could be conserved, and therefore, successful hybridization was possible.

By means of LMD isolation of hybridized cells, a full STR profile could be obtained from samples containing at least 30 cells. Samples with 20 cells yielded a partial profile with one or two locus drop-outs. These results are comparable with earlier published studies [2, 5, 6]. Thus, the entire digestion and hybridization procedure does not significantly influence the sensitivity of DNA profiling.

In summary, successful hybridization can be carried out with both native and dried blood cells. Thus, this method would appear to be suitable for forensic stain investigations. Since male and female cells show different signals, this method can be extended to all mixtures containing biological material from different genders. Clear fluorescent signals greatly facilitate the detection of single cells. Statistical treatment of the mixture becomes unnecessary [7, 10]. However, we are aware that we are still at the beginning. Many other applications like the separation of male and female skin cell mixtures will be tested in further studies.

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