

Laser scanning microdissection - Advantages and pitfalls in forensic diagnosis

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Laser microdissection (LMD) is a technology that has been around for more than 40 years. It combines the amplification power of a microscope with the precision cut of objects allowed by the laser technology. The main use of a Laser Microdissection System (LMS) is for clinical purposes, in order to isolate cells or its components of interest. Only in the last decade has LMD been used for forensic purposes, mainly in sexual assault casework for isolating sperm cells from vaginal swabs.

Sexual assault cases are among the most difficult scenarios that forensic laboratories have to deal with. Several factors impact the achievement of a result that either implicates or exonerates a suspect. Among these factors, the most important are the quality of the sample, the amounts of DNA present or even their relative proportions in a mixture. Sometimes the interpretation of the mixture is complex, mainly without reference samples from the victim and the suspect. The LMD technology allows the selection of individual cells based on morphology (e.g. sperm cells opposed to epithelial) or on labeling with specific fluorescent dyes for posterior analysis. Mixture analysis with an azoospermic or oligospermic contributor is even more challenging. In the absence of sperm cells, male and female cells are indistinguishable, requiring the use of specific fluorescent dyes. The use of these techniques can also pave the way for detection of enough cells for analysis.

Here we try to gather the main options to undertake laser microdissection analysis in forensic casework, highlighting in particular the pros and cons of combining LMD with fluorescence identification of interest specimens to increase accuracy and to speed-up isolation.

Keywords: Laser microdissection; Forensic medicine; Sexual assault; Sperm cells isolation.

1. Laser Microdissection Systems

Laser microdissection (LMS) was first described as a method to isolate and harvest chromosomes from cells of interest in clinical assays (1). However, this technology was seldom used until 1998, when, for the first time, more than 10 papers have been published with the LMS used or being the study object. The turning point was the publication of an article entitled “Laser capture microdissection”, which was published in Science magazine (2).

Laser microdissection has since been increasingly used in the clinical field. Forensic use of LMS has been first described in 2003 (3) as a mean of recovering sperm cells from slide smears of sexual assault cases. The published papers since, focused on the forensic aspects of the LMS, have been scarce (34 in the last 15 years, indexed at PubMed), with all of them concentrated on technical aspects, advantages and drawbacks of the LMS and/or its use.

1.1 Laser Capture Microdissection

Laser capture microdissection consists in harvesting cells of interest by melting a thermoplastic membrane around them. The laser heats a thin plastic layer and cells become attached to it. Generally, such thermoplastic layer becomes embedded in the sampling holder (2, 4).

1.2 Laser Cutting Microdissection

Systems that perform a cutting operation are more often found in laboratories. Their main differences lie in the way cells of interest are collected. The operating principle of these types of laser microdissectors is the identification of interest cells and using the laser to perform clean cuts in the supporting layer around them (5, 6). Collecting the cells of interest can be made by several different methods:

- Gravity – the slide is placed inverted in the microscope stage, the cells simply fall into the sampling holder (usually an eppendorf tube);

- Pressure catapult – after cutting, the laser is readjusted, both in wave length and focus, to rapidly heat the air in front of the cell, creating a low pressure area. The air will be sucked from around that point, dragging the cutout cell, catapulting it towards the collection holder.
- Microscopic tweezers – the cut cells are directly placed in the collection holder with the help of these microscopic tweezers.

Laser capture microdissection, gravity and pressure catapult collection systems do not require the physical manipulation of the cells of interest. Therefore, these systems might be less subject to foreign contamination. However, gravity and pressure catapult systems are less accurate in the way cells are collected, lying in an undetermined location inside the collection tube. On the other hand, laser capture microdissection allows the adhesion of the cell to a specific place of the collection holder (generally, the eppendorf tube's cap), retaining the relative position of the collected cells.

This allows for further verification of the collected cells.

There are systems that combine the best of both capture and cutting techniques such as the Olympus MMI Cell Cut. It combines the capture element by cutting the slide's plastic film around the cells of interest onto a special adhesive tube cap, therefore retaining their integrity and relative positions. The process is completely hands and contamination free, but it requires special PET slides and collection tubes with adhesive caps.

1.3 Staining techniques

In order to analyze cells of interest, one needs first to identify them in a slide. Several staining techniques are used to put interest cells into evidence. These techniques can be divided into chemical staining and immunological staining. Here we will cover only the most commonly used in forensic laboratories. Many laboratories have made different modifications to the standard protocols in order to achieve better results.

1.3.1 Chemical Staining

Chemical staining relies on the stain molecules affinity towards different cellular components, such as membranes, lipids, nucleic acids. Techniques that rely on chemical staining for sperm identification often require more than one dye in order to make sperm cells stand out from the background.

- Hematoxylin / Eosin (H&E). The dyes are used in conjunction to create a contrast. While hematoxylin stains nuclei in blue, eosin stains other structures and cytoplasm in red, pink or even orange. It is widely used to differentiate cell populations, particularly in histological sections. Unfortunately, some studies show that this technique degrades DNA, making difficult a downstream genetic analysis (7).
- Nuclear fast red (NFR). NFR is normally used as a counterstain. It is a one-step coloring procedure and stains nuclei in red. It is used as an alternative for the H&E staining, when hematoxylin obscures the substrate colors (8).
- NFR + Picroindigocarmine (Christmas Tree Staining – CTS). It is a specific technique to stain sperm cells. Picroindigocarmine stains the neck and tail portions of the sperm cells in green, while NFR stains the head and acrosomal piece in red. However, since the tails of the sperm cells degrade very rapidly, only the head portions can be visible and confused with other kinds of cells or nuclei present in the slide (9).
- Methyl Green (MG). MG is also used as a counterstain; however, it is of difficult use. The stain washes out rapidly in both aqueous and organic solutions (10).
- Acridine Orange (AO). Another very useful stain, it binds to cell DNA emitting green fluorescence. This staining is mostly used in clinical assays to determine sperm viability (11).

All these staining techniques are useful to detect any kind of cell in a slide. CTS is the more specific to detect sperm cells, but only if they are well preserved, since it is based on morphological aspects for cell recognition.

1.3.2 Immunofluorescent staining

These techniques rely mainly in the use of DNA probes that can be tailored to bind to specific regions of the DNA or other specific cell structures. The more commonly used probes in forensic genetics are designed to target:

- Y-chromosome in cases of sexual assault. Most cases of sexual assault are perpetrated by male individuals and most victims are female. Therefore, finding the male cells is of utmost importance in order to perform DNA genotyping of the perpetrator (12).
- Human antibodies. Fluorescent antibodies are used to find human cells among blood mixtures or when blood is highly diluted (13).

1.4 Current use of LMS in forensic biology.

The prohibitive cost of a laser microdissector is out of reach for most laboratories' budget, but it has many advantages over the differential lysis, namely the ability to see and collect only the cells of interest. The major drawback is, when only a small number of cells are collected, a low template analysis must be performed. This is generally done by increasing the number of cycles in the PCR reaction. With low levels of template DNA and an increase in the number of PCR cycles, stochastic effects are more prone to occur, which may result in unbalanced peaks for the same profile.

So far, most of the research carried out by laboratories around the world focuses in using the LMS technology to pinpoint advantages and drawbacks in certain special cases. One of such uses is where one finds azoospermic individuals, as reported by McAlister (14). Unfortunately, few laboratories possess this kind of infrastructure; mostly due to its high initial cost and long-term abatement of its running costs. As such, budget constrained laboratories rely on the older, proven and cheaper technique of differential extraction. However, this technique may not be as cheap as initially thought due to:

- Low quality or absence of results on the first run may force a second analysis, using time and not so cheap reagents (amplification kits, etc...);
- More time spent analyzing results;
- A greater percentage of cases may be inconclusive using more traditional approaches.

Forensic laboratories that have access to LMS may use one among countless number of possible combinations for staining/system/protocol. Pathology and clinical services are often unaware of the downstream protocols and choose to stain the slides according to their goals, rendering further analysis very difficult. In the cases where those services deliver samples already mounted in slides, we suggest that the samples are merely fixated and unstained. This allows the forensic laboratories to determine the best course of action by not limiting their options.

2. Forensic Casework

Most modern forensic laboratories are still using a technique developed in 1985 (preferential lysis method) as a "gold standard" to process mixture samples in sexual assault cases. Many other methods have been introduced but they all have weaknesses in cell separation efficiency, such as the yield of genetic material, time and cost effectiveness, difficulties to work with minute amounts of starting material, identification of genotypes on target cells and difficulties to discriminate multiple perpetrators. Up-to-date Laser Microdissection Systems (LMS) have been proposed as most suitable methods for separation of samples (7).

Laser Microdissection has been used in biomedical research since 1996 (2), but the first reported use to improve recovery of DNA from sperm on microscopic slides appeared in 2003 (3). Results from this study have shown that LMS performs significantly better than the preferential lysis technique in obtaining a Short Tandem Repeat (STR) profile, even when few sperm cells are found in a sample.

Azoospermia is the medical condition of a male not having any measurable level of sperm in the semen. It is associated with very low levels of fertility or even sterility. In humans, azoospermia affects about 1% of the male population (15) and may be seen in up to 20% of male infertility situations (16). On the other hand, oligospermia describes a condition where man produces semen with a low concentration of sperm. Therefore, it is not inconceivable that some of the sexual assault cases throughout the world are committed by individuals with some sort of defect in their germinal line. In these cases, conducting a mixture analysis can be difficult (17). By being a very unbalanced mixture and due to stochastic effects, the minor fraction may not be amplified yielding no results (18).

The LMS allows the direct collection of the cells or other material of interest, even in low quantities to allow Low Template (LT) DNA analysis (19). In addition, it is likely to have applications in numerous other sample types where the ratio of female cells to male cells is large, including cases involving penetration without ejaculation (azoospermic and/or oligospermic perpetrators), digital penetration, or oral sex (12). As a consequence, possible contaminants of the problem samples are also eliminated (20), contrary to the imperfect differential lysis method where, often, the victim's DNA is amplified, generating a mixed profile (21). LMS also has the potential to shed new light on old cases, because archived samples can also be used with this technique (22). However, time since intercourse, the age of the slide or even the staining and fixation procedures can be detrimental to results achievement (3, 23).

3. Difficult Samples

DNA degradation is a concerning issue. Whether it is due to a long post-coital interval or to deficient sample preservation, there is a consensus that after 48h to 72h it becomes nearly impossible to obtain a complete genotyping profile from the collected samples (3). A procedure to minimize sample degradation and increase the chances of having a successful amplification remains to be established. This procedure can involve simple cautionary measures, such as placing the sample in the refrigerator or freezer, instead of being air-dried or more permanent fixation protocols.

The use of LMS for solving sexual assault cases is fairly recent (3), and there is only one described case where LMS made a difference in the individualization of the cells from an azoospermic individual (14). Nonetheless, using LMS in

cases where preliminary tests for the detection of the presence of sperm are negative (azoospermic or oligospermic contributors or even low sample available) is worth pursuing. Seminal fluid also contains epithelial and other cells, which can be identified by using fluorescence in situ hybridization (FISH) with Y-chromosome specific probes (12, 24-27).

Several authors are starting to recommend the use of LMS in sexual assault cases, due to its high effectiveness in recovering cells of interest, potential of higher throughput and backlog reduction (3, 7). Authors also recommend a much larger examination of casework to make the final evaluation of the LMS method for forensic casework (7), something that has yet to be done. Furthermore, by individually collecting cells of interest, several steps in sample processing, such as purification (by only collecting cells, leaving the greater part of debris and contaminants in the slide) and quantification (the amount of starting DNA / DNA contained in each cell is well known) could easily be eliminated. Recently, our research group compared LMS-based technology with gold standard routine techniques in terms of cost and effectiveness for forensic sexual assault casework (Costa, Correia-de-Sá, Porto and Cainé, 2016, manuscript accepted for publication). Simulated sexual assault mixture samples were prepared to match the detection limit of our routine methodology for genotyping profiling. Under such extreme conditions, the LMS-based technology to harvest fluorescently-tagged interest cells was highly reproducible and provided end results of better quality when performing mixture genotyping analysis, yet it was more time-consuming (i.e. the time required for analysis increased by 56% mainly due to the DNA extraction procedure). The calculated running cost of LMS methodology was similar to more traditional methods when chemical staining was used (Fig. 1), but the use of immunofluorescent staining kits (Fig. 2) increased the cost per sample by 15 to 25%.

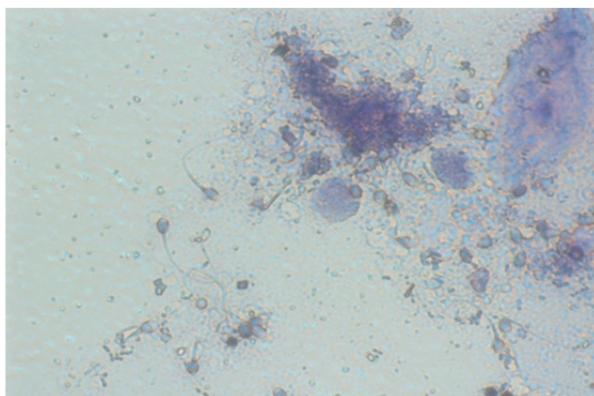


Fig. 1 Simulated sexual assault sample stained with H&E. Several sperm cells are visible, as well as some epithelial cells and cell debris. Images were captured with a 60x objective using a Olympus Cell Cut microdissector coupled to an epifluorescence module. Image capture software: MMI Cell Tools.

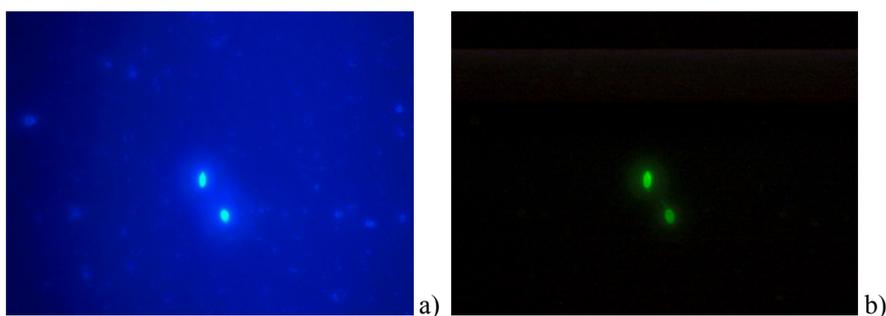


Fig. 2 Simulated sexual assault sample stained with the Sperm Hy-Liter Express commercial kit. Two sperm cells are visible in the centre of the micrographs. a) Photographed with a DAPI compatible filter; b) Photographed with a CFTM488A compatible filter. Images were captured with a 60x objective using a Olympus Cell Cut microdissector coupled to an epifluorescence module. Image capture software: MMI Cell Tools.

More recent studies also encourage the development of LMS-based techniques that enable the analysis of cases where multiple donors are present (e.g. sexual assault cases with more than one perpetrator) (28).

4. Future Prospects

The use of LMS combined with differentiating techniques opens the way for other applications, such as isolation of blood cells from different cell mixtures (13, 29), biological samples mixed with debris (30) or collected with adhesive tape from corpses or cars (31). Beyond the LMS technique, there are other methods in Biology that could benefit from the study and optimization of this technique. Cell and other biological samples staining is one of them. Several authors defend the use or even research of coloring and staining techniques, lysis methods that minimize downstream DNA analysis.

One new promising staining technique involves the use of Quantum Dots (QDs). QDs or semiconductor nanocrystals are tiny crystalline particles that exhibit size-dependent optical and electronic properties. These QDs usually display intense fluorescence. One of the best strategies can be the use of a fluorescent label – a QD – targeted at a protein encoded by the Y-chromosome. This would allow us to identify the male cells present in the microscope slide without tampering with the DNA, increasing the chances of a successful amplification. Furthermore, since QDs can be tailored both in their fluorescence and specific target, multiple QDs can be directed at various isoforms of the same protein, enabling the analysis and interpretation of complex mixture samples (32).

If new labeling techniques such as QDs prove their usefulness and allow the segregation of cells (sperm and non-sperm cells) (23) from different donors, it could be the single major breakthrough in harvesting interest cells for mixture analysis involving several perpetrators (32).

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References

- Berns MW, Floyd AD. Chromosomal microdissection by laser. A cytochemical and functional analysis. *Experimental cell research* 1971 Aug;67(2):305-10.
- Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser capture microdissection. *Science* 1996 Nov 8;274(5289):998-1001.
- Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. *Forensic science international* 2003 Oct 14;137(1):28-36.
- Espina V, Milia J, Wu G, Cowherd S, Liotta LA. Laser capture microdissection. *Methods in molecular biology* 2006;319:213-29.
- Kolble K. The LEICA microdissection system: design and applications. *Journal of molecular medicine* 2000;78(7):B24-5.
- Micke P, Ostman A, Lundberg J, Ponten F. Laser-assisted cell microdissection using the PALM system. *Methods in molecular biology* 2005;293:151-66.
- Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. *Journal of forensic sciences* 2006 Jul;51(4):748-57.
- Leubitz SS, Savage RA. Sensitivity of picroindigocarmine/nuclear fast red (PIC/NF) stain for detection of spermatozoa: a serial dilution study of human ejaculate. *American journal of clinical pathology* 1984 Jan;81(1):90-3.
- Allery JP, Telmon N, Mieusset R, Blanc A, Rouge D. Cytological detection of spermatozoa: comparison of three staining methods. *Journal of forensic sciences* 2001 Mar;46(2):349-51.
- Andersen AP, Lyon H, Jakobsen P, Hoyer PE. Nucleic acid staining with the methyl green-pyronin method. A comparison of the use of pure dyes and commercially available dyes. *Histochemistry* 1986;84(3):279-80.
- Evenson DP. Sperm chromatin structure assay (SCSA(R)). *Methods in molecular biology* 2013;927:147-64.
- Murray C, McAlister C, Elliott K. Identification and isolation of male cells using fluorescence in situ hybridisation and laser microdissection, for use in the investigation of sexual assault. *Forensic science international Genetics* 2007 Dec;1(3-4):247-52.
- Thorogate R, Moreira JC, Jickells S, Miele MM, Daniel B. A novel fluorescence-based method in forensic science for the detection of blood in situ. *Forensic science international Genetics* 2008 Sep;2(4):363-71.
- McAlister C. The use of fluorescence in situ hybridisation and laser microdissection to identify and isolate male cells in an azoospermic sexual assault case. *Forensic science international Genetics* 2011 Jan;5(1):69-73.
- Report on evaluation of the azoospermic male. *Fertility and sterility*;86(5):S210-S5.
- Jarvi K, Lo K, Fischer A, Grantmyre J, Zini A, Chow V, et al. CUA Guideline: The workup of azoospermic males. *Canadian Urological Association Journal* 2010;4(3):163-7.
- Ladd C, Lee HC, Yang N, Bieber FR. Interpretation of complex forensic DNA mixtures. *Croatian medical journal* 2001 Jun;42(3):244-6.
- Clayton TM, Whitaker JP, Sparkes R, Gill P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. *Forensic science international* 1998 Jan 9;91(1):55-70.
- Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic science international* 2000 Jul 24;112(1):17-40.
- Vandewoestyne M, Deforce D. Laser capture microdissection in forensic research: a review. *International journal of legal medicine* 2010 Nov;124(6):513-21.
- Wiegand P, Schurenkamp M, Schutte U. DNA extraction from mixtures of body fluid using mild preferential lysis. *International journal of legal medicine* 1992;104(6):359-60.

22. Burgemeister R. New aspects of laser microdissection in research and routine. *The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society* 2005 Mar;53(3):409-12.
23. Di Martino D, Giuffrè G, Staiti N, Simone A, Le Donne M, Saravo L. Single sperm cell isolation by laser microdissection. *Forensic science international* 2004 Dec 2;146 Suppl:S151-3.
24. Anslinger K, Mack B, Bayer B, Rolf B, Eisenmenger W. Digoxigenin labelling and laser capture microdissection of male cells. *International journal of legal medicine* 2005 Nov;119(6):374-7.
25. Anslinger K, Bayer B, Mack B, Eisenmenger W. Sex-specific fluorescent labelling of cells for laser microdissection and DNA profiling. *International journal of legal medicine* 2007 Jan;121(1):54-6.
26. Murray C, McAlister C, Elliott K. Use of fluorescence in situ hybridisation and laser microdissection to isolate male non-sperm cells in cases of sexual assault. *International Congress Series* 2006 4//;1288:622-4.
27. Vandewoestyne M, Van Hoofstat D, Van Nieuwerburgh F, Deforce D. Suspension fluorescence in situ hybridization (S-FISH) combined with automatic detection and laser microdissection for STR profiling of male cells in male/female mixtures. *International journal of legal medicine* 2009 Sep;123(5):441-7.
28. Li CX, Han JP, Ren WY, Ji AQ, Xu XL, Hu L. DNA profiling of spermatozoa by laser capture microdissection and low volume-PCR. *PLoS one* 2011;6(8):e22316.
29. Anoruo B, van Oorschot R, Mitchell J, Howells D. Isolating cells from non-sperm cellular mixtures using the PALM microlaser micro dissection system. *Forensic science international* 2007 Dec 20;173(2-3):93-6.
30. Lambie-Anoruo BL, Prince DV, Koukoulas I, Howells DW, Mitchell RJ, van Oorschot RAH. Laser microdissection and pressure catapulting with PALM® to assist typing of target DNA in dirt samples. *International Congress Series* 2006 4//;1288:559-61.
31. De Wael K, Gason FGCSJ, Baes CAV. Selection of an Adhesive Tape Suitable for Forensic Fiber Sampling. *Journal of forensic sciences* 2008;53(1):168-71.
32. Oh J-M, Park D-H, Choy J-H. Integrated bio-inorganic hybrid systems for nano-forensics. *Chemical Society Reviews*. [10.1039/C0CS00051E]. 2011;40(2):583-95.