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TECHNICAL NOTE

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Digoxigenin labelling and laser capture microdissection of male cells

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Abstract Laser capture microdissection (LMD) is a relatively new technique for the isolation of single cells. The application in forensic investigations has become more and more widespread, especially to select spermatozoa out of mixtures with vaginal cells. In particular in cases with low numbers of sperm it could be profitable to isolate all male cells (e.g. sperm and male epithelial cells) instead of focussing on the sperm only. Therefore, the specific labelling and detection of the male cells in a male/female cell mixture is necessary. In order to label all cells carrying a Y-chromosome we used a digoxigenin labelled chromosome Y hybridisation probe (Q Biogen). The stained cells were isolated with the SL μCut LMD system from Molecular Machines & Industries AG (MMI). At least ten diploid male cells were required to obtain a partial STR profile, with 20 cells, a full profile could be obtained.

Keywords Laser capture microdissection (LMD) · Chromosome Y specific · Hybridisation

Introduction

Laser capture microdissection (LMD) is a relatively new technique for the isolation of single cells out of larger tissue samples from stained histological slides (Becker et al. 1997). In forensic science, LMD is used for the isolation of spermatozoa from Haematoxylin and Eosin (HE) stained slides containing sperm and vaginal cells (Elliot et al. 2003). These authors could show that the use of LMD greatly improves the recovery of DNA from sperm, compared to the traditional method for the isolation of sperm, the preferential extraction, especially in cases with low numbers of sperm or cases where the original slide is the only remaining evidence.

In order to increase the amount of extracted male DNA in such cases, it could be profitable to isolate all male cells instead of focussing on the sperm only. Since seminal fluid contains not only sperm, but also epithelial cells, there is a good chance of finding further male cells other than sperm. Similarly, the specific detection of male cells in a male/female epithelial cell mixture, for example male cells under female fingernails or male saliva on female skin, could be of real advantage. Therefore, we used a staining method that detects all cells carrying a Y-chromosome (Schwarz et al. 1997) with a digoxigenin labelled chromosome Y cocktail probe from Q Biogen (Q Biogen, Illkirch, France). One Y-specific probe in this cocktail hybridises to multicopy alphoid DNA located at the centromere, a second probe in this cocktail hybridises to short repeated satellites located in the percentric heterochromatin of human chromosome Y. Different mixtures of male and female cell samples were stained, and the labelled male cells were isolated via the SL μCut LMD system from MMI Glattburg, Zurich, Switzerland.

In comparison with other LMD systems the SL μCut works with a different kind of slide. The samples are spread on a membrane which is placed on a special metal holder. The sample is stained on the membrane, and subsequently the sample holder is placed on a glass slide in such a way that the sample side of the membrane is protected by the glass slide. Together, the holder and the slide are placed on the motorised table of the microscope. The laser cuts the membrane around the cells of interest without touching the cell itself (Fig. 1). In contrast to other systems, the cells are not catapulted into a reaction tube by the laser. After cutting the membrane around the cells by the laser power, the isolated cells are securely removed with an adhesive film technology. This adhesive film was fixed on the inner side

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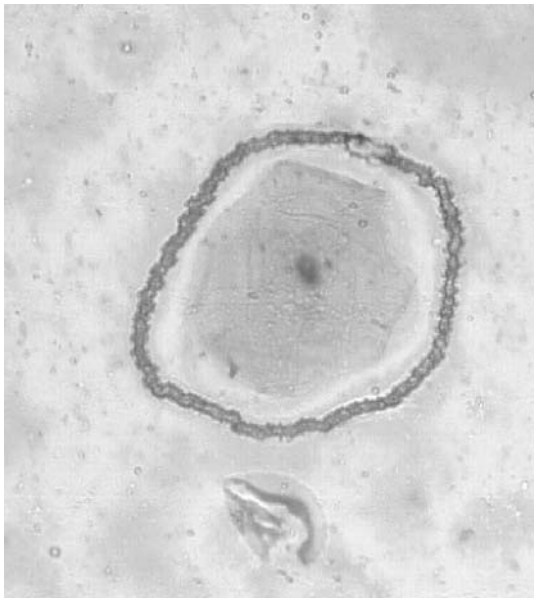

Fig. 1 Buccal cell cut out with the SL μCut from MMI

of the lid of a reaction tube. By lowering the lid down to the membrane only the cut out parts are collected and thus were isolated from the rest of the specimen. The adhesive film only has contact with the reverse of the membrane, avoiding direct contact with the sample side and possible contamination.

In this work, we demonstrate the application of the laser capture microdissection with subsequent STR profiling of mixed samples with different cell types.

Materials and methods

Preparation of the slides For fixation of the cells on the surface of the membrane, the sample was incubated with poly-L-lysine solution P 8920 (Sigma) for 60 min.

Sampling Buccal swabs were taken from a male and a female individual as reference samples. The swabs were incubated in 900 μl 0.9% NaCl. For the unmixed samples, 15 μl of each solution was applied on a slide. For the mixed samples, 10 μl of both the male and the female sample was added to the same slide. The solutions were spread out over an area of 1 cm^2. For the male/female mixtures different sample ratios were prepared. For mixtures of female cells with seminal fluid 100 μl fresh seminal fluid was added to 900 μl female buccal cell solution. After mixing, 15 μl of this mixture was placed on a slide. For the unmixed seminal fluid slides, 15 μl of 1:10 diluted seminal fluid was prepared as well. All slides were air-dried overnight.

Hybridisation The digoxigenin labelled DNA probe, (Q Biogen, Illkirch, France), was resuspended in hybridisation buffer, containing 65% formamide, 2× SSC and 10% dextran sulfate. The DNA probe (1.5 μl/slide) in hybridisation buffer (30 μl/slide) was denatured for 5 min at 75°C and placed on ice until used. The slides were pretreated with hybridisation buffer in the same way. Probe solution (30 μl) was applied to a 22×50 mm hybridisation area, covered with a glass coverslip and sealed with silicon rubber. After hybridisation for 16 h at 37°C, several washing steps were carried out with 0.2× SSC. For the detection of digoxigenin, a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody was used. The HRP was visualised with nitro blue tetrazolium/bromo-chloro-indolyl-phosphate, resulting in a purple colour. To check the reproducibility of the staining technique, all different samples were used at least twice.

LMD and DNA extraction From the stained slides 10, 20, 30, 40 and 50 male cells were isolated with the SL μCut LMD system (MMI). The experiment was repeated three times, respectively. DNA was extracted from these cells using the QIAamp DNA Micro Kit from Qiagen (Hilden, Germany) according to the manufacturers instructions.

In parallel to the LMD procedure, we determined the overall content of DNA and the mixture ratio on the slides. From every dilution step, one slide was swabbed with a wet cotton stick. DNA from these swabs was isolated using the Biorobot EZ1 (Qiagen, Hilden Germany).

Quantification of male DNA The quantification of the male DNA in the different mixtures was carried out using the Quantifiler Human and Quantifiler Human Male DNA Quantifikation Kits (Applied Biosystems, PE Corporation, Foster City, CA) according to the users manual and a 7300 real time PCR sequence detection system from Applied Biosystem.

STR profiling and electrophoresis conditions A multiplex PCR was performed using the AmpFlSTR SGM Plus Kit

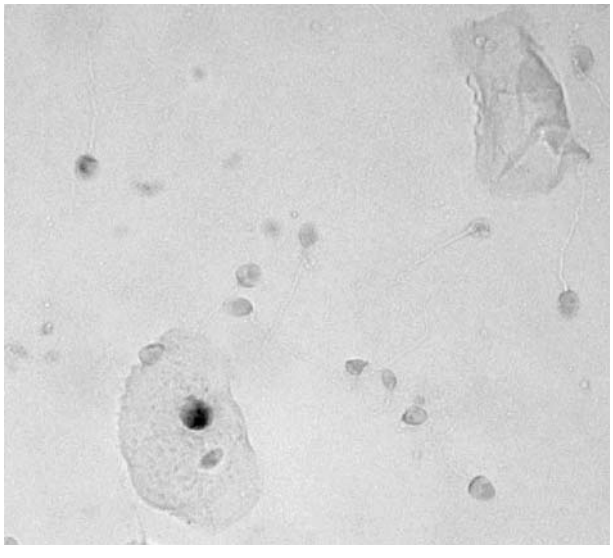

Fig. 2 Example of a stained slide, carrying a female buccal cell/seminal fluid mixture. Beside several sperms a labelled male cell in the lower left corner and an unlabelled female cell in the *upper right corner* of the picture can be seen

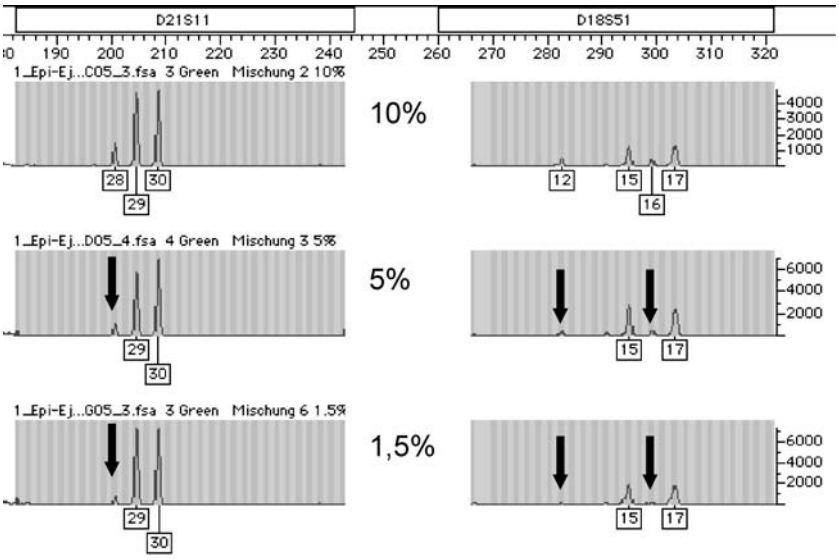

Fig. 3 SGM profiles from a male/female cell mixtures, containing 10, 5 and 1.5% male DNA. The (expected) male alleles are marked with *arrows*

(Applied Biosystems, PE Corporation, Foster City, CA). A DNA sample (1 ng), in a total reaction volume of 25 μl, was used for amplification in a PE 9600 thermal cycler according to the manufacturer's instructions, using 30 cycles. For the LMD dissected samples, the whole DNA extract was added to the PCR reaction (in a volume of 50 μl) and 34 cycles were carried out. The PCR products were analysed on an ABI PRISM 3100 Avant capillary electrophoresis system (Applied Biosystems, PE Corporation, Foster City, CA).

Results

None of the nucleoli from the cells on the female reference slide showed any staining, whereas almost all nucleoli on

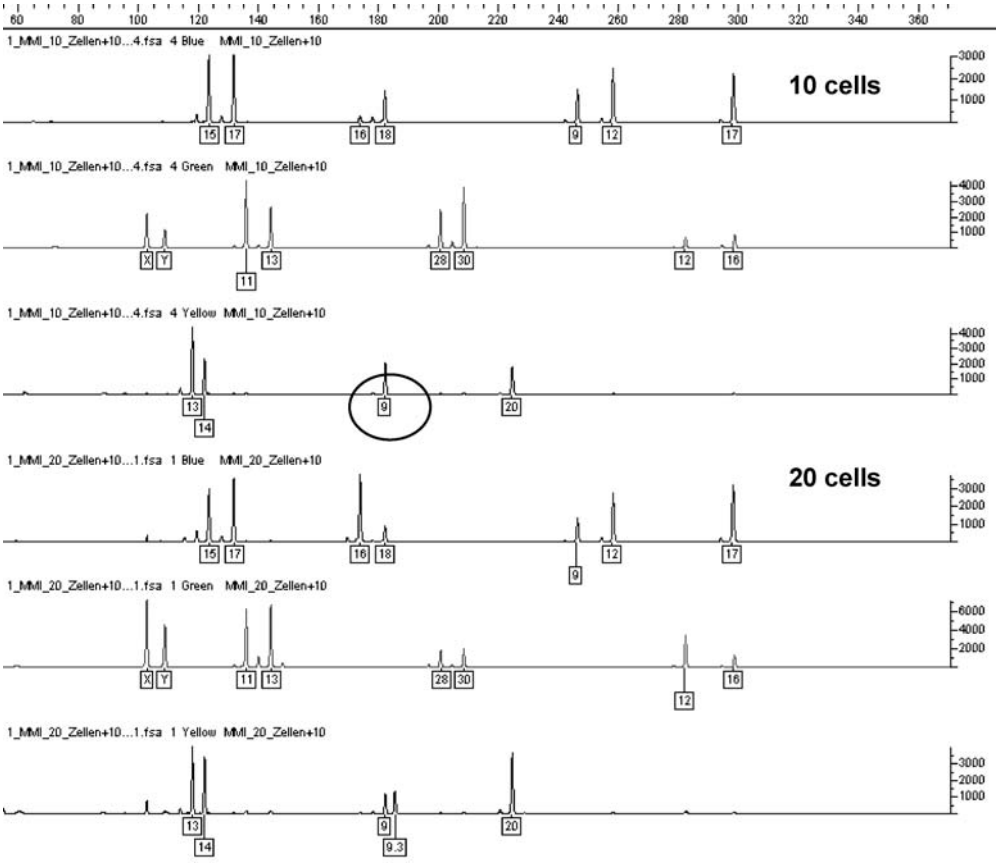

Fig. 4 SGM profiles from 20- and 10-cell samples. The allelic drop out is marked with a *circle*

the male reference slides gave a clear signal. On slides with 1:10 diluted seminal fluid many labelled non-sperm cells beside the sperm could be detected. Similarly, labelled non-sperm cells and sperm were also detected on the slides with the mixed stains (Fig. 2). Because the amount of non-sperm cells in the seminal fluid could differ between male individuals and between semen samples from one male individual, we did not quantify the non-sperm cells in the samples. Because of the small sample size, a reliable estimation of the amount of additional detectable cells is impossible. Y-chromosome carrying sperm cells showed no labelling, because the membrane of the sperm was not disrupted during the staining process and therefore the DNA probe was unable to pass into the cells.

The DNA profiling of the mixed samples without LMD revealed that all alleles of the male component of a mixture could clearly be identified down to a ratio of 5% (Fig. 3). Higher dilutions revealed a profile which showed no male alleles at all, or profiles in which the male alleles could not clearly be distinguished from stutter peaks and/or the female ones (Fig. 3). In contrast, LMD isolation of male cells made it possible to get a full STR profile for a sample that contained only 20 cells (Fig. 4). Even for a ten-cell sample a usable male profile, showing some unbalanced peak heights and one allelic drop out, could be obtained (Fig. 4).

Discussion

In cases where every single cell is important for a successful STR profiling of the male component of a mixture, the new technique presented here can definitely increase the amount of male material that could be extracted out of mixed stains by LMD. Although Y-STRs are a powerful tool for the investigation of mixed stains (Cerri et al. 2003; Sibille et al. 2002: Dettlaff-Kakol and Pawlowski 2002; Parson et al. 2003) their use is limited in cases with unknown suspects. In these cases the goal of each investigation is an autosomal STR profile that can be compared to the respective national DNA database.

Other techniques for the isolation of sperm cells from mixtures, like differential lysis, flow cytometry (Schoell et al. 1999), or even LMD of the sperm (Elliot et al. 2003) make no use of the non-sperm cells that are present in seminal fluid.

Furthermore, our method produces reliable results in cases with aspermatozooic perpetrator or even with men, which have undergone a vasectomy, because in all these cases sperm DNA based methods will fail.

Moreover this technique is suitable to select male cells out of male/female mixtures with identical cell types. On slides carrying the male/female buccal cell mixtures the stained male ones could be clearly identified. Therefore, even in cases with microscopically identical male and female cells, discrimination is possible and statistical treatment of the mixture becomes unnecessary (Egeland et al. 2003; Hu and Fung 2003).

Taken together, the results of our study revealed that this staining method in combination with the LMD seems to be a real advantage when dealing with unfavourable male/female cell mixtures. Unfortunately, the whole hybridisation procedure is time intensive and not easy to handle. However, the microscopical interpretation of the results is very clear and needs no additional experience. Since the staining of the Y chromosome containing cells allows for the selection of male cells from epithelial cell mixtures, the new method has great potential for the investigation of unfavourable male/female mixtures of identical cells.

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