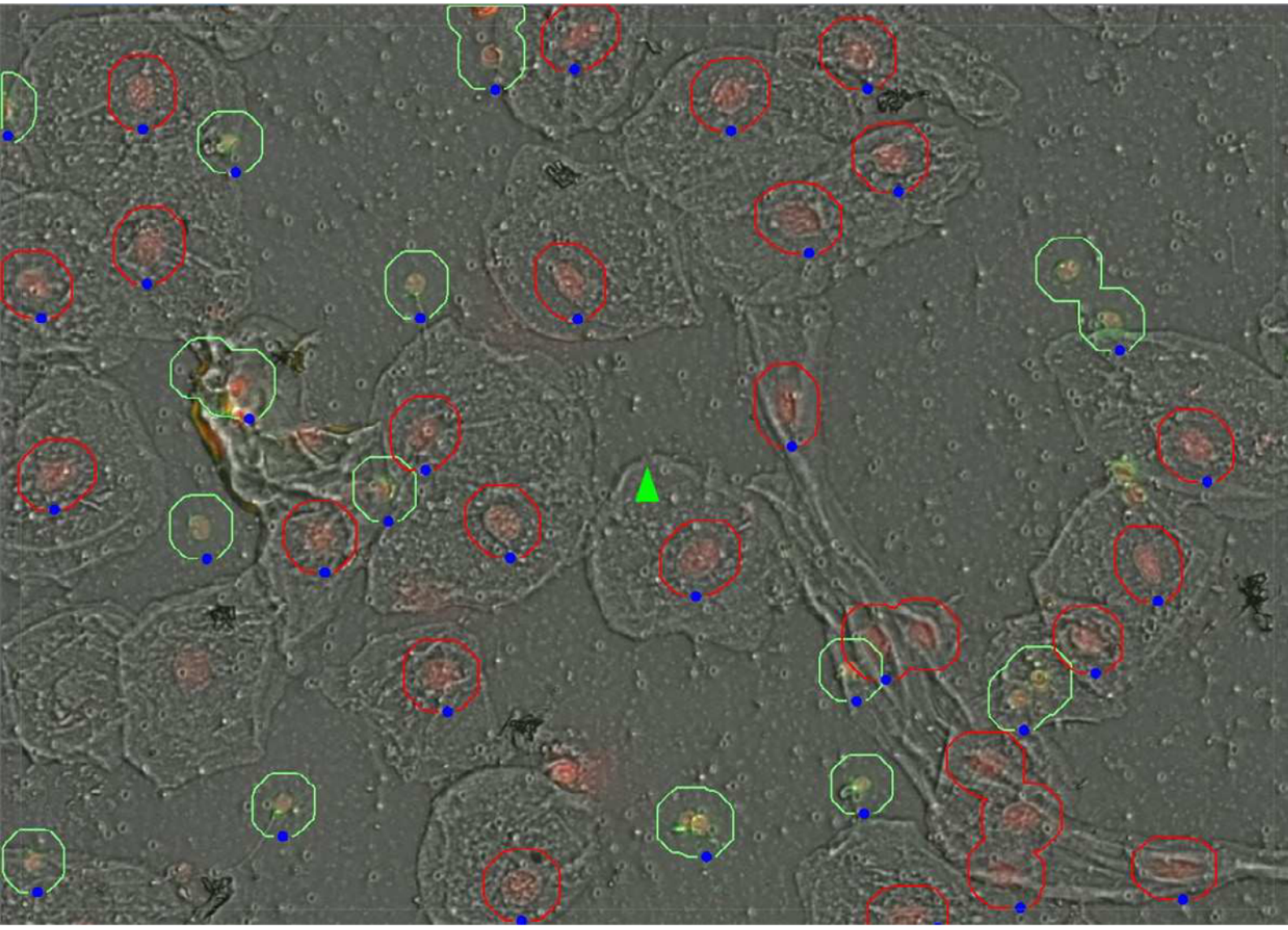
Application Note



Efficient and Reliable DNA Profiling of Spermatozoa from Sexual Assault Evidence.

Efficient and Reliable DNA Profiling of Spermatozoa from Sexual Assault Evidence.

Automated Detection, Isolation and Subsequent DNA Profiling of Spermatozoa Using SPERM HY-LITER™, ZEISS Laser Microdissection and OneTouch LCM™

Authors: Alex Sinelnikov, Jennifer Old, Karl Reich
Independent Forensics

Michael Gögler, Andrea Sigl, Gabriele Friedemann
Carl Zeiss Microscopy GmbH, Germany

Date: February 2017

The methods used to process sexual assault evidence by forensic DNA laboratories are essentially unchanged since the seminal (pun intended) Gill and Jeffries 1985 paper describing differential extraction[1]; a labor intensive and time consuming protocol. Here we present an integrated, efficient, specific and semi-automated workflow that overcomes all of the difficulties inherent in differential extraction. Specificity is provided by an immunofluorescent stain that uniquely identifies human sperm heads; sperm identification is performed using sophisticated computer image analysis; cell isolation is executed by automated laser microdissection and these solutions integrated into a two-step DNA extraction and purification method specifically designed to recover PCR quality DNA from as few as 25 cells.

Introduction

The final goal of the forensic processing of sexual assault evidence is of course to identify the male assailant through DNA profiling (here we assume a female victim and a male assailant, the most common [but not the only] case description). This requires that the DNA from the assailant be isolated from the mixed evidence presented to the laboratory. The detection and subsequent isolation of spermatozoa from sexual assault evidence are among the most time consuming and inefficient processes in forensic analysis. Forensic laboratories seek to identify sperm cells on provided evidence to (1) confirm the allegation of sexual assault and (2) to provide the logical justification for processing the sample through differential extraction, currently the only available method for isolating the DNA from the spermatozoa left by the male assailant.

Sperm cell detection from sexual assault evidence is currently based on the light microscopy identification of sperm cells from stained preparations; the histologically-based stains used by forensic laboratories (typically KPIC or H&E) are of course non-specific and are at best contrast enhancing agents. Forensic evidence is notoriously 'dirty' and identifying sperm cells which have lost their characteristic 'tadpole' morphology, a given from the swabs and fabric stains that make up forensic evidence, is no easy task.

The differential extraction technique makes use of the relative sensitivity to enzymatic digestion of epithelial and sperm cells to sequentially isolate the DNA from a mixture of these two cell types. The method is unfortunately time consuming, requiring between 5 – 7 hours of an analyst's time, and inefficient such that many thousands of sperm cells are required in order to recover sufficient DNA for developing a profile. Although a healthy human ejaculate may have upwards of 50 million sperm, collected evidence can have far fewer sperm cells thus making successful differential extraction unlikely or impossible.

The unfortunate confluence of difficult microscopical identification with inefficient cell and DNA isolation is such that

the success rate of generating a searchable DNA profile from sexual assault evidence processing is approximately 50 %. Here we demonstrate a comprehensive, integrated, semi-automated solution to (a) positively identifying sperm cells from sexual assault evidence, (b) isolating a sufficient number of positively identified sperm (only between 25 – 50 cells are required for our technique) and (c) successfully processing the identified and isolated sperm to generate robust DNA profiles suitable for database searching.

The workflow solution incorporates a variant of an immunofluorescent staining method for the identification of human sperm heads (SPERM HY-LITER™ PI), semi-automated screening of stained laser capture microdissection membrane slides on the ZEISS LCM system (PALM MicroBeam) followed by computer controlled dissection of positively identified sperm heads and a novel, but simple to implement, molecular biological-based DNA purification method designed to recover PCR quality DNA from as few as 25 sperm cells.

Sample preparation

Extracts from post-coital swabs or stains on fabric or laboratory generated mixtures of buccal cells and semen on swabs were prepared using PBS and cell pellets recovered using a spin-basket and centrifugation. Briefly, swabs or cuttings were soaked in PBS in a standard 1.7 ml microcentrifuge tube at room temperature for a minimum of 30 minutes in sufficient buffer to immerse the swab or fabric cutting. Swabs or cuttings were placed in a spin-basket and the entire extract + cell pellet recovered by centrifugation at ~13,000 x g for 5 minutes. After removal of the supernatant, the cell pellet was resuspended in 20 – 40 µl of PBS and added to an LCM membrane slide and allowed to air dry.

SPERM HY-HILITER™ PI staining followed manufacturer's directions (short fixation step followed by sample preparation buffer, blocking buffer and staining solution with brief washes with 1× washing buffer between each addition).

Imaging and automated image analysis

SPERM HY-HILITER™PI stained semen – buccal cell mixtures and post coital samples prepared on PEN MembraneSlides were imaged with a LD Plan NeoFluar 20x objective and an Axiocam MRm. For imaging, a region of interest was identified manually on each slide. Tile images (see Fig. 1) were acquired in 3 channels (brightfield, Alexa 488 & PI); the

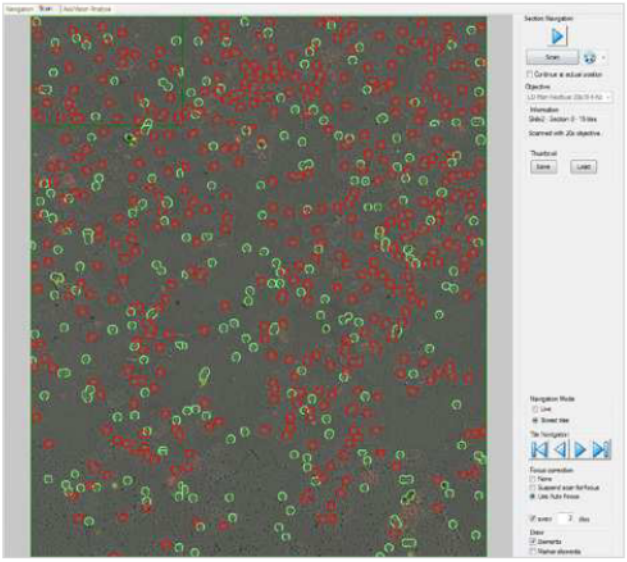


Figure 1 *Overview scan of semen - buccal cell mixture in 3 channels (brightfield, Alexa488, PI). Each tile is analyzed using an image analysis script. Detected sperm cells are outlined in green. Detected buccal cells are outlined in red.*

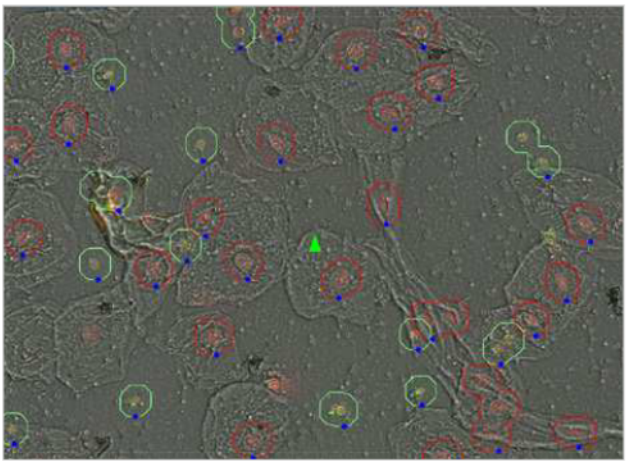


Figure 2 *Sperm cells (outlined in green) and buccal cells (outlined in red) have been automatically detected in a semen - buccal cell mixture.*

nuclear PI stain identifies all nuclei in the preparation (i.e., both sperm and epithelial cells are labeled with this dye) while sperm cells are specifically labeled with Alexa 488 (the sperm-head specific antibody in SPERM HY-LITER™ has been derivatized with Alexa 488). Differentiation between epithelial cells, released nuclei and sperm is therefore possible by fluorescent image analysis.

The acquired images were analyzed automatically via an image analysis script designed to identify and differentiate sperm and epithelial cells. In the present work two computer scripts were used, one for the semen – buccal cell mixtures

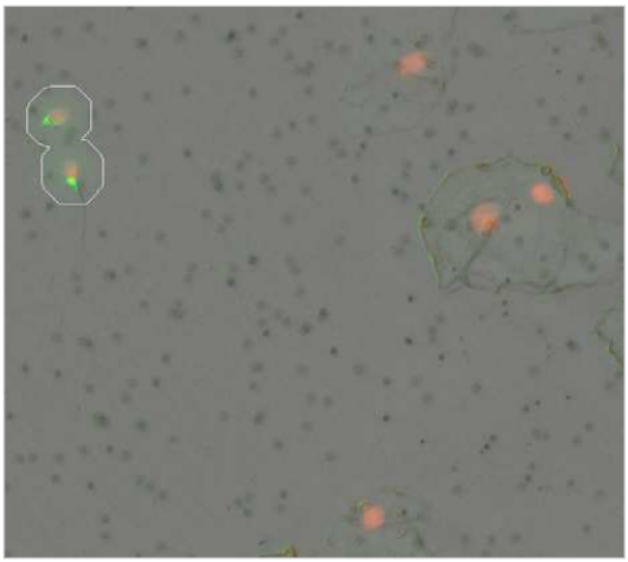


Figure 3 *Sperm cells (outlined in white) in a post coital sample are automatically detected by image analysis.*

(see Fig. 2) and one for post coital samples (see Fig. 3). Software identification labels sperm cells in green and epithelial cells in red on the processed image.
A complete list of all detected cells is generated by the image analysis routine and provided in an Element List. Users can review individual features (identified cells) and choose which are to be microdissected from this list.

Automated laser microdissection

From the Element List users select defined elements or features and choose which are to be dissected and isolated. Identified elements (sperm or epithelial cells) are then automatically and individually dissected and catapulted to separate adhesive caps for downstream processing mimicking the F1 and F2 fractions from a differential extraction.
The fully automated dissection and catapulting is contact-free and contamination-free.

Dissected cells isolated on adhesive caps can be visualized post catapulting and recorded such that identification, dissection and cell collection can be fully documented (see figures 4 & 5).

Downstream Analysis

Identified, dissected and catapulted cells collected on adhesive caps were processed using OneTouch LCM kit. Briefly, closed cap tubes were incubated with ProK and DTT at elevated temperature and the DNA purified through Xs subtractive spin columns as per manufacturer's protocol. Purified DNA can be used directly or concentrated by vacuum centrifugation before being added to multiplex STR-PCR kits. PCR reactions are analyzed by capillary electrophoresis. If required, post-PCR concentration and purification of amplicons can be used to further boost the capillary electrophoresis signal.

Results

DNA Profiles were obtained from SPERM HY-LITER™ PI stained preparation, from image analyzed identified sperm, dissected and subsequently processed via OneTouch LCM (½ volume PP16 reaction used).

Discussion

It has been estimated that up to half of the effort expended in forensic DNA laboratories is devoted to processing and analyzing sexual assault evidence. The DNA profile success rate from this type of evidence is less than satisfactory due, for the most part, to the inefficient differential extraction method.

The combination of immunofluorescent detection with modern image analysis and laser capture microdissection provides an unparalleled increase in specificity, sensitivity and

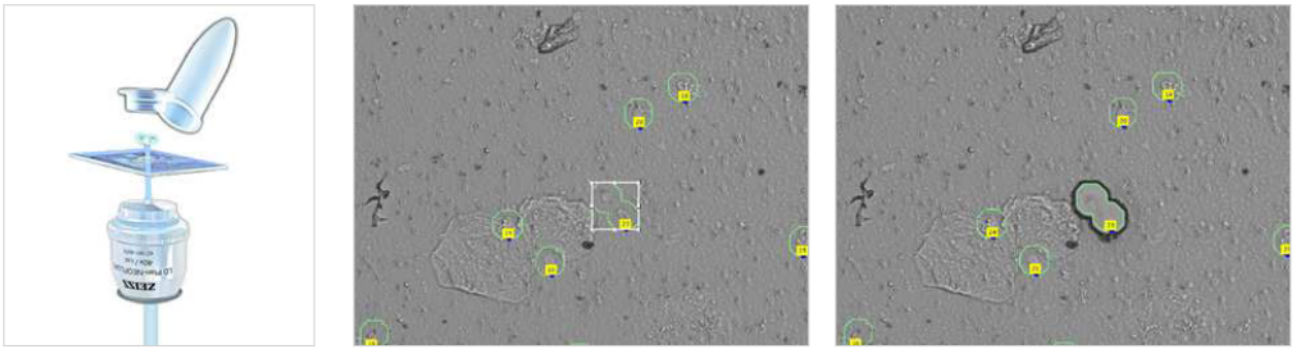


Figure 4 *Principle of ZEISS non-contact laser microdissection technology: a laser isolates the desired sample and transfers it into a collection cap. Auto-documentation of sperm cells before LCM and after LCM, indicating the successful transfer to the collection cap.*

efficiency: sperm cells can be positively stained, identified by automated image analysis, individually chosen by an analyst, dissected from the stained preparation and then quickly processed for DNA profiling. The sensitivity of the combined protocol is such that consistent, robust DNA profiles can be obtained from as few as 25 sperm cells. By implementing this procedure, forensic DNA laboratories are guaranteed to improve their DNA profile success rate from sexual assault evidence.

The described method incorporates well established and proven methods and technology (immunofluorescence, laser capture microdissection, automated image analysis, spin-column based DNA purification) that are quickly validated and will withstand any level of court or legal challenge.

This approach could be easily adapted to increase the throughput of successful analysis of sexual assault evidence; by assigning two DNA analysts working in tandem on an LCM instrument, over 1,000 sexual assaults could be processed in a year (assumes 200 working days and 6 samples processed per 8 hour day). This cost and resource efficient solution could eliminate case backlogs and insure the prompt, accurate and effective processing of submitted sexual assault cases.

It is worth noting that a slight modification of our approach would allow the processing of a class of sexual assault evidence that is currently impossible to process by differential

Figure 5 *View with 5x objective in the collection cap. The PEN membrane exhibits strong autofluorescence when excited with light around 400nm (e.g. DAPI-Filter). This allows easy identification and verification of the isolated and captured sample in the cap. Here, 25 sperm cells have been successfully captured via LCM.*

extraction and thus analyze successfully by somatic / autosomal DNA profiling, i.e., evidence entirely lacking sperm cells. While this class of evidence is not numerous, it often includes cases with juvenile victims which are particularly important to the criminal justice system. By adapting Y-chromosome fluorescent in situ identification, male cells from non-sperm cases can be visualized, identified and dissected[2] and again extracted using the OneTouch LCM method and the recovered DNA used to generate a searchable DNA profile. These types of cases are currently beyond the scope of the DNA laboratory.

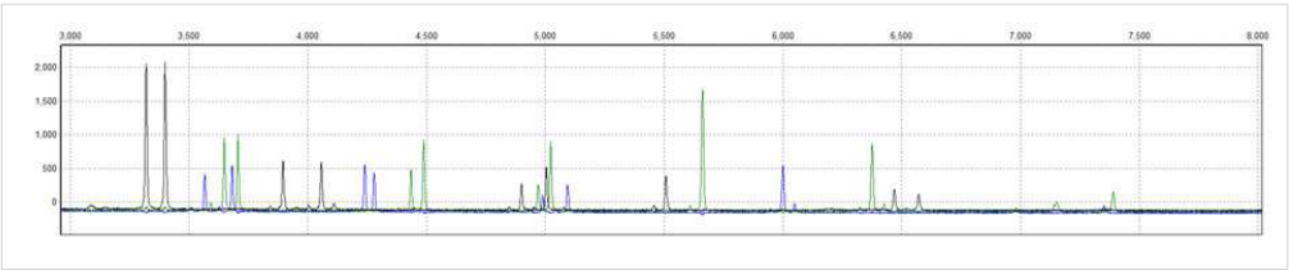


Figure 6 *PEN membrane – 500 µL cap tube, sperm and buccal cells on slide, full profile obtained. Total RFU: 20,765*

References:

[1] Gill P., Jeffreys AJ., Werrett DJ., Forensic application of DNA 'fingerprints'; Nature. 1985 Dec 12 – 18; 318 (6046):577-9.

[2] Mado Vandewoestyne & David Van Hoofstat & Filip Van Nieuwerburgh & Dieter Deforce, Suspension fluorescence in situ hybridization (S-FISH) combined with automatic detection and laser microdissection for STR profiling of male cells in male / female mixtures; Int J Legal Med (2009) 123:441 – 447

Specific forensic processes are to be determined by forensic analysts. The specialist bears full responsibility for results and interpretation.
Further detection methods may be required.

Not all products are available in every country. Use of products for medical diagnostic, therapeutic, or treatment purposes may be limited by local regulations.
Contact your local ZEISS representative for more information.

EN_42_013_227 | CZ 02-2017 | Design, scope of delivery and technical progress subject to change without notice.

Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
www.zeiss.com/microscopy