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REVIEW ARTICLE

Laser capture microdissection in forensic research: a review

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Abstract In forensic sciences, short tandem repeat (STR) analysis has become the prime tool for DNA-based identification of the donor(s) of biological stains and/or traces. Many traces, however, contain cells and, hence, DNA, from more than a single individual, giving rise to mixed genotypes and the subsequent difficulties in interpreting the results. An even more challenging situation occurs when cells of a victim are much more abundant than the cells of the perpetrator. Therefore, the forensic community seeks to improve cell-separation methods in order to generate single-donor cell populations from a mixed trace in order to facilitate DNA typing and identification. Laser capture microdissection (LCM) offers a valuable tool for precise separation of specific cells. This review summarises all possible forensic applications of LCM, gives an overview of the staining and detection options, including automated detection and retrieval of cells of interest, and reviews the DNA extraction protocols compatible with LCM of cells from forensic samples.

Keywords Laser capture microdissection · Forensics · Sexual assault · Cell separation techniques

Introduction

DNA analysis has become a crucial tool in suspect identification. Successfulness of the analysis depends on the ability to obtain interpretable DNA profiles. Different factors may lead to genotypes that are difficult to interpret: low DNA yields due to DNA damage or degradation and/or presence of PCR inhibitors may give rise to low intensity profiles, whereas presence of biological material of different persons may result in mixed profiles.

Biological stains from two or more individuals will result in a mixed genotype if they cannot be separated prior to DNA analysis. Mixture interpretation is often very complex [1]. Moreover, mixed genotypes have a lower probative value and are difficult to convince a jury. Therefore, in biological evidence containing cells from different individuals, successful separation of the offender's cells from those of the victim is very helpful for unambiguous genotyping. Even in a two-person mixture, when there are shared alleles between the major and the minor profiles, the interpretation becomes difficult—especially in mixtures where the minor profile is less than one third of the level of the major profile [2]. Therefore, the development of separation methods reduces the need for mixture interpretation.

The laser capture microdissection (LCM) technology represents a significant improvement in cell separation methods [3]. It combines existing light microscopic instrumentation with laser beam technology and allows targeting of specific cells or tissue regions that need to be separated from others. Isolation of these cells or tissue sections occurs, under direct visualisation, into separate tubes for direct DNA extraction and analysis.

There are two main classes of LCM systems: ultraviolet (UV) cutting systems [4–6] and infrared (IR) capture systems [7, 8]. After visualization via microscopy, the cells of interest are isolated by focused laser energy (UV systems) or transferred to a thermoplastic polymer with formation of a polymer-cell composite (IR systems) [3]. In contrast to the IR systems, there is no heating or cooling of

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a plastic membrane in the UV systems, thus avoiding the theoretical disadvantages of melting and solidifying of plastic that occur in the IR systems [9]. The UV systems are completely non-contact systems and are to be preferred in the forensic context because there is no risk of contamination from cells non-specifically adhering to the thermoplastic film, which is inherent to the IR systems. An overview of the different features of UV and IR systems is given in Table 1.

Low-template (LT) DNA analysis typically refers to less than ~100 pg of input DNA into a PCR. This technique is sensitive enough to analyse just a few cells [15], which is often the amount of cells that will be recovered by LCM in forensic case samples. Nevertheless, all methods used to analyse LT DNA suffer from several disadvantages, primarily derived from stochastic variation: Allele drop out because of preferential amplification of one allele from a heterozygote locus, stutter peaks falsely considered as alleles and risk for contamination leading to the amplification of alleles that are not associated with the crime stain [16]. For example, Sanders et al. showed that allelic imbalance may occur in LT samples of LCM isolated spermatozoa (http://www.ncjrs.gov/pdffiles1/nij/grants/217268.pdf). This is not surprising, as spermatozoa are haploid cells. Nevertheless, one of the challenges of LT DNA analysis lies in the generation of reliable DNA profiles. LCM makes it possible to specifically isolate selected cells, resulting in pure samples for LT analysis. As only intact cells are microdissected, it is also useful to avoid cell-free DNA originating from lysed cells, which may be problematic in LT DNA analysis. Moreover, the DNA of the LCM-isolated cells can be extracted in very low volumes which is also beneficial for LT analysis.

In forensics, not only freshly prepared but also archived samples can be used for LCM. Even years after a crime, a genetic fingerprint obtained from specific cells can help to identify the culprit [17]. This review will summarise all forensic applications of LCM, give an overview of the staining and detection options, including automated detection and retrieval of cells of interest, and review the DNA extraction protocols compatible with LCM of cells from forensic samples.

Physical and chemical separation methods

Until now, most studies have concentrated on the development of separation methods for sexual assault crimes. In these cases, spermatozoa are typically the biological material of interest. However, these cells are often only present in minute amounts, in contrast to the overwhelming amount of vaginal, rectal or buccal cells from the victim.

The differential lysis method [18–20] has long been the gold standard for separating spermatozoa from epithelial cells. Although this method can theoretically provide two fractions, one comprising offender's DNA and the other containing victim's DNA, the separation is not always complete, resulting in mixed genotypes [20].

As an alternative, the use of Y-chromosome STR analysis has been proposed to detect the male component in mixed stains when the DNA of the male contributor is present in very small amounts [21, 22]. Nevertheless,

Table 1 Overview of the different features of UV and IR laser capture microdissection systems

	Ultraviolet	Infrared
Operating wavelength	320–400 nm	812 nm
Focusing width	Shorter wavelength allows focusing of the laser light in the sub-micron range [10]	Focusing diameter of the laser beam can be adjusted from 7.5 to 30 μm [9, 10]
	More precise cutting enables single cell and subcellular microdissection	Subcellular microdissection is impossible
Sample retrieval	Photovolatilization of cells surrounding a selected area [11]; subsequently: ejection against gravity, falling by the force of gravity or separation by electrostatic forces (depending on the system) [10]	Transfer of laser energy to a thermolabile polymer thus forming a polymer-cell composite [11]
	Contact-free	Not contact-free (higher risk of contamination with non-selected material)
Impact on cellular biomolecules	High photon density (cold laser)	Generated heat (90°C) may potentially be harmful, but the thermal effect is transient [12, 13]
	Minimal heat generation [11]	Alterations in DNA, RNA and protein content are not measurable [3, 9, 11]
	Absorption maxima of DNA, RNA and proteins lie outside the operating wavelength	
	No harm to DNA, RNA and proteins [14]	

unlike autosomal STRs, Y STR profiles are identical for all paternal relatives [23]. Moreover, due to the lack of Y-chromosome recombination, Y STR haplotype diversity is lower than that of similar autosomal STR panels. Therefore, the observation of a Y STR match does not possess the same power of discrimination as an autosomal STR match. Some are of the opinion that Y STR typing can only be used for exclusion and not for inclusions [24].

Alternatively, various physical separation methods have been reported. In 1998, Chen et al. developed a filtration method to separate spermatozoa from epithelial cells based upon differences in size and shape [25]. Most of the spermatozoa (70%) present in the sample cross the filter, while the epithelial cells remain on top of it. Nevertheless, about 1% to 2% of the epithelial cells also cross the filter, as well as nuclei from lysed epithelial cells, resulting in mixed genotypes.

Fluorescence-activated cell sorting is another cell separation method in which cell populations are sorted based on immunolabelling. The major disadvantage is that it is only applicable on fresh vaginal lavages and not on vaginal smears or archived material [26]. Moreover, although this technique is well suited for cells in suspension, it does not lend itself to separation of regions of interest in tissue preparations [3] e.g. for parentage testing on abortion tissue.

In 2001, researchers from the University of Virginia started developing a cell separation method based on magnetic-activated cell sorting (http://www.healthsystem.virginia.edu/internet/news/Archives01/forensic.cfm). The biggest challenges of this antibody-based approach lie in the identification of specific monoclonal antibodies that target the sperm cell surface and in the stability of the sperm cell surface antigens in degraded forensic samples. A monoclonal antibody specific for the sperm head antigen, equatorial segment protein and a monoclonal antibody specific for the sperm flagellar antigen, calcium binding tyrosine phosphorylation-regulated protein were formulated in this study (http://www.ncjrs.gov/pdffiles1/nij/grants/220289.pdf).

More recently, Horsman et al. described a microfabricated device for the separation of sperm and epithelial cells. This method exploits the differential physical properties of these cells, resulting in sedimentation and adsorption of epithelial cells to the bottom of an inlet reservoir on a glass microdevice. A buffer flow causes the migration of the sperm cells towards the outlet reservoir while the epithelial cells remain in the inlet reservoir [27]. However, the sperm recovery was only about 25% or less.

All methods described above have disadvantages relating to the efficiency of mixture separation, yield and ability to work with very minute amounts of starting material or archived material. Therefore, most researchers have been concentrating on the use of LCM to address these drawbacks. LCM offers the possibility of specific collection of the target cells from mixed samples. Moreover, microdissection is performed under direct microscopic visualization. Using the most recent UV and IR LCM systems, microscopic inspection of the collection device after LCM makes it possible to verify that the correct cells have been isolated.

Forensic applications of laser capture microdissection

Most reports have been concentrating on the use of LCM to isolate spermatozoa in sexual assault cases [28–33]. The first application of LCM in the investigation of sexual assault was described by Elliott et al [28]. These authors used an IR system to separate the spermatozoa from the victim's cells. In a comparative study, 16 pairs of slides were processed by either LCM or differential lysis [18]. This comparison conclusively demonstrated that LCM performed significantly better than differential lysis, since in 15 out of 16 sample pairs, LCM resulted in the greatest likelihood ratio, usually by several orders of magnitude. However, male/female mixed genotypes were still relatively common. Possibly, this was due to adherence of intact epithelial cells to the thermoplastic film or to adherence of female DNA from lysed epithelial cells to the sperm heads [34]. Time since intercourse (TSI) appeared to affect the success rates (in terms of percentage of the male profile recovered), independently of the number of spermatozoa isolated or the age of the slide. The longer the period of TSI, the more apoptosis occurs, resulting in degraded DNA, notwithstanding the morphologically normal appearance of the sperm heads [28].

Additionally, Di Martino et al. state that although the sperm heads may appear to be intact, the DNA inside may be degraded as a consequence of the fixation and staining procedure [29]. These authors used an UV system for the microdissection of 10, 20 and 30 cells from semen smears. They obtained useful DNA profiles, but at any number of cells isolated, the profiles showed at least a few typical allelic drop-outs. In contrast, Seidl et al. sampled epithelial cells using an UV system and obtained complete DNA profiles if a minimum of 10 diploid cells were isolated [30].

Sanders et al. used a UV system in combination with membrane-coated slides. In this setup, the laser beam is used to cut the membrane film around the cell(s) of interest and the material is collected by gravity into the cap of a PCR tube which is mounted below the stage [31]. The use of membrane-coated slides comes with several disadvantages: the membrane slides are quite expensive in comparison to normal glass slides and are difficult to use in combination with traditional staining techniques [35]. Moreover, they can only be used in new sexual assault

cases, as the vaginal smears need to be made directly on the membrane slides. This is a major disadvantage, as LCM frequently needs to be performed on archived material, where the smears have been made on routine glass slides. In contrast, our group showed that LCM can be performed on normal glass slides, allowing its use in existing preparations from sexual assault cases [32].

As spermatozoa are haploid cells, they contain only half the genetic material of the donor organism. Lucy et al. calculated the theoretical number of cells needed for a full representation of the alleles comprising the donor profile. To obtain the highest possible probability for a complete DNA profile, between 15 and 20 intact and not-degraded haploid cells need to be pooled [36]. Using LCM, robust DNA profiles without allelic drop-out could consistently be obtained from as little as 30 spermatozoa recovered from postcoital samples [32].

Since seminal fluid does not only contain spermatozoa but also epithelial and other cells, isolation of male cells is a good alternative for azoospermic offenders. Similarly, specific detection and isolation of male cells in male/female mixtures such as fingernail scrapings, bite or licking traces and male/female blood mixtures could be a major advantage. Therefore, several groups developed staining methods to perform sex-specific labelling of cells for LCM [37–41]. To distinguish male from female cells, fluorescence in situ hybridization (FISH) is performed using Y chromosome specific probes.

Anslinger et al. showed that DNA profiling of mixed samples without LCM could identify all alleles of the male component of the mixture down to a ratio of 5%. Below this ratio, profiles 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 alleles were recovered. In contrast, LCM of digoxigenin labelled, diploid male cells in a male/female epithelial cell mixture, made it possible to obtain complete DNA profiles from a sample that contained only 20 male cells [37]. These data demonstrate that LCM greatly improves the recovery of male DNA, especially in cases with low amounts of male cells in mixtures containing excessive numbers of female cells. Conversely, isolation of female cells from mixtures containing many male cells may also be relevant in some cases, e.g. for the detection of female cells on post-coital penile swabs or post-coital condoms. Then, the sole use of a Y chromosome specific probe is not the method of choice, since false negatives can be caused by incomplete hybridization [37, 38]. In this case, both X and Y chromosome-specific probes, labelled with different fluorochromes, need to be used. Male cells will be recognised by the presence of two different FISH signals, while female cells will contain two FISH signals of the same colour [38–40]. Figure 1 shows the difference between cells only labelled with a Y chromosome specific probe (Fig. 1a) and cells labelled with both an X and a Y chromosome specific probe (Fig. 1b).

Most reports on sex-specific identification use standard FISH techniques, requiring fixation of the cells on the microscope slide prior to the FISH procedure. Our group reported the use of suspension FISH (S-FISH) [41]. Here, the whole FISH procedure is performed with the cells in suspension, which makes it possible to perform on vaginal and rectal washings and soaked off biological stains. After the S-FISH procedure, the cells are cytospun on a microscope slide. The most important advantage of this procedure is that the cells are less tethered to the microscope slide in comparison to the traditional FISH protocols, because only one short fixation step is performed instead of the ethanol series that are used in the traditional FISH protocols. As a consequence, the cells detach easier upon LCM, resulting in a higher LCM efficiency and the

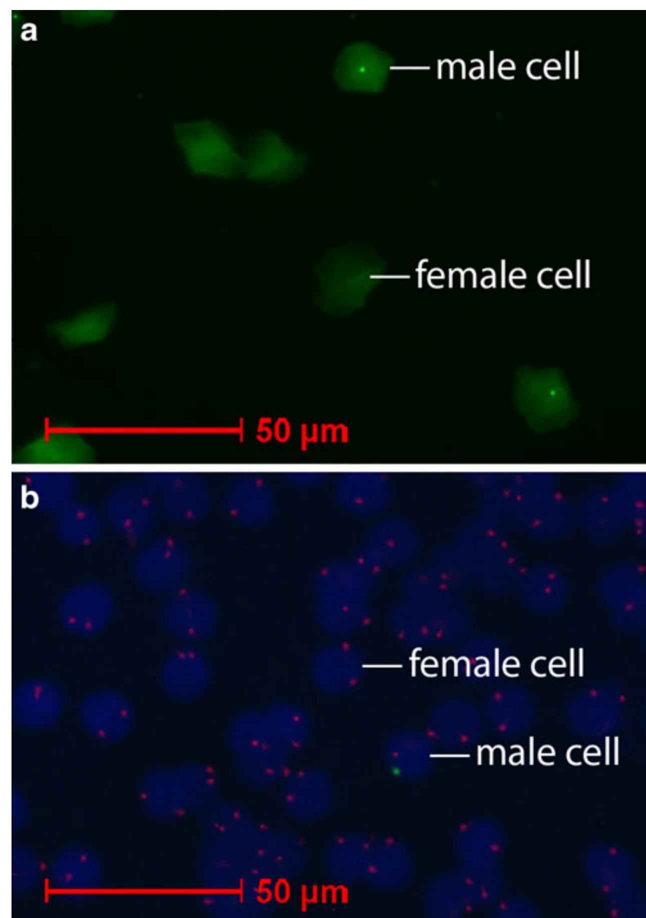

Fig. 1 Fluorescence in situ hybridization: identification of male and female cells. **a** Fluorescent image of male and female buccal cells, after FISH with a Y-chromosome specific probe (*green dots*). **b** Fluorescent image of male and female lymphocytes, after FISH with an X-chromosome specific probe (*red dots*) and a Y-chromosome specific probe (*green dots*); additionally, a DAPI-staining was performed to stain the cell nuclei (*blue*)

need for fewer cells to be isolated to obtain complete DNA profiles. Full DNA profiles could consistently be obtained from as little as ten male buccal cells.

Three case reports communicate on the use of LCM in DNA-based paternity testing on abortion material of sexual assault victims [42–44]. Traditionally, this is accomplished using the foetal remains as a source for foetal DNA [45]. Unfortunately, foetal tissues cannot always be easily distinguished from maternal tissues. Nevertheless, maternal decidua can be discriminated microscopically from foetal chorionic villi. LCM can be used to separate the latter for subsequent DNA typing.

In addition to its well-established use in sexual assault crimes, LCM can also be used in other forensic applications. It can for example be very useful to collect single hair follicles, in order to perform a more efficient DNA extraction without contaminants and inhibitors, such as keratin, that could interfere with STR amplification and DNA typing [46]. Another option is to microdissect dandruff that is adhering to the hair for DNA profiling.

Two groups have reported the use of LCM for isolation of blood cells from different cell mixtures [47, 48]. Anoruo et al. used LCM to separate cellular mixtures of blood and saliva. Haematoxylin and eosin (H&E) staining was used to assist identification of white blood cells (WBCs) and buccal cells. Single-source DNA profiles of each of the cell types were generated from the majority of the mixtures. However, in some cases, the blood donor's DNA profile contained some alleles from the saliva donor. The most likely explanation for this observation is that nuclei from partially lysed buccal cells were mistaken for WBCs [47]. The use of a more discriminative staining than H&E would lead to a better differentiation of the WBC from disrupted buccal nuclei, leading to pure DNA profiles. Therefore, Thorogate et al. developed an immunofluorescence-based technique for the detection of individual WBCs and the DNA contained within them. This technique also proved to be useful on older blood stains, which is important in forensic casework [48].

LCM may also be used to obtain DNA profiles from biological samples when these are mixed with debris, such as dirt. Lambie-Anoruo et al. reported the use of LCM to isolate buccal cells from a saliva stain that was mixed with soil [49]. Dirt samples containing biological material usually pose difficulties for traditional DNA extraction methods. LCM, however, shows to be useful in casework samples in which the cells are overwhelmed by PCR inhibiting substances as the microlaser permits isolation of cells from the environmental debris, this way avoiding the need for complex DNA extraction and clean-up protocols.

Unpublished data from our group show that LCM can also be used on forensic adhesive tape. These tapes are mainly used for sampling in cars and on corpses [50]. Our data show that these tapes can be attached to frame slides (i.e. metallic frames with the dimensions of a normal microscope slide) and target cells, such as dandruff or flakes of skin, can be microdissected directly from the tape, eventually after a histological staining for better discrimination of the different cell types. Figure 2 shows a brightfield image of part of a forensic tape before (Fig. 2a) and after (Fig. 2b) laser microdissection of the cell aggregate lying in the middle of the image. The cells are catapulted directly into the cap of a standard microfuge tube, containing proteinase K. DNA extraction is performed in the same tube. Profile recovery depends on the cell type that has been isolated. Skin cells from the epidermis, for example, may have lost their nucleus, due to apoptosis and DNA degradation by several enzymes during keratinisation [51]. Therefore, no exact statement can be made on how

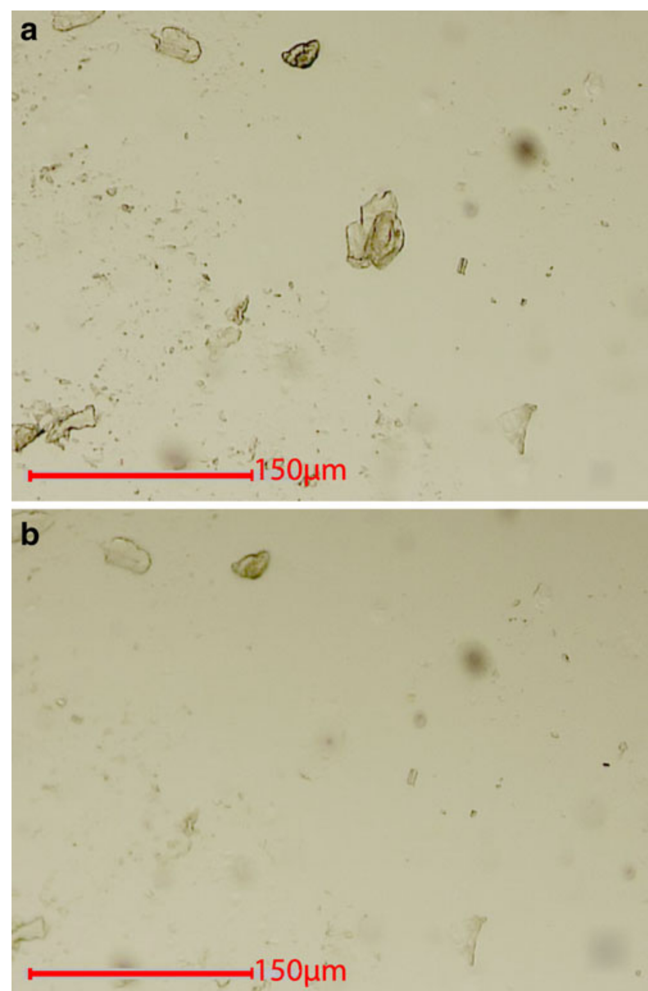

Fig. 2 Laser capture microdissection on forensic adhesive tape. **a** Brightfield image of part of a forensic tape before LCM of the cell aggregate lying in the middle of the image. **b** Brightfield image of part of a forensic tape after LCM of the cell aggregate lying in the middle of the image

many skin cells need to be isolated by LCM to obtain a useful DNA profile.

Staining and detection of target cells for LCM

One of the biggest challenges of mixture separation by LCM is to discriminate different cell types. Several specific and non-specific staining techniques have been proposed. In general, immunofluorescent stainings have a higher discrimination power than cytological stainings. The major disadvantage of the first is that they require the availability of a fluorescent module in the LCM system, which involves an additional investment for the forensic laboratory.

Another issue lies in the fact that some stains, used for the discrimination of different cell types, may have an influence on DNA recovery after LCM. While some staining protocols may cause DNA damage, e.g. those based on the use of picric acid [31] and reduce the DNA quality, others do not interfere with DNA amplification and typing because they can be used without any fixation step and lack aggressive chemical agents [29]. When a new histological stain is taken into consideration for LCM, the effect on downstream DNA analysis needs to be evaluated. Although a stain might provide good discrimination of the cells of interest, it may negatively influence DNA quality. For sperm cell detection, the three most commonly used cytological stains in forensic routine are H&E, Christmas tree stain (CTS or nuclear fast red and picroindigocarmine) and alkaline fuchsin. Comparative studies showed that CTS outperforms both others [31, 52]. Moreover, several reports state that H&E staining results in DNA degradation [30, 31, 40, 53–55]. Nevertheless, even the CTS staining remains non-specific and depends on morphological features to distinguish the spermatozoa from other cell types. Therefore, the commercially available specific sperm stain Sperm HY-LITER™ (Independent Forensics, Hillside, IL, USA) is for the time being and in our opinion the preferred option for sperm cell detection. This staining does not rely on morphological characteristics for sperm identification but utilises fluorescently labelled monoclonal antibodies directed against a protein contained in the human sperm head. Moreover, it has been shown not to interfere with DNA quality when combined with LCM and can be combined with software tools for the scanning of the microscope slide and the automatic detection of the stained spermatozoa [32]. This way, time-consuming and labour-intensive manual detection of the spermatozoa is avoided.

As microdissection needs to be performed without coverslips or immersion oils, this results in a tissue that lacks refractive index-matched image qualities [3]. Nevertheless, this limitation can be overcome by the use of water soluble mounting media. Once the cells of interest have been detected on the glass slide, the coverslip is removed and the mounting medium is easily washed off [41]. Subsequently, the target cells are easily relocated based on their XY coordinates. When an image is acquired and before the coverslip and mounting medium are removed, the non-target cells and cellular debris that surround the target cells can be outlined using a software tool. This makes it possible to visually check under brightfield illumination whether the target cells are correctly relocated by the software after removal of the coverslip, as the relative distance between the outlined non-target cells and the relocated target cells stays the same. If a shift has occurred during relocation, the same shift will also appear on the non-target cells. Repositioning of the outlined non-target cells will ensure the correct relocation of the target cells. After this visual control, the target cells are isolated by LCM.

Automated cell recognition

The search for desired cells on a slide can often be time consuming and exhausting, especially if the cells of interest are rare, e.g. in cases of sexual assault. Image analysis software modules can be very helpful for the automatic detection of the target cells [56]. LCM can be combined with automated scanning software such as Metafer P™ (MetaSystems, Altlussheim, Germany), Cellenger™ (Definiens AG, Munich, Germany) or others [17]. Reliable object recognition by intensity, colour and shape analysis allows the generation of a list with coordinates for cell relocation. After relocation and visual re-inspection to make sure the detected cells are not false positives, the target cells are isolated and collected by LCM. Therefore, automated cell recognition can be a very helpful tool for rapid screening of biological samples and fast access to the desired cells.

Thus far, only two publications mention the combination of LCM and automated cell recognition in forensic mixtures [32, 41] although several commercial groups offer integrated staining and detection systems for forensic laboratories (http://www.ifi-test.com/laser_capture.php; http://www.laser-microdissection.com/news/free_trial_-_new_mmi_cell_id_software_october_2009.html).

DNA extraction from laser microdissected samples

An important challenge lies in the development of a DNA isolation method appropriate for laser microdissected cells. Routinely applied tools to extract DNA cannot be used without adaptation to the few cells isolated by LCM.

When an extraction method is evaluated for use after laser microdissection, different features need to be

addressed: can the method remove PCR inhibitors from the sample, does the method maximally conserve the DNA and does it provide a concentrated extract such that the entire volume can be used for PCR? The latter is especially important for the recovery of very minute evidence samples for subsequent low copy number analysis, which is often the case in forensic samples [31].

Spin columns, containing a silica membrane that binds nucleic acids, have been used to isolate DNA from forensic casework after LCM [29, 31]. The advantage of these columns is that proteins and other contaminants, which can possibly act as PCR inhibitors, are removed through a series of washing and elution steps, resulting in a relatively pure DNA extract. Conversely, the high amount of manipulations increases the chance of contamination and sample loss.

Independent of the extraction method, maximal recovery of DNA can be obtained by extracting the samples directly in the cap of the collection device [30]. Moreover, the DNA is not lost due to organic extractions or to the high amount of washing and elution steps when spin columns are used. These single-tube methods require little manipulation and prevent contamination. Although Chelex™ (Bio-Rad Laboratories, Hercules, CA, USA) is widely used in forensic casework, Sanders et al. [31] and our group (unpublished data) demonstrated that it was difficult to use in a low-volume format. Moreover, it was hard to completely separate the extraction liquid from the resin beads, which resulted in non-interpretable DNA profiles. As the Chelex™ resin withdraws multivalent metal ions, contamination of the DNA extract with this resin can inhibit the PCR process as the Taq polymerase requires Mg^{2+} as a cofactor to bind the negatively charged DNA [57]. The most widely used, single-tube, DNA extraction method after LCM is the Proteinase K based method [28, 30, 32, 39–41, 43] In general, very good results are achieved using this method.

Finally, as an alternative to the single-tube methods, a recently developed technology needs to be mentioned. The AmpliGrid slide (Advalytix AG, Germany) is a standard microscope slide sized amplification platform for ultra low volume applications in the 1 µl range, based on a chemically structured microscope slide [58]. After laser microdissection of (single) cells into one of the hydrophilic reaction sites on the slide, DNA extraction and PCR can be performed directly on these reaction sites. Although combination of this new technology gives highly sensitive results in combination with regular micromanipulation [59], no amplification was achieved by Daniel et al. from any number (between 1 and 50) of laser microdissected cells [60]. This disappointing result might be due to the fact that the laser microdissection system utilised during these preliminary trials did not allow for the delivery of the cells directly from the object slide to the AmpliGrid slide. The cells were first transferred by LCM to the cap of a tube and then to the AmpliGrid slide by pipetting. However, when the laser-microdissected cells are collected directly onto the AmpliGrid slide, interpretable profiles can be obtained from as few as one cell [61].

Conclusions

Taken together, we can state that LCM provides an answer to the need for a technology for efficient separation of cells from difficult forensic samples and for rapid isolation of pure cell populations from heterogeneous samples. It can be used for a wide range of applications, mainly but not restricted to sexual assault casework. This review summarises all published forensic applications of LCM. Nevertheless, it is certain that new applications can and will be developed in the future. The use of LCM should be considered in every forensic case where minute traces need to be separated from a mixture.

When working with LCM, special care needs to be taken for staining and DNA extraction protocols. Traditional protocols need to be adapted for the combination with LCM. Despite these technical peculiarities, LCM offers a valuable tool for the investigation of unfavourable forensic mixtures.

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