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TECHNICAL NOTE

CRIMINALISTICS

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Single Cells for Forensic DNA Analysis—From Evidence Material to Test Tube

ABSTRACT: The purpose of this project was to develop a method that, while providing morphological quality control, allows single cells to be obtained from the surfaces of various evidence materials and be made available for DNA analysis in cases where only small amounts of cell material are present or where only mixed traces are found. With the SteREO Lumar.V12 stereomicroscope and UV unit from Zeiss, it was possible to detect and assess single epithelial cells on the surfaces of various objects (e.g., glass, plastic, metal). A digitally operated micromanipulator developed by aura optik was used to lift a single cell from the surface of evidence material and to transfer it to a conventional PCR tube or to an AmpliGrid® from Advalytix. The actual lifting of the cells was performed with microglobes that acted as carriers. The microglobes were held with microtweezers and were transferred to the DNA analysis receptacles along with the adhering cells. In a next step, the PCR can be carried out in this receptacle without removing the microglobe. Our method allows a single cell to be isolated directly from evidence material and be made available for forensic DNA analysis.

KEYWORDS: forensic science, forensic DNA analysis, single cells, evidence material, micromanipulation

The method most commonly used in forensic DNA analysis is the amplification of short tandem repeats (STRs) of nuclear DNA (1). Particularly, in cases for which there is insufficient material for STR typing, the forensic analysis of mitochondrial DNA (mtDNA) is standardly used as a supplementary measure (2–6). For small amounts of trace evidence, or evidence that is badly preserved, the success of standard STR and mtDNA analyses is, however, limited. A further problem that arises for extremely small amounts of cellular material is the additional loss of DNA during preparation and DNA extraction procedures. This problem could be sidestepped by directly taking single cells for DNA analysis from the evidence material. Single-cell analyses would also allow the separation of mixed traces (7) into the traces of individual contributors.

Several studies have demonstrated that the analysis of forensically relevant DNA segments from single cells is also possible (8,9). However, in these cases, the single cells were obtained from blood samples either by flow cytometry or by means of a capillary from a gel in a Petri dish. Both methods have in common that they can only be applied for sufficiently large amounts of cell or trace material. The option of being able to isolate single cells for forensic DNA analysis would, however, be of particular interest in cases where only very small amounts of trace material are available or where traces are mixed.

Our investigative approach for this project was influenced by the experience that standard procedures frequently did not yield results in forensic casework. This was because of either the small amounts of available cells or traces being mixed. We first began to experiment with the isolation of cells using the equipment used for in vitro fertilization (8). Although we were successful in isolating single cells with this method, the necessary step of transferring the cells from the material evidence to the Petri dish involved the risk of losing material. We therefore developed the method presented here to allow the isolation and transfer of single cells directly from material evidence to the reaction tube.

The purpose of this study was thus to develop a method that would enable the detection, morphological assessment, and lifting of a single cell directly from evidence material, without the necessity of intermediary steps in which cells or DNA are lost, such as in the preparation of swabs from evidence material. The aim was to lift cells directly from evidence material and to transfer these to a PCR tube for later forensic DNA analysis in one quality-controlled step.

Materials and Methods

Equipment

A SteREO Lumar.V12 high-resolution stereomicroscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), equipped with a UV light unit, was used to detect the individual cells and to visually control their transfer. The UV light unit contained several filters that allowed the lighting to be adjusted for different surface materials. For this study, aura optik developed a motorized micromanipulator that was microscope compatible for the transfer of cells (aura optik GmbH, Jena, Germany). The system could not only be steered via joystick but could, optionally, also be programmed for automated workflows. The manipulator could be moved in four directions (horizontally: forward–backward [*y*-axis] and left–right [*x*-axis]; vertically: up–down [*z*-axis]; diagonally: along the axis of the attached micro-instrument [*m*-axis]) (Fig. 1). As an additional

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function, the attached microtweezers could be opened and closed. Dissecting tweezers (type N5) from Dumont were used. Commercially available microglobes made of various materials (glass, dextran, polysterol, polymethylmethacrylate) were used as carriers to lift the cells from the evidence material (Table 1). The principle underlying the use of microglobes here is that of physical adhesion. In choosing a microglobe, it was important to observe that the adhesive force of the microglobe surface was greater than the adhesive force of the surface on which the cell rested. The cells were then deposited either in MicroAmp Reaction Tubes with cap, 0.2 mL from ABI (Foster City, CA), or on AmpliGrid® microscope slides from Advalytix (Concord, MA) (10).

Preparatory Measures and Contamination Prevention

Buccal samples were taken from test persons with sterile swabs. Then, to create "evidence material," the swabs were smeared across the surfaces of conventional glass slides as well as the metal blades and plastic handles of standard scissors. The scissors had been cleaned beforehand with a 70% alcohol solution, to which dishwashing detergent had been added (1–2 drops per 100 mL); they were then treated with sodium hypochlorite (2%), finally rinsed with demineralized, decontaminated water (ddH_2O), and dried.

Before and after working with the "evidence material," the stereomicroscope used for the preparation of the single cells, the steering module, the micromanipulator, and the microtweezers were first treated with DNA Exitus Plus from Applichem (Boca Raton, FL), following the manufacturer's instructions, and were then wiped down, using a 70% alcohol solution as a disinfectant.

During all preparatory work, protective clothing, disposable gloves, and masks were worn so that during the trial run testing, the technical aspect of the procedure was conducted as if DNA analysis of evidence material was already being performed. Test persons had no access to the work bench where the samples were prepared.

Preparation of Single-Cell Specimens—Position of the Microglobe

In a first step, a microglobe was picked up with the tweezers, which were fixed to the microscope stage at an angle of 32°. The microglobes were on a glass microscope slide with a central well (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). It was important to ensure at 150× magnification that the microglobe was positioned far enough forward between the tweezer tips so that it protruded below them, while still being firmly gripped. A mirror, which was placed on the microscope stage at an angle of 45°, was used to monitor the best position of the microglobe (Fig. 2).

Preparation of Single-Cell Specimens—Finding and Lifting Cells from Evidence Material

A number of days or even weeks lay between the preparation of the artificial "material evidence" and the isolation of single cells from it; consequently, the surface of the material evidence was thoroughly dried. Immediately prior to preparation, the surface of the material evidence was slightly moistened by spraying it with aqua dest. from an atomizer. With the aid of UV light, a nucleated cell was then found and brought into focus. The manipulator was adjusted so that the microglobe held with the tweezers was as close as possible to the cell and could be seen just above the surface of the evidence material (Fig. 3). Then, the manipulator was started to

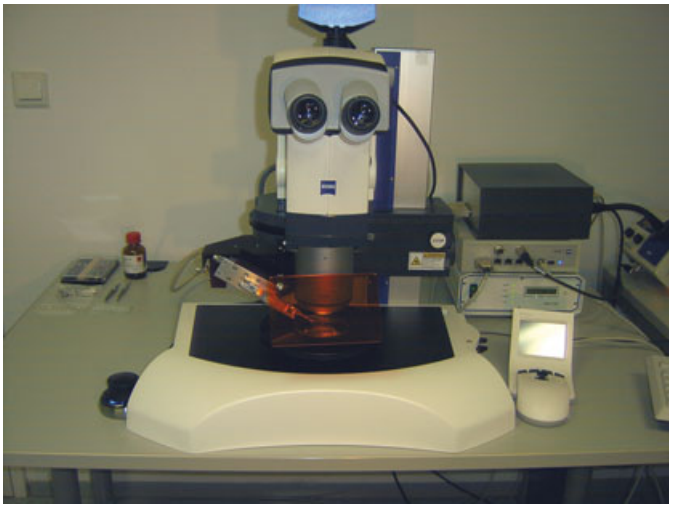

FIG. 1—*Work place: SteREO Lumar.V12 from Zeiss with the digital micromanipulator from aura optik.*

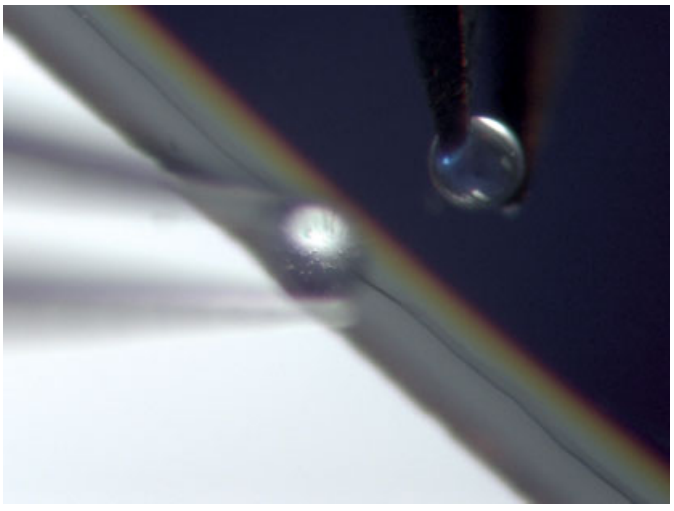

FIG. 2—*Controlling the position of the microglobe (glass) between the tweezer tips with the aid of the mirror.*

TABLE 1—*Types of microglobes tested in this study.*

Name	Manufacturer	Material	Diameter (μm)	Fluorescence of the Microglobes	Costs
Solo Hill Microcarrier	SIGMA®	Polystyrene, Pronectin F coated	125–212	Strong	152€ per 10 g
PLPM-100	G.KISKER GbR	Polymethylmethacrylate	50–100	Weak	29 € per 10 g
PGB-20	G.KISKER GbR	Glass	100–200	Weak	20 € per 100 g
Cytodex™	SIGMA®	Dextran	131–220	None	74 € per 5 g

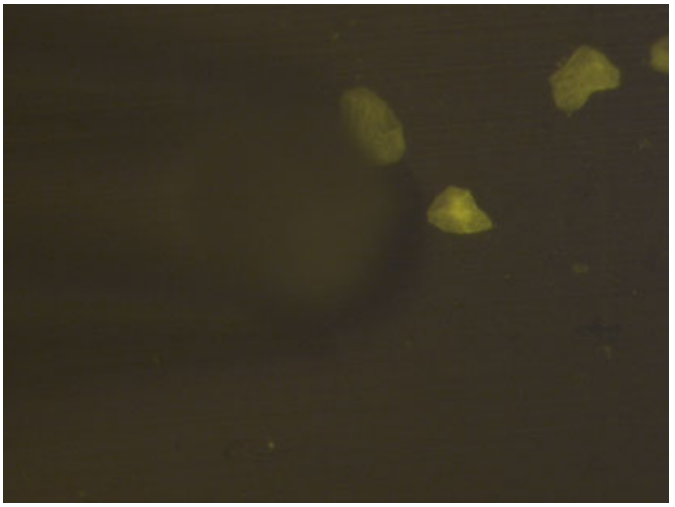

FIG. 3—*Finding a cell on the metal blades of scissors.*

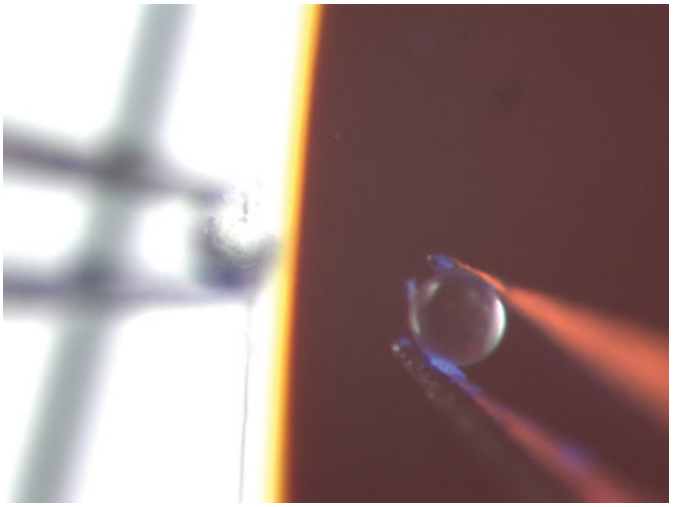

FIG. 5—*Viewing the lifted cell on the microglobe (polymethylmethacrylate) with the mirror.*

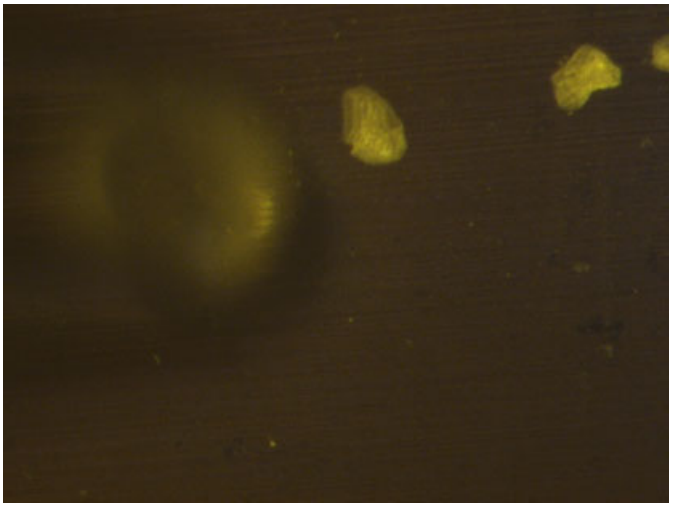

FIG. 4—*Lifting a cell with the microglobe (polymethylmethacrylate).*

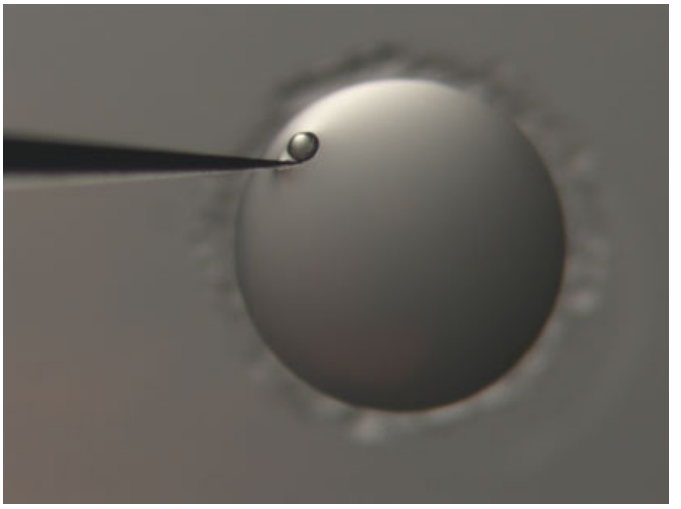

FIG. 6—*Depositing the microglobe (glass) with the adhering cell on a drop of water on the AmpliGrid®.*

perform a series of programmed, automated movements: the globe moved from left to right and back again for a defined length on the horizontal axis. At each point in which the direction of movement changed, the microglobe was lowered by a programmed distance. This way, the microglobe could be brought closer in small steps until it touched the surface of the evidence material and the cell adhered to it (Fig. 4). When the cell could no longer be seen on the surface of the material evidence through the microscope, it had been picked up by the microglobe (Fig. 4). At precisely this moment, the movement was stopped and the manipulator was lifted up, so that the evidence material could be safely removed from the microscope stage without touching the microglobe again. The adhesion of the cell to the bottom surface of the microglobe was once again controlled with the mirror (Fig. 5).

Preparation of Single-Cell Specimens—Transfer into PCR Tube

The microglobe along with the adhering cell was deposited either in a PCR tube (MicroAmp Reaction Tube with cap; 0.2 mL; ABI) or on an AmpliGrid® microscope slide from Advalytix. For this purpose, a drop of water (≤1 μL) was placed in the PCR tube or, respectively, on the AmpliGrid®. The AmpliGrid® was placed directly on the microscope stage, and the microglobe was deposited on top of the water drop. This procedure was monitored through the microscope (Fig. 6).

To facilitate placing the cell in the PCR tube, the tube was aligned with the manipulation tool by a holding device. The microglobe could then be placed in the tube using the diagonal movement of the micromanipulator (Fig. 7*a*,*b*).

Results

Using the available lighting variants, it was possible to find epithelial cells on all of the tested surfaces. The UV light unit was particularly useful in discerning the nucleus and the cell edges.

Single cells could repeatedly and easily be lifted off glass, metal, and plastic with all types of microglobes used. However, with respect to manageability, dextran microglobes had the disadvantage of being soft and of being deformed by the pressure of the tweezer tips, which made lifting of the cells more difficult at times. The glass microglobes, for their part, were delivered by the

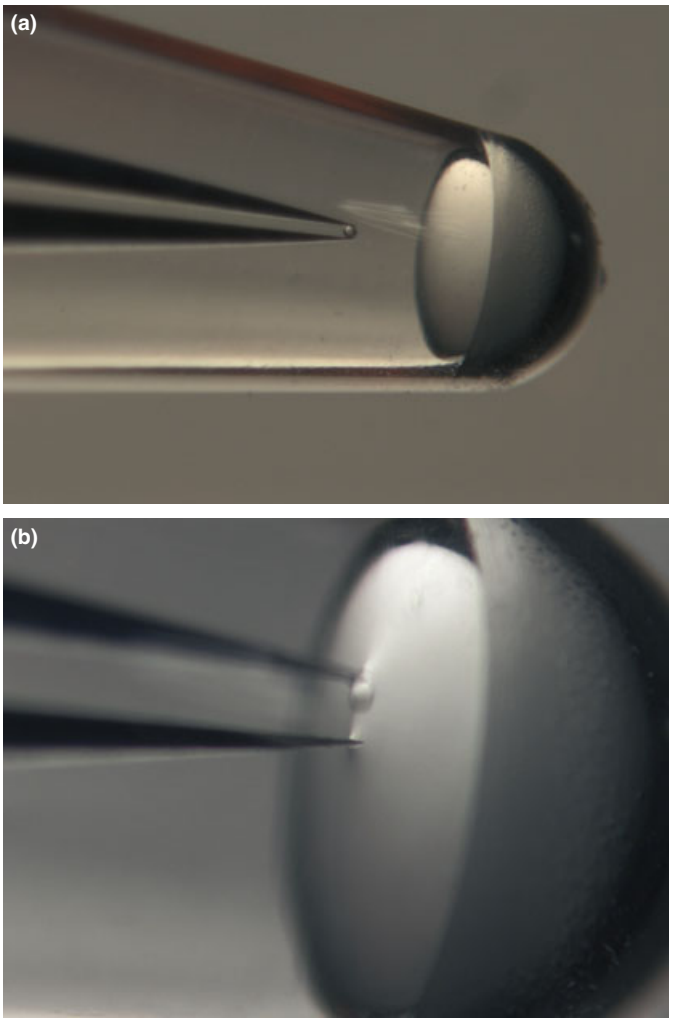

FIG. 7—*(a, b) Depositing the microglobe (glass) with the adhering cell on a drop of water in the PCR tube.*

manufacturer along with glass fragments and oval shapes, so that usable microglobes had to be painstakingly selected first. Overall, the microglobes made of polystyrol and polymethylmethacrylate were best. An additional advantage of the polystyrol microglobes was their somewhat larger diameter (Table 1).

The following values for programming the micromanipulator were determined as being best for lifting cells from the surfaces tested in our study: length of movement along the *x*-axis: 300 μm and approaching distance per run along the *z*-axis: 1 μm.

A single cell transferred to the drop of water on the AmpliGrid® could be seen adhering to the microglobe on the AmpliGrid® after the water had evaporated (Fig. 8). This was not possible for the cell transferred to the water drop on the bottom of the PCR tube.

Discussion

In this study, it was possible to develop a technique of securing single epithelial cells directly from glass, metal, and plastic surfaces. It was also possible to transfer the isolated cells directly into reaction tubes or onto a chip (AmpliGrid® from Advalytix). The final step of the procedure could be programmed to be fully automatic.

The stereomicroscope with its UV light unit, an integrated part of the developed system, not only allowed finding cells on the surfaces but also allowed the morphological assessment of the edges and nucleus of single cells.

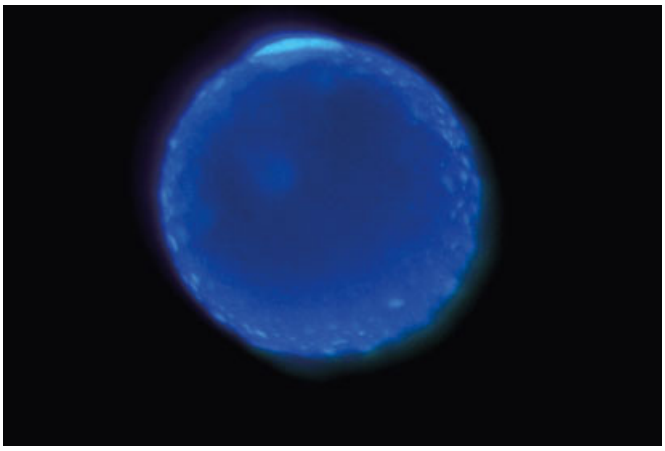

FIG. 8—*Monitoring the presence of a cell adhering to a microglobe (polystyrene) on the AmpliGrid® after evaporation of the water.*

For the first time in forensic DNA analysis, this method opens up the possibility of identifying single cells on evidence material, of selecting these on the basis of morphological criteria, of lifting them off the surface, and of directly transferring them to a reaction tube. This method could be used for evidence material with only small amounts of cellular material. By directly obtaining single cells from evidence material, the necessity of swabbing the surface of evidence material—at present a common procedure in forensic analysis, accompanied by the danger of losing trace material—is no longer required. The amount of suitable cells for STR analysis can also be increased if several cells isolated in this way are transferred to the same reaction tube.

A further application of the method could be single-cell forensic DNA analysis (8) sequencing the D-loops of mtDNA. This could be a last resort in cases for which the usual mtDNA analysis did not yield results, indicated mixed traces, or in which there were not enough cells for conventional mtDNA or STR analyses right from the start. If, for example, 15 cells were needed for PCR, 15 microglobes, each with an adhering cell, could be placed in the reaction receptacle. It is not to be expected that the microglobe material would interfere with the PCR; there could, however, be a volume problem when using an AmpliGrid®.

Last but not least, this method offers the possibility of isolating single cells from a mixed sample. In this case, several single cells would have to be isolated and analyzed until both, or all, originators of the biologic trace were determined. The exact number of cells that would have to be analyzed in such cases would depend on the ratio of mixing. With our procedure, picking up a cell from the surface of material evidence and placing it in a reaction tube or on an AmpliGrid® take about 10 min. We expect that the procedure time will be reduced even further by the automated placing of microglobes in reaction tubes or on AmpliGrids®; this method is currently being developed.

In cases of mixed traces, where only one man and one woman are involved, the traces can be separated following the use of fluorescence *in situ* hybridization to decipher between female and male epithelial cell donors (11,12). Epithelial cells from two male donors are more difficult to decipher. Several cells are then needed for PCR.

Of the two transfer options, PCR tube or Ampligrid® microscope slide, the latter has the advantage of allowing the presence of the

cell on the slide to be verified. It is also possible to visualize a cell adhering to a microglobe in a PCR tube, but this is only possible before the microglobe touches the water and is complicated. To visualize the cell on the microglobe in the PCR tube, it is best to apply a small drop of silicon glue to the wall close to the bottom of the tube instead of a water drop and to stick the microglobe onto this so that the cell is projected into the space of the tube. This method is, however, laborious and error prone, as the microglobe slips off when too much glue is used and falls off when too little glue is used.

No particular advantages or problems were found in regard to the surface materials of the material evidence. Although the red plastic grip of a pair of scissors showed the highest fluorescence in UV light, cells could be distinguished both on the basis of morphology and through differences in the fluorescent intensity. On the whole, the detection and lifting of cells was somewhat easier for metal surfaces. Further studies with additional surfaces and with other types of biologic fluid, such as semen or blood, are still necessary.

All steps of the procedure, from finding the cell on the objects/evidence material to lifting and transferring it to a receptacle for a subsequent PCR, take place at the very same work place. This, along with the fact that all steps can be visually controlled through the microscope, provides the certainty that only one cell is being analyzed, rather than none, several, or an unknown number.

The density of cells on the material surfaces was low in our experiment, which should emulate the situation found on ''real'' material evidence in casework. In cases with high cell densities, it could prove difficult to pick up only one cell with a microglobe. Microglobes with multiple adhering cells would, however, be discovered through the visual control. In such cases, the microglobe would have to be disposed of because there is no means of removing individual cells from its surface. In cases with high cell densities on material evidence, conventional preparation methods for a forensic DNA analysis would be preferable anyway.

Single cells can also be reliably isolated and visually controlled with laser microdissection. This method is applied in forensic identity analysis but, in contrast to the method presented here, has the drawback that tissues either have to be fixed in formalin or have to be paraffin-embedded and microscope slides have to be treated before cells can be transferred to them (11–15). The use of laser microdissection as a method of isolating cells directly from evidence material is therefore currently not feasible.

We hope that the technique presented here will find application in forensic DNA analysis in cases with problematic evidence material. Our study has shown that obtaining single cells from evidence material is technically feasible. In a next step, we will conduct an experimental series in which we will analyze the DNA of cells isolated in this manner. Preliminary trials have been successful.

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