Laser Microdissection as a Technique to Resolve Mixtures and Improve the Analysis of Difficult Evidence Samples

Grant # 2006-DN-BX-K032

Final Technical Report

Authors:

Robert Driscoll, MFS
Dane Plaza, BS
and
Robert Bever, Ph.D.

Bode Technology
Lorton VA

October 9, 2008

ABSTRACT

Laser microdissection (LM) has proven to be an effective method for cell mixture separations in the forensic laboratory. Its adaptation to the forensics field has provided a means of physically separating the components of assault mixtures as well as improving the collection and DNA analysis associated with low copy number (LCN) samples. The work performed for NIJ Grant # 2006-DN-BX-K032 has focused on the further investigation of the various aspects and benefits of this burgeoning technology. Numerous facets of the processing of evidence containing LCN samples with LM methods were examined including: the best technique to prepare and transfer cells from the evidence to the slide, the enhancement of the extraction process, optimization of the STR amplification procedure, using Fluorescent In Situ Hybridization (FISH) techniques to resolve difficult mixtures, and the effectiveness of laser microdissection across a wide range of evidentiary samples. Findings indicated that the swabbing of substrates followed by a cellulase aided elution did increase the number of cells recovered for LM slide preparation. The QIAamp® Micro Extraction kit was found most efficient and reliable for extracting low level samples collected by LM. Amplification results indicate that the Applied Biosystems AmpF*l*STR MiniFiler™ kit is the optimal choice for amplifying LCN samples collected with LM instruments. Additional research was also completed on the cellular separation of problematical sample mixtures. While sperm and epithelial cell sexual assault mixtures can easily be separated based upon morphological differences, combinations of the same cell type are more difficult to separate. For problematic samples like these, a protocol was developed that is capable of successfully separating male/female cellular mixtures of similar morphology using chromosome X/Y

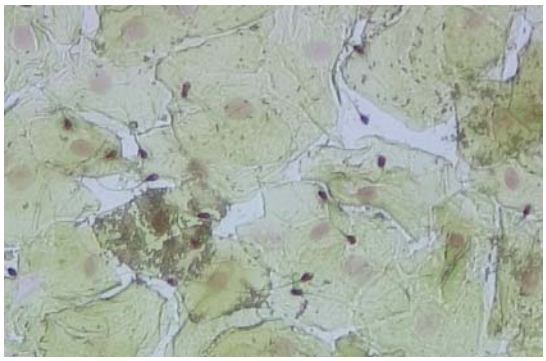
FISH probing. During the course of performing all of these LM evaluations, the PixCell® and Zeiss PALM® Microbeam instruments were assessed to determine optimal processing protocols and were shown to be suitable for LCN and mixture evidence processing. By using what was learned during this work, forensic labs can now successfully and efficiently resolve various LCN sample mixtures by incorporating FISH, LM, and optimized elution/extraction/amplification methods into their standard operating protocols. The completed results are currently being disseminated through the law enforcement and scientific communities via seminars, journal articles, and poster presentations.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The generation of clean, single source genetic profiles from sexual assault and touch evidence cellular mixtures continually proves to be a difficult challenge in the field of forensics. Evidence of this nature can contain trace amounts of human DNA from mixtures of cell types of various morphologies (Figure A). The key objectives of our work on NIJ Grant # 2006-DN-BX-K032 was to develop and implement different techniques utilizing laser microdissection (LM) for the improved separation of these forensic mixtures. Our research also sought to increase the number of methods related to DNA analysis of low copy number (LCN)/ touch evidence samples and to enhance the separation of cells in forensic mixtures.

Figure A: Sexual Assault Mixture Containing Epithelial and Sperm Cells Visualized at 630X Magnification



The research conducted can be subdivided into four distinct sections:

- Determination of the best evidence collection technique for both sperm and touch evidence, i.e. establish the most efficient way to prepare and transfer the cells from the evidence to the slide for LM analysis.
- Optimization of the DNA extraction process for epithelial and white blood cells collected from LM.

- Establishment of the best amplification procedure for a single cell or low number of cells for sperm and touch evidence.
- Separation of cells of similar morphology by gender using Fluorescent In Situ Hybridization (FISH) and specifically CEP X® and CEP Y® satellite III probes.

Successful resolutions of various LCN sample mixtures were continually performed at the conclusion of testing by integrating the FISH, LM, and optimized extraction/amplification techniques ascertained in each of these studies.

I. Cell Collection Studies and the Use of Cellulase to Increase Collection Efficiency

Different techniques of cellular collection were investigated to determine the best collection method for the recovery of cells from evidentiary substrates. The three methods of collection evaluated included direct transfer of evidence to slides, the cutting of substrate target areas followed by elution in buffer, and the swabbing of interest areas followed by elution in buffer. In conjunction with these studies, research was also conducted on increasing the efficiency of cellular elutions from cotton swabs by use of the enzyme cellulase. Blood, sperm, and epithelial sample cell collections were each evaluated from a variety of substrates.

Elution Evaluations of Evidence Swabs Using Various Cellulases

For the initial studies involving mock evidence and cellulases, epithelial and sperm cells were each separately spotted onto photocopy paper. The trials conducted utilized three different types of cellulases extracted from *Aspergillus niger, Trichoderma reesei,* or *Trichoderma viride.* Mock evidence areas, dried and aged for two days, were

wet swabbed and the swabs were eluted into phosphate buffered saline (PBS) containing different varieties of cellulase. Cellulase extracted from *A niger* worked the best out of the three cellulases releasing approximately twice as many cells as the control swabs based on DNA quantification. *T reesei* also seemed to improve cell release, but *T viride* appeared to disrupt the structural integrity of the epithelial cells and resulted in fewer cells being eluted from the cotton swab as compared to the control sample.

Based upon initial results, a second study was run with only *A niger* cellulase compared to non-cellulase swabs on one month old samples. The results associated with epithelial cells show an overall drop in DNA recovery when compared to the first study, but show the same correlation with cell numbers from cellulase recoveries being higher than control swabs. The results associated with the elution of sperm from cotton swabs in the presence or absence of cellulase indicated minimal differences regardless of the use or non-use of cellulase. Additional tests were conducted with sperm and white blood cell samples spotted on substrates such as metal, cotton, and leather. The results from these studies also suggested that the addition of *A niger* cellulase during elution increases the overall DNA yield received from swabbed samples. In summary, our findings indicate that cellulase does increase the total number of cells eluted from cotton swabs following the swabbing of various evidence substrates.

Mock Evidence Elution Evaluations of Epithelial and Sperm Cells

Mock evidentiary samples were created by spotting epithelial cells and sperm separately on cotton, paper, steel, and brass surfaces. Time points of zero days, one month, and in some cases, six months were eluted. Collection techniques of wet

swabbing and cutting were both used for the cotton and paper mock evidence samples. Only the swabbing technique was utilized for the metal surfaces. Because our cellulase examinations demonstrated improved cellular elution, the enzyme *A niger* cellulase was utilized for swabbing and cutting techniques. A set of control swab elutions without cellulase was also performed in conjunction with the other tests. Swabbing evidence in conjunction with the enzymatic cellulase digestion proved to be the most efficient method of collection. Those samples swabbed and subjected to enzymatic digestion consistently outperformed cuttings and control swabs not exposed to cellulase.

Mock Evidence Elution Evaluations of Blood Samples

Mock evidentiary samples were created by spotting whole blood onto cotton, paper, steel, and brass surfaces. The spots were allowed to dry and were then collected by wet swabbing with cellulase aided elution. The biological material was lightly pelleted, and a white blood cell isolation protocol was performed to eliminate the presence of any other components of the blood. Cells were collected via LM and were amplified using low volume, high cycle number MiniFiler™ reactions. Full 9 loci STR profiles were obtained from low numbers of white blood cells collected from all the surfaces.

II. Slide Preparation Techniques for LM Processing

Once sample cells have been successfully removed from evidence substrates and eluted into a collection buffer, application to standard glass microscopic slides can ensue. For some biological fluids, blood samples in particular, a brief purification step may be

required prior to affixation onto slides. Any residual materials remaining from collection may obstruct the capture of target cells when processing with an LM instrument. A cytogenetic cellular separation technique involving multiple buffers and wash steps was found to be highly effective for preparing white blood cell samples for application to glass slides to be used in LM practices.

The preparation of slides for LM is a delicate practice. Sample cells must be adhered lightly enough to glass slides for unproblematic removal but also firmly enough to avoid nonspecific capture. Standard slide preparations typically utilize air and/or heat fixations which can be too harsh and will not release cells during LM. A graded ethanol and xylene fixation technique was found most suitable for affixing the cells to glass slides for LM purposes.

III. Laser Microdissection Systems Overview

Laser microdissection instruments provide for the separation and removal of cells from mixtures in order to obtain a single DNA profile. The two LM systems utilized for our research can be categorized as either non-automated, Arcturus PixCell® II, or automated, Zeiss PALM® MicroBeam. Each machine is able to selectively remove cells of interest from biological samples. The two mechanisms by which these systems capture samples are discussed in detail in this section.

Sample Processing with the Arcturus PixCell® II System

The Arcturus PixCell® II laser capture microdissection system utilizes laser energy and caps comprised of a thin thermoplastic film to remove tissues or individual

cells. Prepared slides are mounted onto the scope and a CapSure® laser capture cap is placed gently onto the sample. The cap does not actually touch the material in the target area but rests just above. After the cell of interest is targeted with the laser, the laser is fired, melting the thermoplastic film on the base of the cap to the targeted material. The cap can then be removed with the selected material adhered to the base of the cap. In cases where low cell numbers are captured, the slides are checked after capture to ensure the cells are removed; the cap is checked to ensure that the cells are attached and that no other cells have adhered to the cap. Throughout the course of research, practical techniques have been optimized for sample processing with this machine.

Sample Processing with the ZEISS PALM® MicroBeam System

In addition to processing samples with the PixCell® II, samples were also microdissected with the Zeiss PALM® MicroBeam system. The basis for laser microdissection with the PALM® MicroBeam system is non-contact cellular and tissue catapulting via a high-powered UV laser into a collection vessel. The PALM® system uses a high energy UV laser to transfer sample cells from glass slides into collection vessels. The laser utilized in this system makes direct contact with target cells and pressure catapults them into collection caps. Throughout the course of our research, we have developed practical techniques to optimize these laser settings for sample processing.

IV. Extraction and Concentration of LM Processed Cells

Multiple methods for DNA extraction and amplification were evaluated in order to select a protocol that was consistently compatible with PixCell® II laser capture functions and Zeiss PALM® laser microdissection pulse catapulting (LMPC) operations. The QIAamp® Micro Extraction kit and Arcturus PicoPure® DNA extraction kit extraction were evaluated to determine the best system for achieving robust DNA yields and quality downstream STR profile generation when using the Applied Biosystems AmpF*l*STR Identifiler® kit, the Applied Biosystems AmpF*l*STR Yfiler® kit, and the Applied Biosystems AmpF*l*STR MiniFiler™ kit. Epithelial, blood, and sperm samples were tested during these examinations. At the conclusion of this evaluation process, the QIAamp® Micro Extraction kit was found most efficient and reliable for processing low number of cells (5 -10) collected by laser microdissection.

Qiagen QIAamp® Micro Kit Evaluation

In order to extract the low amount of DNA that is present in the LM collected cells on the caps, low volume DNA extractions were utilized when testing the QIAamp® Micro Kit. For LCN testing of the QIAamp® Micro Kit, cell collections were performed with the PixCell® II. Collections of 1, 2, 5, 10, and 20 cells were performed in triplicate. Following extraction, amplification of the STR loci was accomplished using the MiniFiler™ kit in a low volume/high cycle number reaction. All 10 and 20 cell captures yielded full profiles, the 5 cell captures yielded a full and a high-partial profiles of 6 loci, and the 1 and 2 cell captures yielded low partial profiles of 4 alleles or less. The

QIAamp® Micro protocol for the "Isolation of Genomic DNA from Laser-Microdissected Tissues" was found to be highly efficient when compared the PicoPure® method.

PicoPure® DNA Extraction Evaluation

The PicoPure® DNA extraction kit is an *in situ* method which permits extraction of DNA from cells without requiring transfer of the lysate to different collection tubes. Once the extraction is complete, a PCR reaction can be set-up in the same microcentrifuge tube. The first set of extraction evaluations involving this kit were performed with elevated cellular amounts (≥ 55 cells) to ensure that the kit would work at levels higher than those typically used in the LCN laser captured samples. Following extraction, samples were amplified using low volume/high cycle MiniFiler™ reactions. The DNA extracted with the PicoPure® method was suitable for subsequent amplification and did produce full 9 locus profiles.

LCN evaluations with the PicoPure® kit were performed with low cellular amounts (≤20) collected via the PixCell® II. Following extraction, samples were amplified using low volume/high cycle MiniFiler™ reactions. One amplification from each of the three cellular amounts resulted in a poor DNA profile (zero or one locus). The 20 cell collections yielded full profiles, but the 10 cell and 5 captures yielded low-partial profiles of 6 loci. The results indicate that the PicoPure® technique did not perform as well as the QIAamp® Micro kit when processing LCN DNA samples.

V. Comparison of STR Amplification Systems

There were several amplification kits evaluated during this study to test the effectiveness of generating STR profiles from laser captured biological materials at low copy number levels. The Identifiler® kit, Yfiler® kit and the Minifiler™ kit were all individually examined for performance output. These kits have been tested on different types of cells with extractions performed with QIAamp® Micro kits.

Identifiler® Testing

LCN evaluations of the Identifiler® kit were performed with low cellular amounts (≤20) collected via the PixCell® II. Following extraction, samples were amplified using low volume/high cycle amplification reactions. Full 16 loci profiles were generated from 33% of the samples, 50% of the samples generated partial profiles, and a 17% failed/low amplification rate was observed in these tests. At low levels of epithelial cells, it appears that full 9 locus profiles are achieved in higher numbers with Minifiler™ as compared with the 16 locus Identifiler®. However, with the difference in loci present in kits of 9 with Minifiler™ and 16 with Identifiler®, it is possible to amplify more alleles in a high partial Identifiler® profile as compared to a full Minifiler™ profile.

Yfiler® Testing

LCN evaluations of the ABI AmpF*l*STR Yfiler® kit were performed with low cellular amounts (≤30) collected via the PixCell® II. Following extraction, samples were amplified using low volume/high cycle amplification reactions. Multiple counts of 5, 10, and 30 epithelial cells were collected using the PixCell® II instrument, and Yfiler®

amplifications were performed using low volume/high cycle number reactions. The 10 and 30 cell captures yielded high partial and/or full 17 loci Yfiler® profiles while each of the 5 cell amplifications yielded low partial profiles. Subsequent amplifications at 5 epithelial cells as well as 5, 10, and 20 spermatozoa yielded full 17 loci Yfiler® profiles.

MiniFiler™ Testing

Our results indicate that the ABI AmpF*l*STR MiniFiler™ amplification kit is the best amplification system for yielding full STR profiles from low levels of cells collected via LM. This kit was designed for the amplification of degraded or inhibited samples by reducing the size of the amplification targets. LCN evaluations of the MiniFiler™ kit were performed with low cellular amounts (≤20) collected via the PixCell® II. Following extraction, samples were amplified using low volume/high cycle amplification reactions. Multiple counts of 5, 10, and 20 epithelial cells were collected using the PixCell® II and PALM® MicroBeam instruments and amplifications were performed using low volume/high cycle number MiniFiler™ reactions. Examinations demonstrated that the MiniFiler™ amplification kit can consistently produce a full 9 locus STR profile from 5 buccal cells. The MiniFiler™ amplification kit outperformed the Identifiler® kit at low (5-10) epithelial cell levels when compared to the Identifiler® amplifications results.

Evaluation of the Effect of Cellulase on Laser Capture Samples

The incorporation of cellulase during our elution studies demonstrated a greater release of cells from cotton swabs than PBS elution alone. To ensure that the cellulase would not affect LCN sample extractions and amplifications, LM was performed on

cellulase manipulated samples. When utilizing low volume/high cycle MiniFiler™ amplifications, both cellulase treated and non-treated samples yielded full 9 locus STR profiles, thus revealing that cellulase at the concentration used has no detrimental effects on yielding full STR profiles.

Microdissection of Epithelial and White Blood Cells with the Zeiss PALM® MicroBeam

Varying numbers of epithelial and white blood cells were separated and collected using the PALM® MicroBeam. The MiniFiler™ kit was used to amplify the LCN samples. STR amplifications of 5, 10, 15, 20, and 25 LM collected cells were performed using both epithelial and blood samples. Sample sets of nine were collected for each cell count. Full profiles were consistently seen from the 15, 20, and 25 cell count collections. Approximately 75% of all the 10 cell count samples produced full or high partial profiles (one or two loci drop-out). Of the 5 cell samples, greater than 50% of these samples returned partial profiles (~50% drop-out) and approximately 25% returned high partials.

Laser Captures of White Blood Cells, Spermatocytes, and Sperm/Vaginal Epithelial Cell Mixtures Using the PixCell® II System

PixCell® II collections were performed on mixtures of sperm and vaginal cells to simulate the processing of sexual assault evidence. Calculated one-to-one mixtures of sperm and epithelial cells were combined in microcentrifuge tubes prior to application to glass slides. PixCell® II collections were performed at spermatozoa count levels of 5, 10, and 20 and vaginal cells counts of 5 and 10. All samples were amplified using low volume/high cycle number MiniFiler™ reactions. The 5 spermatozoa sets displayed a full

9 locus STR profile. The vaginal 5 and 10 cell extractions yielded full STR profiles with no visible contamination from the male contributor. All the spermatozoa extractions had full profiles or 17 out of 18 loci with only one replicate displaying a female component. Of the 10 male replicates, only one of the replicates displayed a weak profile containing a female component. The other nine male replicates exhibited only indications typical of a male profile.

Multiple sets of white blood cells were also collected using the PixCell® II instrument. Sets of 5 and 10 white blood cells were collected and amplified using low volume/high cycle number MiniFiler™ reactions. The 5 cell captures yielded high partial profiles (11-14 alleles) while all 10 cell captures yielded full profiles.

VI. The Use of Fluorescent in Situ Hybridization Techniques to Resolve Male and Female Epithelial Cell Mixtures

Fluorescent In Situ Hybridization (FISH) is a traditional cytogenetic technique used to detect the presence or absence of specific chromosomes and/or sequences of an individual's genome. This method utilizes fluorescent probes which are designed to bind to the targeted conserved DNA sequences of individual chromosomes. Fluorescence compatible microscopes are typically employed to visualize the multicolor probes used in these hybridizations. Interphase FISH techniques incorporate probes which pass through cellular membranes and into the nucleus, eliminating the need to lyse cells during processing. The absence of membrane lysis during interphase FISH techniques is desirable for the purposes of forensic operations. This method allows for the visual identification of male and female cells from sexual assault evidence using X chromosome

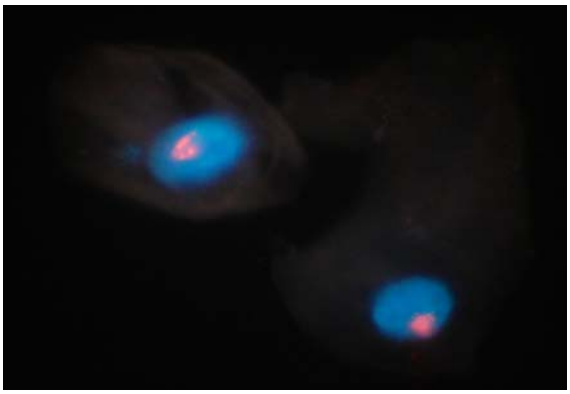
and Y chromosome probes. Following fluorescence processing, *intact* sample cells can be removed from the slides via laser-microdissection methods and extracted for further STR interpretation.

FISH Protocol Development

In attempt to separate male/female mixtures using interphase FISH techniques, Vysis CEP X® alpha satellite and CEP Y® satellite III probes were purchased and employed to visually identify the sex origin of each cell. An initial working protocol for the hybridization of Vysis CEP X® alpha satellite and CEP Y® satellite III probes was developed by merging the manufacturer's recommended procedure, those procedures published in scientific literature, and those techniques learned through personal scientific experience into one complete protocol encompassing all aspects of this type of analysis.

Sex chromosome labeling was successful with both epithelial and white blood cell varieties. Processing samples with this technique has allowed us to visually identify the male and female contribution to each sample mixture. For these evaluations, prepared one-to-one mixtures of epithelial and white blood cells were combined and applied to glass slides. Sex chromosomes were easily identified from epithelial and white blood cell mixtures. An X chromosome is visually identified by the presence of a green fluorescent marker while a Y chromosome can be readily detected by the presence of an orange fluorescent marker (Figure B).

Figure B: CEP-Y® Labeled Male Epithelial Cells with DAPI Counterstain Visualized at 630X Magnification As Viewed Through DAPI/FITC/TRITC filter



Amplification of FISH Treated Epithelial and White Blood Cells

Once the gender of target cells were visually identified, laser microdissections were performed using the PixCell® II and PALM® instruments. Collections of 5, 10, 20, 25, 30, and 40 cells were performed according to the optimized operating protocols for each LM system. Following extraction, amplifications using low volume/high cycle Identifiler® reactions were executed. The 20 cell captures displayed full to high partial STR profiles (8-14 loci) with the higher cellular amounts displaying full STR profiles or profiles missing only one or two alleles. For the purposes of MiniFiler™ processing, probe hybridized epithelial cells were captured in groupings of 5, 10, and 20 cells and then amplified in low volume/high cycle reactions. The 5 cell captures yielded high partial profiles of 7-8 loci and the 10 and 20 cell captures displayed full 9 locus MiniFiler™ STR profiles.

FISH Processing of Aged Forensic Samples

Interphase FISH processing was tested on varying aged forensic sample types. FISH processing and collections were performed on aged epithelial and white blood cell samples. Samples originating from aged post-coital swabs were also included in this evaluation. Target cells were located, identified, and separated using the PALM® instrument. Probe hybridization was successful on all of these samples. While it is unclear how the age of these samples affected profiling results, it appears that the age of a sample does not negatively affect the responsiveness of samples to interphase FISH processing. The collections of cotton substrates containing epithelial cells that were aged for 12 months generated low to mid-partial STR profiles (2-8 loci), and one sample did not generate any profile. The collections of glass substrates containing white blood cells aged for 16 months also generated low and mid-partial STR profiles (2-8 loci). The cellular collections of two year aged epithelial cells originating from post-coital swabs generated low (1-4 loci) profiles. It should be noted that moderate evaporation of samples was observed following amplification. Evaporation is always a concern when amplifying samples in lower reaction volumes. The evaporation witnessed may have adversely affected the results of this particular study.

VII. Summary

Many new and/or improved techniques for the processing of LCN evidence mixture samples were examined and developed during our sixteen months of work with LM instruments. Methods for the collection of cells from paper and cotton substrates using cellulase were evaluated and found to be highly efficient in comparison to other

methods. Basic protocols for collecting cells using LM with the PixCell® II instrument and the Zeiss PALM® MicroBeam system were utilized and improved upon. A standard method to extract DNA from laser captured cells using the Micro QIAamp® kit was established. The AmpF*l*STR Identifiler® kit, AmpF*l*STR Yfiler® kit and the AmpF*l*STR MiniFiler™ kit were each assessed and found to be suitable for the amplification of the DNA extracted from LM collected cells. A method to resolve mixtures of cells that have the same morphology but contain male and female contributors was developed. This method uses Fluorescent In Situ Hybridization (FISH) via Vysis CEP X® and CEP Y® probes to separate the male and female cells. Protocols and protocol enhancements were examined and implemented for the successful collection of FISH stained cells using the PixCell® II and the Zeiss PALM® MicroBeam instruments. By using what was learned during the work on NIJ Grant # 2006-DN-BX-K032, forensic labs can now successfully and efficiently resolve various LCN sample mixtures by incorporating FISH, LM, and optimized elution/extraction/amplification methods into their standard operating protocols.

The combination of these methods allows forensic scientists to identify male and female contributors from mixed gender assault cases in which the evidence consists of similar cell morphology. The resolution of these mixtures is based on genetic gender identification using the FISH method combined with LM and optimized DNA extraction and amplification techniques.

I. INTRODUCTION

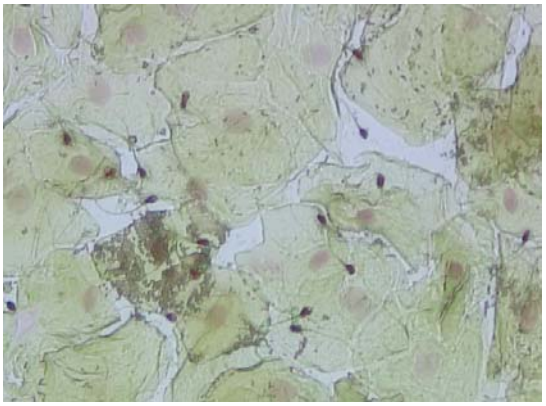
Statement of Problem and Literature Review

Laser microdissection (LM), a micromanipulation procedure that isolates individual cells for subsequent molecular analysis, is a well-established clinical laboratory technique [1-4]. The adaptation of this method to the forensic field has provided an additional means of physically separating sperm from epithelial cells, separating mixtures of cells, and enhancing touch evidence analyses. The ability to target single cells or parts of cells is invaluable to the study of cellular dynamics, biochemistry, and genetic analysis. Although some authors have investigated its forensic use [5-9, 10], much of the research required to meet the rigorous standards of casework has yet to be completed, including optimization of the cell transfer technique, determination of the most efficient DNA extraction and amplification methods, separation of difficult mixtures, and support that the procedure works for a wide range of evidentiary samples.

LM systems can be categorized as either non-automated (e.g. Arcturus PixCell®) or automated (e.g. Arcturus Veritas™ and Carl Zeiss, Inc. PALM® MicroBeam), and each is able to selectively remove the cells of interest. The benefit of the automated systems is that they have scanning devices that recognize cells based on size, color, shape, or fluorescent signal and can process up to three slides with less human interaction. Multiple scanning programs have been developed and utilized for examining and detecting sperm cells removed from sexual assault evidence. It is within these two types of systems that one of the strengths of LM lies – the automated systems can be adopted by high throughput laboratories and the less costly non-automated systems by smaller laboratories.

An area of forensics that has benefited most from the use of LM technology is the separation of sperm cells from epithelial cells associated with evidence from sexual assaults (Figure 1). Common methodology involves an initial screen for semen followed by a visual confirmation of the presence of sperm and a differential extraction to isolate the DNA. Preferential lysis (i.e. differential DNA extraction) has been the benchmark on obtaining separate male and female DNA profiles from sexual assault evidence [11]. However this technique is extremely laborious and often results in DNA mixtures consisting of a major and minor DNA profile.

Figure 1: Sexual Assault Mixture Containing Epithelial and Sperm Cells Visualized at 630X Magnification



With the development of LM techniques, researchers can now separate sperm cells and epithelial cells resulting in clean single DNA profiles. Elliott et al. [6] investigated the effectiveness of using laser microdissection versus differential extraction on post-coital vaginal smears. A comparison of likelihood ratios generated from DNA analysis of sexual assault evidence consisting sperm and epithelial cells, derived from laser microdissection versus preferential lysis, showed that LM performed better than

differential lysis in 15 of the 16 sample pairs analyzed [6]. The researchers continuously obtained the best results, with regards to clean DNA profiles, from the LM samples. In their study, partial DNA profiles (43% of the expected 10 loci and amelogenin profile) were obtained from as little as two sperm cells. These results demonstrate great potential for the development of low copy amplification in combination with LM in order to obtain a discriminating DNA profile from forensically relevant evidence.

An example of mixture resolution is demonstrated by Robino et al. [9] who utilized LM for the purposes of determining incestuous paternity. In this case, morphologically different cells were dissected from aborted material to reveal an STR profile indicative of a child conceived by the mother and her own brother [9]. The ability to resolve a mixture containing fetal cells from the aborted maternal tissue by using LM made it possible for the researchers to provide convincing evidence to prove the paternity of the products of conception. Overall, LM has great potential as a standard tool to resolve mixtures of morphologically different cells. The body fluids often encountered in casework (blood, saliva, semen, and vaginal fluid) contain different types of cells. By using LM, as in the study of Robino et al., specific cell types from mixed fluids may be collected separately and analyzed to determine an STR profile from each individual contained in the mixture.

One of the major challenges in LM is being able to differentiate between male and female cells. For morphological differences between sperm and epithelial cells, hematoxylin/eosin staining performs best for its ability to morphologically differentiate sperm cells from epithelial cells and has a minimal effect on further downstream analysis. Christmas tree stain (nuclear fast red/picroindigocarmine) does differentiate sperm cells

from epithelial cells; however it exhibited more of an inhibitory effect on PCR than the hematoxylin/eosin staining method [12]. The acridine orange stain was shown to totally inhibit the amplification reaction [12]. This study only examined sperm and epithelial cells. Other methods should be implemented to differentiate mixtures of donor cells that have similar morphology.

Techniques are needed for the separation of mixtures of cells of that originate from donors that have different genders but have similar cell morphology. For example, sexual assault evidence may contain male epithelial cells derived from saliva mixed with female epithelial cells from the skin. Fluorescent In Situ Hybridization (FISH) is a traditional cytogenetic technique used to detect the presence or absence of specific chromosomes and/or sequences of an individual's genome. This method utilizes fluorescent probes which are designed to bind to the targeted conserved sequences of individual chromosomes. Interphase FISH techniques incorporate probes which pass through cellular membranes and into the nucleus, thus eliminating the need to lyse cells for hybridization and allowing for intact cell separation by LM methods. In this case, labeling the X and Y chromosomes with different labels would be a way of determining the sex of a donor of a particular cell which then can be targeted for analysis. A recent study utilized a digoxigenin labeled chromosome Y hybridization probe to distinguish male epithelial cells in a mixture. Using this differentiation technique, it took ten diploid male cells to obtain a partial STR profile and 20 cells for a full profile [13]. Techniques of this nature can also use multiple probes for both X and Y chromosomes to better assure a scientist of the sex of the donor of a certain cell. Collins et al. [14] utilized multicolor FISH probes using dual X- and Y chromosome probes with 4'-6 diamidino-2-

phenylindole (DAPI) counterstaining to differentiate male from female epithelial cells obtained from post-coital penile swabs.

Analysis of touch evidence, or evidence containing a low number of cells, would benefit greatly from the implementation of LM. Currently, touch evidence is analyzed by swabbing the area of interest and using a low copy number protocol to extract and amplify the DNA. However, if only a few cells are present they can be missed by the swab or lost in the extraction process. LM collection of cells followed by extraction and amplification directly in the collection tube would maximize the amount of template DNA leading to a more definitive DNA profile. It would also provide the benefit of removing the cells from environmental contaminants that may inhibit DNA polymerases.

Furthermore, in the case of objects handled by more than one individual, LM may be able to help resolve the mixture. As mentioned above, LM would allow the isolation and possible amplification of DNA from a single cell. It has been previously demonstrated that a 6 locus DNA profile can be obtained from a single cell [15], and optimization of the extraction and amplification processes should allow for additional loci. With the background knowledge gained from the aforementioned studies [6, 10], we are on a path to potentially develop methods to select single cells from evidence using laser microdissection and obtain a discriminating profile from that cell, whether it is a spermatocyte, epithelial cell, or white blood cell.

The efficacy of removing evidentiary cells from swabs from such crimes as rape or burglaries is also critical. If cells cannot successfully be eluted from their substrates, then downstream analysis will not be as successful. Cellulases from organisms such as *Aspergillus niger* have produced twofold enhancements in sperm cell elution over elution

buffer alone. Even higher activity cellulases from the organisms *Trichoderma reesei* and *Trichoderma viride* are currently showing some initial promise. This research indicates that cellulase-digesting enzymes may enhance evidentiary cell release from a cotton swab over standard buffers alone [16].

DNA extraction protocols from these low copy number (LCN) is another crucial step which must be addressed. Both QIAamp® and Lyse-N-Go™ have been proven for recovery of DNA from LM-collected sperm cells [12]. Also Proteinase K digestion has been proven to be superior for LCN DNA extractions as compared to alkaline lysis procedures [17].

When dealing with very low amounts of DNA, it can be very difficult to obtain a full STR profile regardless of the amplification kit utilized. The first time that single cells were typed using modern forensic techniques, only six forensic STR markers were utilized [15]. In several cases, with the proper kit and cycling parameters, STR profiles were seen when amplifying as little as 10 cells [10]. This is extremely encouraging in attempting to obtain profiles from touch evidentiary samples. If only a few cells are needed in order to obtain a full STR profile, then mixture samples could be analyzed with greater ease. Typical multiplex STR typing PCR for forensics uses 1 ng of DNA coupled with 28 amplification cycles. If you are working with much lower DNA amounts, cycle numbers must be increased.

It has been demonstrated that by increasing the PCR amplification to 34 cycles, it is possible to analyze less than 100 pg of DNA [18]. Other studies have shown similar results by using 34 PCR cycles with 20-30 unstained, DAPI stained nuclei, and/or FISH treated male cells to achieve full 11 locus profiles [19]. However there are limits to the

number of amplification cycles that a DNA sample can maintain before amplification artifacts are observed. Further increase of the number of PCR cycles may not result in enhanced sensitivity and may have a highly negative effect on the balance of multiplex PCR systems. Exposing a sample to too many amplification cycles (55 cycles) showed that one could not obtain an accurate STR profile [18, 20].

One avenue to obtain a DNA profile from minimal cell amounts (5-20 cells) is to utilize low volume amplification reactions instead of the higher volume and manufacturers recommended amplification volumes. Low volume amplification has been demonstrated to significantly improve the ability to obtain DNA STR profiles from low amounts of DNA [21]. Gaines et al. showed that greater sensitivity was achieved when using 5 µl amplification reactions (in comparison to the manufacturer's standard 25 µl) for DNA amounts of less than 250 pg (the equivalent of about 50 cells) [21]. The researchers were able to obtain partial (5-8 loci) STR profiles using Applied Biosystems AmpF*l*STR® Profiler Plus® with as little as 30 pg DNA [21].

In 2007, Applied Biosystems released the AmpF*l*STR® MiniFiler™ PCR Amplification Kit which is the first commercially available 9-plex miniature STR amplification kit. This kit includes the D13S317, D7S820, D2S1338, D21S11, D16S539, D18S51, CSF1PO, and FGA loci along with sex-typing amelogenin. As in other STR kits, it will run the standard 5-dye technology and is designed to work on degraded or low level samples (125 pg) to achieve full profiles [22]. By relocating the PCR primers extremely close to the tandem repeats of each locus, improving the amplification buffers, and modifying the amplification reaction conditions, this kit provides profiles from samples that were unobtainable previously. We propose that further optimization of the

amplification reaction can be accomplished by evaluating the effect of low volume reactions on different multiplex kits. If found to be effective and efficient, the incorporation of the MiniFiler™ PCR Amplification Kit into LCN and LM procedures may prove to be an invaluable asset for profile generation.

Developing and implementing the aforementioned methods may improve the ability to separate forensic male/female mixtures of cells with the same morphology. Additionally, these methods may improve the ability to obtain a DNA profile from forensic evidence with a trace number of cells. Many of these aforementioned published techniques and methods have not been evaluated for their optimal use in conjunction with LM technologies. Improvements in processing could include optimization of cell transfer techniques, determination of the most efficient DNA extraction and amplification methods for LM collected cells, provide a method for the separation of difficult mixtures (epithelial/epithelial, white blood cell/white blood cell), and verification that these procedures work for a wide range of evidentiary samples. If shown to be successful, these techniques could be used in combination for a protocol to visually identify cells using fluorescent sex chromosome markers, knowingly collect low numbers of cells (via LM), utilize efficient DNA extractions, and perform low volume amplification for high quality short tandem repeat (STR) analysis. Protocols of this nature could be of extremely high value to forensic scientists in all laboratories.

Statement of Hypothesis

Laser microdissection methods provide for the separation and removal of cells from mixtures in order to obtain a single DNA profile. The key objectives of the research

were to develop, optimize, and implement different techniques utilizing LM for improved separation of sperm from epithelial cells, develop a method to resolve mixtures of male and female cells that have the same cell morphology, and improve methods related to DNA analysis of low copy number (LCN)/ touch evidence samples. The planned objectives of the research grant were as follows:

1. Determine the best evidence collection technique for both sperm and touch evidence, such as to establish the most efficient way to prepare and transfer the cells from the evidence to the slide for LM analysis.
2. Enhance the DNA extraction process from epithelial and white blood cells collected from LM.
3. Determine and optimize the best amplification procedure for a single cell or low number of cells for sperm and touch evidence.
4. Using what was learned in the above tasks, demonstrate the effectiveness of LM to isolate cells from a range of evidence samples (sperm and touch evidence).
5. Investigate methods to separate cells of similar morphology by using Fluorescent In Situ Hybridization (FISH) and specifically CEP Y® and CEP X® satellite III probes. These chromosomal stains will indicate the sex of the donor of a specific cell by fluorescing in the presence of a genetic marker found specifically on the X and Y chromosomes. It was the goal of this study to create a functional protocol in which 10-30 cells from sexual assault and touch evidence mixtures could be visually identified using FISH labeling of sex chromosomes and manually separated using an LM system, ultimately resulting in the generation of a full, single source STR profiles.

Each of these objectives focuses on the various sequential stages encountered in the processing of evidence with LM technologies. By examining and optimizing each stage, more can be learned about what strategies can and cannot be successfully integrated with cells collected by LM. It is theorized that by ascertaining and incorporating what was learned during the this investigation of overall LM processing, forensic labs could successfully and efficiently resolve various LCN sample mixtures by incorporating FISH, LM, and optimized elution/extraction/amplification methods into their standard operating protocols.

II. METHODS

In order to accomplish the targeted goal of resolving LCN assault mixtures using LM, the research was subdivided into four distinct sections. First, the best evidence collection technique for both sperm and touch evidence, i.e. establish the most efficient way to prepare and transfer the cells from the evidence to the slide for LM analysis, was determined. Once evidence collection evaluations were completed, an optimization of the DNA extraction process for epithelial and white blood cells collected from LM was performed. Following the extraction examinations, the best amplification procedure for a single cell or low number of cells for sperm and touch evidence was established. Lastly, cells of similar morphology by gender were separated using Fluorescent In Situ Hybridization (FISH) and specifically CEP X® and CEP Y® satellite III probes. At the conclusion of testing, it was expected that successful resolutions of various LCN sample mixtures could be continually performed by using the FISH, LM, and optimized extraction/amplification techniques ascertained in each of these studies.

A. Cell Collection Studies and the Use of Cellulase to Increase Collection Efficiency

Different techniques of cellular collection were investigated to determine the best collection method for the recovery of cells from evidentiary substrates. The three methods of collection evaluated included direct transfer of evidence to slides, the cutting of substrate target areas followed by elution in buffer, and the swabbing of interest areas followed by elution in buffer. In conjunction with these studies, research was also conducted on increasing the efficiency of eluting cells from cotton swabs by use of

cellulase. Blood, sperm, and epithelial sample cell collections were each evaluated from a variety of substrates.

Evaluation of Cellulase to Improve the Recovery of Cells from Evidence Swabs

For the initial studies involving mock evidence and cellulases, 400 cells (epithelial and sperm) were each separately spotted on photocopy paper. This cell amount was selected to simulate mock touch evidence. A hemocytometer was employed for estimating cell counts during the original preparation of these samples. However, this method was found to be unreliable when counting the number of cells recovered from each cellulase processed samples. The low recovery numbers and presence of residual cotton and/or paper fibers left in the bottom of elution tubes made it difficult to accurately count cells with a hemocytometer. Due to these factors, cell counts were not performed after collection form the substrate. DNA extractions and quantifications were used to measure the post-experimental data for this experiment to determine the optimum method for cell recovery.

Voorhees et al. had concluded that the enzymatic digestion of cotton swabs by low grade cellulases increased sperm elutions [16]. Their research indicated that the *Aspergillus niger* cellulase was the most efficient at digesting the cotton fibers and thereby improving the collection of sperm. Our investigation utilized the same three cellulases that were tested in the publication: *Aspergillus niger, Trichoderma reesei,* and *Trichoderma viride.* Mock evidence areas, dried and aged for two days, were wet swabbed and then eluted in 300 µl of 1X phosphate buffered solution (PBS). Cellulase enzymes were tested at concentrations of 7.02 units (U) (*A. niger)*, 16.32U (*T. reesei*),

and 37.4U (*T. rivide*). The cellulases were mixed with the PBS before the swabs were added. Control swabs were eluted in 300 µl of phosphate buffered solution (PBS) not containing with any cellulase. Data returned from those swabs subjected to enzymatic digestion were compared to the control swabs.

Mock Evidence Elution Evaluations of Epithelial and Sperm Cells

Mock evidentiary samples were created by spotting 4,000 epithelial cells and spermatozoa separately on cotton, paper, steel, and brass surfaces. Time point of zero (1-2 days after spotting and allowed to dry), one month, and six months were eluted. Collection techniques of wet swabbing and cutting were both used for the cotton and paper mock evidence samples. Only the swabbing technique was utilized for the metal surfaces. Because our cellulase examinations demonstrated improved cellular elution, cellulase (*A. niger*), at a concentration of 7.02U per reaction, was utilized in the evaluation. A set of control swab elutions without cellulase was also performed in conjunction with the other tests. Each collection technique was performed in triplicate and elutions were performed in 300 µl of PBS (20 µl cellulase (7.02U) added when applicable) at 37°C with 400 rpm agitation for one hour. Two sets of counts were taken from each replicate to determine the amount of material recovered.

Mock Evidence Elution Evaluations of Blood Samples

Mock evidentiary samples were created by spotting 20 µl of whole blood onto cotton, paper, steel, and brass surfaces. The spots were allowed to dry overnight and were then collected by wet swabbing with cellulase aided elution. The biological

material was centrifuged (6,000 rpm), and the white blood cell isolation protocol (*described in detail in the following section)* was performed using a hypotonic solution followed by the acetic acid and methanol stops. The cells were then spun down, suspended in low volume solution, stained with hematoxylin, and then spread on glass slides and prepared for LM according to protocol. DNA was extracted from the cells collected by laser microdissection and subsequently amplified using the MiniFiler™ kit, 32 cycles in a 6 µl reaction volume.

B. Slide Preparation Techniques for LM Processing

Once sample cells have been successfully removed from evidence substrates and eluted into collection buffer, application to microscopic slides can ensue. For some biological fluids, blood samples in particular, a brief purification step may be required prior to affixation to slides. Any residual materials remaining from collection may obstruct the capture of target cells when processing with an LM instrument. A cytogenetic cellular separation technique involving multiple buffers and wash steps was found to be highly effective for preparing impure samples for application to glass slides to be used in LM practices. This method is described in detail in the following section.

Regardless of the instrument utilized, the preparatory steps for slides to be used for LM processing require meticulous technique. Sample cells must be adhered lightly enough to glass slides for unproblematic removal but also firmly enough to avoid nonspecific capture. Standard slide preparations typically utilize air and/or heat fixations which can be too harsh and will not release cells during LM. The graded ethanol and

xylene fixation technique listed in this section has been found most suitable for affixing the cells to glass slides for LM purposes.

Separation of White Blood Cells from Whole Blood

Performing LM on blood samples can prove to be arduous due to the sheer number of red blood cells in comparison to white blood cells. When examining whole blood samples with LM instruments at the microscopic level, it can be extremely complicated to find and separate white blood cells from the remainder of the fluid's components. To resolve this problem, we utilized techniques typically used in clinical cytogenetic laboratories for cell harvesting and chromosome preparation [24]. The basis for these techniques lies in the osmotic lysing of red blood cells and separation of components by high speed centrifugation.

The following protocol was implemented to prepare white blood cells from whole blood:

1. Add 1-2 ml of whole blood to a 15 ml conical centrifuge tube containing 10 ml of hypotonic solution (0.075 M KCl buffer) pre-warmed to 37°C. Incubate tube in 37°C water bath for 20 min.
2. Add 4-5 drops of freshly prepared *Carnoy's Fixative* (3:1 Methanol/Glacial Acetic Acid) to stop the reaction. Mix well and centrifuge for 5 minutes at 1,200 rpm.
3. Remove the supernatant, leaving 0.5 ml of solution in the tube. Add 5 ml of freshly prepared fixative and mix well by inverting the tube so no clumps of cells remain. Centrifuge 5 min at 1,200 rpm. Repeat this step two more times.
4. By this point, a solid white clump of cells should be observed at the bottom of the tube in a clear solution. Remove the supernatant, leaving 0.5 ml of solution in the tube. Add 1 ml of freshly prepared fixative to the clump of cells and mix well. Transfer the 1.5 ml cell suspension to a 1.5 ml micro centrifuge tube and store at 4°C.

The resulting product of this protocol should be a purified pellet of white blood cells with a minimal presence of red blood cells. This technique for the purification of white blood cells has allowed us to conduct systematic LM and FISH examinations on

specimens of this type. This specific method was developed to systematically collect and process white blood cells from whole blood during the initial stages of this project.

Graded Ethanol and Xylene Cellular Fixation Technique

Preparing slides for laser capture microdissection is a critical step in the process. Cells need to adhere lightly to glass slides and be very dry for optimal capture. Standard microscopic slide preparation techniques utilizing air and /or heat fixation are not appropriate for the recovery of cells using LM. The cells adhere to the glass slides and are difficult to collect using LM. Because of these problems, we investigated different protocols to affix the cells to glass slides for downstream LM, FISH, and STR amplification. The gradual alcohol dehydration protocol listed below was found to provide an appropriate level of cellular attachment. This method allows for the cells to attach firmly to the glass slide while still permitting for relatively easy removal with LM instruments. The following protocol was found to work best for sample preparation:

-Graded Ethanol and Xylene Cellular Fixation Protocol-

1. Suspend sample cells in 25–50 µl of *Carnoy's Fixative* or 1X PBS
2. Spread low volume of suspended cells onto the slide (5-15 µl) and air dry
3. Immediately place slide in 75% ethanol for 30 seconds
4. Place slide in 95% ethanol for 30 seconds
5. Place slide in 100% ethanol for one minute
6. Place slide in xylene for two minutes
7. Air dry slide until all xylene has evaporated (~10 minutes)

Slides and samples prepared using this protocol resulted in the successful collection of cells using two different laser microdissection instruments, and the collected cells were found suitable for STR analysis.

C. Laser Microdissection Systems Overview

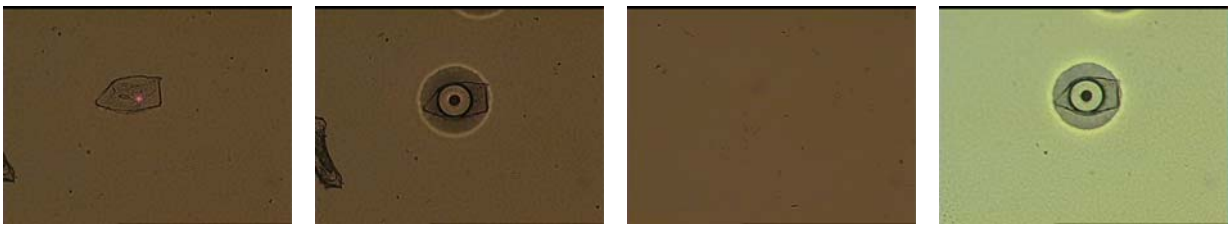
Laser microdissection instruments provide for the separation and removal of cells from mixtures in order to obtain a single DNA profile. The two LM systems utilized for our research can be categorized as either non-automated, Arcturus PixCell®, or automated, Zeiss PALM® MicroBeam. Each machine is able to selectively remove cells of interest from biological samples. For the purposes of this research, a direct comparison of the two instruments was not performed. These two machines were evaluated for their suitability in the processing of forensic mixtures. The two mechanisms by which these systems capture samples as well as the protocols developed for their efficient operation are discussed in detail in this section.

Sample Processing with the Arcturus PixCell® II System

The Arcturus PixCell® II laser capture microdissection system (Figure 2) utilizes laser energy and caps comprised of a thin thermoplastic film to remove tissues or individual cells. Prepared slides are mounted onto the scope and a CapSure® laser capture cap is placed gently onto the sample. The cap does not actually touch the material in the target area but rests just above. After the cell of interest is targeted with the laser, the laser is fired, melting the thermoplastic film on the base of the cap to the targeted material. The cap can then be removed with the selected material adhered to the base of the cap. In cases where low cell numbers are captured, the slides are checked after capture to ensure the cells are removed; the cap is checked to ensure that the cells are attached and that no other cells have adhered to the cap. This process is described below in Figure 3.

Figure 2: The PixCell® II Laser Capture Microdissection System

Figure 3: Cell Collection Process with the Arcturus PixCell® II. From left to right: cell targeting, cell capture, image of the slide with cell removed, and presence of the cell on the cap alone.



The protocol below outlines the developed method for configuring the optimal laser and duration settings of the PixCell® II system.

-Protocol: Optimizing Laser Intensity and Duration using the PixCell® II System-

This protocol is further described in the Arcturus PixCell® II system technical manual [25].

1. Mount slide to microscope stage and lock in place using the vacuum slide holder.
2. Remove CapSure® cap from base using the swing arm and set cap gently onto the slide surface.
3. Activate laser energy (~72 milliwatts (mW)) and focus the beam until a fine point can be seen with a minimal halo around the beam.
4. As a test, fire a single laser pulse onto an area of the slide where there is no material. Ensure that the film melts down to the slide surface by verifying a dark center to the laser fire.

5. Vary intensities and duration of laser energy until slide contact can be achieved by a single pulse. Typical power ranges from 72-80 mW with 1.8-2.4 millisecond (ms) pulse duration.
6. Target first cell and fire laser. Lift cap gently to ensure cell is removed from slide surface and is attached to the CapSure® cap.

DNA extraction can then be performed on the material with the addition of an ExtracSure™ device. This creates barrier around the collected tissue to which extraction buffer can be added.

Sample Processing with the ZEISS PALM ® MicroBeam System

In addition to processing samples with the PixCell® II, we also microdissected samples with the Zeiss PALM® MicroBeam system (Figure 4). The basis for laser microdissection with the PALM® MicroBeam system is non-contact cellular and tissue catapulting via a high-powered UV laser into a collection vessel. The PALM® system uses a high energy UV laser to transfer sample cells from glass slides into collection vessels. The laser utilized in this system makes direct contact with target cells and pressure catapults them into collection caps. Because the laser comes in direct contact with sample cells, it is extremely important to adjust energy levels and focal settings to the lowest effective levels to limit damage to cellular DNA sequences when removing samples from a glass slide. Throughout the course of our research, we have developed practical techniques to optimize these laser settings for sample processing.

Figure 4: The PALM® MicroBeam LM System

Optimization of Catapulting Energies

Based on feedback we received from PALM® Microlaser Technologies of Germany, we implemented a technique to adjust the laser of the Zeiss accurately to obtain optimal forced focus light (catapult) energy levels achieving optimal laser focus settings for glass slides. This technique employs marking a clean 1 mm thick glass slide with a black ink fine tipped marker. The black ink smear provides a visual representation of the focal plane of the slide. The below protocol outlines the developed method for configuring the optimal laser and focal settings of the PALM® instrument using black ink marked slides.

-Protocol: Optimization of the Laser Energy and Focus Settings for Laser Cutting-

1. Mark a clean 1 mm thick glass slide with a black ink fine tipped marker. The black ink smear provides a visual representation of the focal plane of the slide. Set the glass slide under the microscope into the optical focal plane.
2. Start cutting lines into the ink smear and lower the energy to the point where the laser scarcely scrapes the ink surface (minimal ablation).
3. Adjust upwards with the laser focus (left lower laser panel) until the visual cutting line disappears. You should note this value; this is the upper laser focus limit.

4. Adjust downwards again and find the lower laser focus limit, which is the point where the visual cutting line disappears again. The mean value between the upper and lower focus limit is the optimal focal plane for your sample.
5. Starting from the optimized values obtained above, increase the delta energy as much as necessary (typically around 20 to 40 points, but may be lower), and the delta focus should be set around plus or minus 4 points.
6. The main setting to obtain optimal catapulting conditions is the delta focus. Find out which combination of delta energy and focus leads to catapulting with the lowest amount of energy. This is done by catapulting single cells with one set of values. After this first optimization step, these values tend to be valid for all similar specimens.

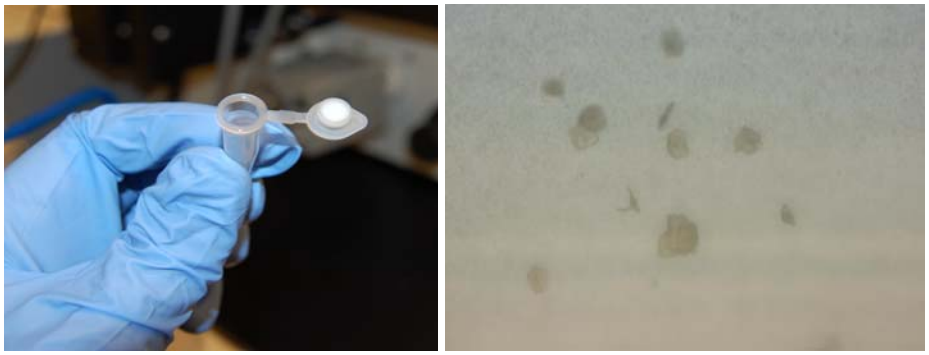
Using the optimal laser setting technique described above, we established an optimal focus value at 45 units which allowed us to catapult at an energy level of 60 mW, the lowest possible setting to consistently lift sperm cells from the slide. When performing Laser Microdissection Pulse Catapulting (LMPC) on epithelial and white blood cells on glass slides, always target the laser pulse directly at the middle of the cell. This is a good way to assure complete cells heads have been catapulted into the collection device.

Collecting Catapulted Samples with Zeiss Adhesive Caps

When catapulting samples with the Zeiss PALM® MicroBeam instrument, the microscopist is given the choice of collecting the processed cells in the cap of a 0.5 ml micro centrifuge tube containing 40 µl of lysis buffer or a cap containing adhesive glue, PALM® Adhesive Caps, manufactured by Zeiss (Figure 5a). Through a series of short studies, we have determined that optimal collection of samples is achieved by utilizing the caps containing adhesive rather than those with buffer. The micro centrifuge caps containing the adhesive glue secured and retained catapulted cells much more efficiently and reliably than those caps containing buffer. It is also important to note that visual

confirmation of successful sample microdissection was easily achieved when using the adhesive caps (Figure 5b).

Figure 5: (a) Photograph of a Zeiss Adhesive Cap for LM collection. (b). Image of Zeiss adhesive cap collection surface after LM collection of hematoxylin stained epithelial cells.



(a) (b)

D. Extraction and Concentration of LM Processed Cells

Multiple methods for DNA extraction and amplification were evaluated in order to select a protocol that was consistently compatible with PixCell® II laser capture functions and PALM® LMPC operations. The QIAamp® Micro Extraction kit and PicoPure® DNA extraction kits were evaluated to determine the best system for achieving robust DNA yields and quality downstream profile generation when using the AmpF*l*STR Identifiler® kit, the ABI AmpF*l*STR Yfiler® kit, and the ABI AmpF*l*STR MiniFiler™ kit. Epithelial, blood, and sperm samples were tested during these examinations. At the conclusion of this evaluation process, the QIAamp® Micro Extraction kit was found most efficient and reliable for processing low level (5-10 cells) LM samples.

Qiagen QIAamp® Micro Kit Evaluation

In order to extract the low amount of DNA that is present in the LM collected cells on the caps, low volume DNA extractions were utilized when testing the QIAamp® Micro Kit. The QIAamp® Micro protocol for the "Isolation of Genomic DNA from Laser-Microdissected Tissues" was found to be highly efficient when compared to other methods. In this protocol, 15 µl of ATL Buffer and 10 µl of Proteinase-K are used for the initial lysis at 56°C for one hour. Additional lysis using AL Buffer follows which contains carrier RNA. This enhances the binding of low levels of DNA to the QIAamp® membrane. At the end of the extraction, the sample is eluted with deionized H_2O in a 50 µl volume twice with five minute incubations each for a total of ~100 µl of eluate. The volume of this eluate is subsequently adjusted to 5 to 10 µl using Microcon® YM100 filters.

For LCN testing of the QIAamp® Micro Kit, PixCell® II captures were executed as described in the previous sections. Captures of 1, 2, 5, and 10 cells were performed in duplicate and 15 µl aliquots of ATL Buffer and 10 µl aliquots of Proteinase-K were added to the LCM ExtracSure® devices. Extractions and Microcon® concentrations were performed and amplification of the STR loci was accomplished using the MiniFiler™ kit in an amplification volume of 6 µl. A 32 cycle amplification procedure followed.

PicoPure® DNA Extraction Evaluation

The PicoPure® DNA extraction kit is an *in situ* method which permits extraction of DNA from cells without requiring transfer of the lysate to different collection tubes. Lysis buffer and Proteinase-K are added to the collection vessel to lyse and digest cellular

proteins. The reaction is a 3 hour, 65°C incubation with sample and the reconstituted Proteinase-K solution. The protease reaction is halted by a 10 minute, 95°C inactivation step. Once the Proteinase-K is heat inactivated, a PCR reaction can be set-up in the same microcentrifuge tube.

The first set of extraction evaluations was performed on higher cellular amounts of 55, 110, 219, and 438 cells to ensure that the kit would work at levels higher than those typically used in the laser captured samples. These counts were based on an average of multiple hemocytometer counts taken from a cleaned buccal cell suspension. Each cellular amount was suspended in 2 µl of PBS, and 15 µl of the prepared extraction buffer were added to the samples. After the extraction was finished, samples were concentrated via Microcon® and then amplified in a 6 µl, 32 cycle MiniFiler™ reaction.

The PicoPure extraction method for low copy number samples was evaluated with cells collected by PixCell II® laser capture microdissection. Captures of 5, 10, and 20 cells were performed in duplicate and 15 µl of the prepared extraction buffer was added to the LCM ExtracSure® devices. Extractions and Microcon® concentrations were performed and amplification of the STR loci was accomplished using the MiniFiler™ kit in an amplification volume of 6 µl and 32 cycle amplification procedure.

Microcon® YM-100 Sample Concentrations

Sample volumes in low volume amplifications should be reduced to 2-3 µl in order to utilize all genomic material present in each extraction. Millipore Microcon® YM-100 columns were used to accomplish this task. Samples volumes are increased

with TE^{-4} (10 mM Tris HCL, .1mM EDTA) to 300 µl and then the sample is spun at low speed (600 x g) until the desired volume is reached.

E. Comparison of STR Amplification Systems

There were several amplification kits evaluated during this study to test the effectiveness of generating STR profiles from laser captured biological materials at low copy number levels. The ABI AmpF*l*STR Identifiler® kit, ABI AmpF*l*STR Yfiler® kit and the ABI AmpF*l*STR Minifiler™ kit were all individually examined for performance output. These kits have been tested on different types of cells with extractions performed with QIAamp® Micro kits.

Identifiler® Testing

Initial Identifiler® evaluations employed 6 µl 32 cycle amplifications with an amplification reaction utilizing 1/3 of the primer that the manufacturer recommends. LM collections of five cells per DNA extraction were performed using the PixCell® II system, extracted, and concentrated using the QIAamp® Micro Kit and concentrated using Microcon® YM-100 columns. An additional set of five cell amplifications was performed with enzyme volumes scaled down from manufacturer's recommendations. A subsequent study was performed with three donors and four replicates utilizing the reduced units of DNA Polymerase in 6 µl, 32 cycle Identifiler® reactions. Five cell captures were performed using the PixCell® II system and extracted/concentrated using the QIAamp® Micro Kit and Microcon® YM-100 columns. Additional testing was also

performed using ten spermatozoa and epithelial cell levels as well as 75 pg of control DNA.

Yfiler® Testing

For initial Yfiler® trials, male buccal cells were purified, adhered, and dehydrated on slides as described in the previously listed protocols. Captures at 5, 10, and 30 epithelial cells were performed on the PixCell® II followed by QIAamp® Micro DNA extraction and Microcon® concentration. Yfiler® amplifications were performed using a reduced volume 6 µl, 32 cycle reaction based upon manufacturer's recommendations. Subsequent amplifications were also performed with 5 epithelial cells as well as 5, 10, and 20 spermatozoa. Yfiler® amplification from collected sperm cells is currently still in progress.

MiniFiler™ Testing

For the initial testing of MiniFiler™ paired with LM, an abundant number of cells were collected to ensure proper amplifications. Trials of 10, 20, 50, 100, and 200 epithelial cells and 100 and 200 sperm cells were captured. QIAamp® Micro was run with a single 20 µl elution, and no concentration was performed. For the amplification in this initial run, a 13 µl, 30 cycle amplification was run using 5.5 µl of template.

Additional sets of captured cells utilized several modifications to the standard amplification procedure. The samples were amplified in a 6 µl reaction with 32 cycle amplification. These techniques have all previously shown to work better on LCN samples. Cell captures of 1, 2, 5, and 10 cells were used for this trial. After extraction

with a single 20 µl elution, these samples were quantified and then concentrated using Microcon® in an attempt to amplify all available templates. Alongside these amplification, 9947A control samples were prepared at concentrations of 3, 6, 12, and 25 pg.

With the threshold of five cells needed to get a complete profile, cell capture numbers lower than that were investigated more in depth. Increasing amplifications to 34 cycles was attempted in order to accurately generate more alleles in low cell capture extractions. Captures of 1 cell, 2 cells, and 3 cells were performed, extracted, and concentrated as previously described. Six microliter amplifications were run at 34 cycles, and 3100® analysis followed.

An additional study was conducted at a 5 epithelial cell level with Minifiler™ to ensure full 9 loci profiles could be obtained over multiple trials. Three separate, 5 cell captures, each from a single donor, were performed with the PixCell® II followed by QIAamp® Micro DNA extractions and Microcon® sample concentrations. Six microliter, 32 cycle amplifications were run and analyzed with an ABI 3100® genetic analyzer.

In a multiple person study at the 5 buccal cell level, three individuals were sampled at four separate trials each. Laser captures were performed using the PixCell® II system and extracted/concentrated using the QIAamp® Micro Kit and Microcon® YM-100 columns. Additional testing was performed with various levels of spermatozoa and epithelial cells as well as 75 pg of control DNA. Minifiler™ in conjunction with various extraction techniques as well as cellular treatments has been optimal in amplifying LCN samples. These results are discussed in the subsequent sections of the report.

Evaluation of the Effect of Cellulase on Laser Capture Samples

The incorporation of cellulase during our elution studies demonstrated a greater release of cells from cotton swabs than PBS elution alone. To ensure that the low grade cellulase would not affect LCN sample extractions and amplifications, LM needed to be performed on cellulase manipulated samples. A 0.351 U/µl *A. niger* cellulase solution was prepared in dH2O. Twenty microliters of the cellulase solution were added to a 280 µl buccal cell suspension in PBS, and samples were incubated at 37°C for one hour. Cells were adhered to slides using the graded ethanol and xylene protocol, and PixCell® captures were performed at the five cell level along with non-cellulase exposed cells as controls. QIAamp® Micro extractions, Microcon® concentrations, and 6 µl, 32 cycle Minifiler™ amplifications were performed.

Microdissection of Epithelial and White Blood Cells with the Zeiss PALM® MicroBeam

Varying numbers of epithelial and white blood cells were separated and collected using the PALM® MicroBeam. Samples were catapulted onto Zeiss adhesive caps using an energy level of 65 mW. Samples were then spun down from the adhesive at 14,000 rpm into QIAamp® lysis buffer. The QIAamp® Micro kit was utilized for all extractions. After extraction, these samples were concentrated using Microcon® YM-100 columns in an attempt to amplify all available templates. The MiniFiler™ kit was used to amplify the LCN samples. Sample template was amplified in 6 µl amplifications were performed using 2.5 µl PCR master mix, 1.25 µl primer, and ~2.25 µl sample. Amplifications were run at 32 cycles due to the low template amount. Samples were analyzed with ABI 3100® Genetic Analyzers and Genemapper™ ID software. Using the MiniFiler™ kit, STR

amplifications of 5, 10, 15, 20, and 25 LM collected cells using the PALM® MicroBeam were performed using both epithelial and blood samples. Sample sets of nine were collected for each cell count.

Collection of White Blood Cells, Spermatocytes, and Sperm/Vaginal Epithelial Cell Mixtures Using the Laser Capture Microdissection PixCell® II System

White blood cells were purified for testing by using the white blood cell isolation procedure, which successfully lysed all red blood cells while leaving white blood cells intact. The resulting solution of methanol, acetic acid, and intact white blood cells evaporated almost immediately upon placement on glass slides for fixation. The standard dehydration procedure was followed and during the LM process, it was noticed that the cells adhered better than the PBS cellular suspensions. Cells were still able to be removed from the glass slide, but no additional cells would bond to the cap as in some of the PBS cellular slides. Captures of 5 and 10 white blood cells were performed and then extracted using QIAamp® Micro followed by Microcon® concentration. Amplifications with 6 µl, 32 cycles MiniFiler™ amplifications were then performed.

The collection of spermatocytes using LM with the PixCell® II system, extracted using the QIAamp® Micro kits, concentrated with Microcon® filters, and amplified with the MiniFiler™ system was further evaluated to determine the limit of sensitivity (minimum number of cells producing an 8 locus STR profile). Spermatocytes were isolated by combining 60 µl of semen and 400 µl PBS. Five minute spins at 3,000 rpm were used to pellet the spermatocytes, and the supernatant was removed. The pellet was re-suspended in 400 µl PBS and the spin/cleaning procedure was repeated. After two

washes, the pellet was re-suspended in 20 µl PBS and 2 µl of Arcturus® hematoxylin stain was added. Ten microliters were placed onto glass slides and the graded ethanol/xylene dehydration protocol was performed. Five and 20 spermatozoa were collected by the PixCell® II procedure. Dithiothreitol (DTT) was added to the extraction buffer in order to properly lyse the spermatocytes. The lysis solution for the QIAamp® Micro kit contained 15 µl ATL Buffer, 5 µl Proteinase K, and 5 µl 0.39M DTT. The rest of the DNA extraction was carried on according to standard operating protocol and was followed by Microcon® concentrations and 6 µl, 32 cycle MiniFiler™ amplifications.

Forty microliters of semen and vaginal suspensions were purified separately. The fluid was mixed with 200 µl PBS and spun at 3,000 rpm for 5 minutes. Pellets were re-suspended in fresh 200 µl PBS. This sample cleaning protocol was repeated a total of three times. Final pellets were re-suspended in 20 µl PBS and 2 µl of Arcturus® stain. Approximately 22 µl of each solution were combined together and spotted onto glass slides. The graded ethanol/xylene dehydration protocol was performed to ensure proper adhesion. PixCell® II captures were performed for cell counts of 5 vaginal cells and 5, 10, and 20 spermatozoa. Modified Qiagen® Micro DNA extractions were performed followed by Microcon® concentration and 6 µl, 32 cycle MiniFiler™ amplifications.

F. The Use of Fluorescent In Situ Hybridization Techniques to Resolve Male and Female Epithelial Cell Mixtures

Fluorescent In Situ Hybridization (FISH) is a traditional cytogenetic technique used to detect the presence or absence of specific chromosomes and/or sequences of an individual's genome. This method utilizes fluorescent probes which are designed to bind to the targeted conserved sequences of individual chromosomes. Fluorescence

compatible microscopes are typically employed to visualize the multicolor probes used in these hybridizations. FISH examinations can typically be divided into two categories: Metaphase and Interphase analysis.

Metaphase FISH analysis normally involves the culturing of various cell and tissue types, fracturing cellular membranes for the purposes of DNA release, and the systematic spreading of chromosomes for visual interpretation. Interphase FISH techniques incorporate probes which pass through cellular membranes and into the nucleus, eliminating the need to lyse cells during processing. The absence of membrane rupture during interphase FISH techniques is desirable for the purposes of forensic operations. This method allows for the visual identification of male and female cells from sexual assault evidence using X chromosome and Y chromosome probes. Following fluorescence processing, *intact* sample cells can be removed from the slides via laser-microdissection methods and extracted for further STR interpretation. For these reasons, interphase FISH techniques were employed during the course of the research.

FISH Protocol Development

In attempt to separate male/female mixtures using interphase FISH techniques, Vysis CEP X® alpha satellite and CEP Y® satellite III probes were purchased and employed to visually identify the sex origin of each cell. An initial working protocol for the hybridization of Vysis CEP X® alpha satellite and CEP Y® satellite III probes was developed by merging the manufacturer's recommended procedure, those procedures published in scientific literature, and those techniques learned through personal scientific experience into one complete protocol encompassing all aspects of this type of analysis.

Listed below is the protocol that was initially developed and employed for all subsequent testing:

-Initial FISH Sample Slide Preparation-

- Before applying cell samples to glass slides, be sure to pre-warm a water bath to a temperature of 75 - 80°C, a slide warmer to a temperature of 45 - 50°C, and a biological oven to a temperature of 60°C.

1. Pipette 10–20 µl of sample cell solution onto a glass slide, 1 mm in thickness, and spread gently using the pipette tip.
2. Place the slide above the steam of a water bath for 5 seconds to allow for further cell membrane spreading.
3. Place slides on a slide warmer until completely dry.
4. Fix the cells to the slides by placing the sample slides in a biological oven heated to 60°C for three hours.

-Initial FISH Protocol for CEP X and Y Probe Hybridization-

-Before beginning these FISH procedures, be sure to pre-warm a water bath to a temperature of 75 - 80°C, a slide warmer to a temperature of 45 - 50°C, and a biological oven to a temperature between 37 - 42°C.

-A pre-warmed humidity chamber can be prepared for the hybridization step by placing a moistened paper towel inside a small airtight container and inserting it into the heated oven.

-Note: Fluorophores are readily photobleached by exposure to light. To limit the degradation of signal, handle all solutions and slides containing the probes in reduced light. All washes and incubations should be performed in complete darkness.

1. Sample slides are denatured by placing them in a Coplin jar containing denaturant solution (Formamide, 20x SSC, and dH2O) warmed to a temperature of 75-80°C. The solution is pre-warmed in the Coplin jar by immersion in the above mentioned water bath. Slides are submerged in the denaturant bath for five minutes.
2. Upon removal from the denaturant bath, slides are placed in a series of ethanol washes (70%, 85%, and 100%) for one minute each. These ethanol washes are performed at room temperature. Following ethanol washing, slides are placed on a slide warmer until dried and ready for probe application.
3. Prepare the CEP X® and Y® probes for each slide by first combining 7 µl of CEP Hybridization Buffer®, 1 µl of CEP X® probe, 1 µl of CEP Y® probe, and 1 µl of dH2O for a total of 10 µl in a 0.5 µl microcentrifuge tube. This mixture is vortexed and then briefly centrifuged for 1–3 seconds. Place the tube in 75-80°C

water bath for five minutes to denature the probe. Remove from the heated bath and place on a 45-50°C slide warmer until ready to apply to the target DNA.

4. Probes are hybridized to the target DNA by applying 10 µl of the probe mix directly to the slide and immediately concealed with a cover slip. Seal the cover slip to the slide with rubber cement. Place slides in a pre-warmed, humidified container. Place the container in a biological oven heated to a temperature between 37-42°C for six hours.
5. Following the six hour incubation period, the slides are removed from the oven and placed into a 0.4X SSC/0.05% Tween 20 solution bath (pre-warmed to ~73°C in a Coplin jar). The slides should be agitated for 1–3 seconds while soaking in the solution and then left to incubate for two minutes.
6. The slides are removed after two minutes and immersed in a 2x SSC bath for one minute at room temperature. The slides should be agitated slightly during this wash. Following the completion of this step, the slides should be removed from the Coplin jar and left to air dry in darkness.
7. Once dry, apply 10 µl of Vectashield® 4'6-diamedino-2-phenylindole (DAPI) mounting medium (Vector, UK) to the target area of the slide and place a cover slip onto the stain. The DAPI fluorophore will stain the nuclei of each cell. All three fluorophores may be visualized using a fluorescence capable microscope with DAPI, FITC (Fluorescein), and TRITC (Rhodamine) filters.

FISH Protocol Revisions

Multiple examinations of the initial protocol revealed that cells were bound too tightly to the glass substrate following FISH processing and were not releasing during LM procedures. Several variations of cellular application, slide preparation, and sample counterstaining methods were examined and incorporated into our pre-existing protocol that work more efficiently with LM methods.

Improved Slide Preparation Buffer

Borrowing yet another tool out of cytogenetic literature, 3:1 Methanol/Glacial Acetic Acid buffer, or *Carnoy's Fixative (Fix)* as it is commonly referred, was examined as a functional elution and application buffer for the purposes FISH processing and LM manipulations. *Fix* is an isotonic solution that can be used to store cellular material long

term at appropriate temperatures. Cellular suspensions prepared in this fixative are easily applied, spread, and dried on glass slides prior to fixation by heat or alcohol. When applied to slides, cells suspended in this buffer adhered to the glass surface firmly but not stringently. The utilization of this buffer has been found to drastically decrease sample loss during the graded ethanol and xylene fixation procedure described in the following section.

New DAPI Counterstaining Application

For the purposes of counterstaining, DAPI nucleic acid stain was chosen to enhance probe visualization. DAPI will typically stain all double stranded nucleic acid material blue. For the purposes of FISH processing, a DAPI counterstain will stain all nuclear material blue except for those sequences hybridized with probes. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes. A DAPI counterstain will essentially block out the fluorescence of all material that is not intended to be observed. We originally chose to use Vectashield® mounting medium (Vector, UK) as our counterstaining agent. Vectashield® is a viscous mounting medium containing DAPI nucleic acid stain. While this medium counterstained brilliantly and provided us with excellent images under the microscope, it was not practical for the purposes of LM processing. It left our samples quite moist and submerged under thick oil. Traditional LM applications require for target samples to be dry and accessible to the instrument of manipulation.

The above mentioned difficulties lead us to employ Invitrogen® DAPI Nucleic Acid Stain. The initial success observed with this stain led to its utilization for the

remainder of the testing. This DAPI stain is much more water soluble and workable than Vectashield®. At the conclusion of FISH processing, this counterstain can be washed off with deionized H2O or 1X PBS. Sample slides were then dehydrated and placed on an LM instrument for cellular separation.

The following is the protocol found to work best for sample staining:

1. Before applying stain, briefly rinse the sample slides in dH2O to remove residual buffer salts from the slide to reduce nonspecific background staining.
2. Dilute the DAPI stock solution to 30 nanomoles (nM) in 1X PBS.
3. Pipet 100 µl of the staining solution directly onto the specimen.
4. Use a plastic coverslip to distribute the dye evenly on the slide.
5. Incubate the specimen in the dark for 30 minutes at room temperature.
6. Carefully remove the coverslip and rinse the specimen briefly with 1X PBS or dH2O to remove unbound dues.
7. Remove excess liquid from the slide by gently blotting around the sample with an absorbent tissue.
8. Dehydrate the slides with the graded ethanol and xylene protocol listed in the previous section.
9. Once the slides have dried, view the samples using a fluorescence equipped LM microscope with appropriate filters.

Slides and samples processed with this protocol were found to be manipulated with multiple LM devices without difficulty.

Revised FISH Processing Protocol

Incorporating all of the changes previously discussed, a revised and compressed protocol was designed. Listed below is the optimized protocol that was employed for all subsequent testing:

-Final FISH Sample Slide Preparation Protocol-

- Before applying cell samples to glass slides, be sure to pre-warm a water bath to a temperature of 75-80°C, a slide warmer to a temperature of 45-50°C, and a biological oven to a temperature of 60°C.

1. Pipette 10–20 µl of sample cells suspended in 3:1 Methanol/Glacial Acetic Acid onto a glass slide, 1 mm in thickness, and spread gently using the pipette tip.
2. Place the slide above the steam of a water bath for 5 seconds to allow for further cell membrane spreading.
3. Immediately place slide in 75% ethanol for 30 seconds.
4. Place slide in 95% ethanol for 30 seconds.
5. Place slide in 100% ethanol for 1 minute.
6. Place slide in xylene for 2 minutes.
7. Air dry slide until all xylene has evaporated (~10 minutes).

-Final FISH Protocol for CEP X and Y Probe Hybridization-

-Before beginning these FISH procedures, be sure to pre-warm a water bath to a temperature of 75-80°C, a slide warmer to a temperature of 45-50°C, and a biological oven to a temperature between 37-42°C.

-A pre-warmed humidity chamber can be prepared for the hybridization step by placing a moistened paper towel inside a small airtight container and inserting it into the heated oven.

-Note: Fluorophores are readily photobleached by exposure to light. To limit the degradation of signal, handle all solutions and slides containing the probes in reduced light. All washes and incubations should be performed in complete darkness.

1. Sample slides are denatured by placing them in a Coplin jar containing denaturant solution (Formamide, 20x SSC, and dH2O) warmed to a temperature of 75-80°C. The solution is pre-warmed in the Coplin jar by immersion in the above mentioned water bath. Slides are submerged in the denaturant bath for five minutes.
2. Upon removal from the denaturant bath, slides are placed in a series of ethanol washes (70%, 85%, and 100%) for one minute each. These ethanol washes are performed at room temperature. Following ethanol washing, slides are placed on a slide warmer until dried and ready for probe application.
3. Prepare the CEP X® and Y® probes for each slide by first combining 7 µl of CEP Hybridization Buffer®, 1 µl of CEP X® probe, 1 µl of CEP Y® probe, and 1 µl of dH2O for a total of 10 µl in a 0.5 µl microcentrifuge tube. This mixture is vortexed and then briefly centrifuged for 1 – 3 seconds. Place the tube in 75-80°C water bath for five minutes to denature the probe. Remove

from the heated bath and place on a 45-50°C slide warmer until ready to apply to the target DNA.

4. Probes are hybridized to the target DNA by applying 10 µl of the probe mix directly to the slide and immediately concealed with a cover slip. Seal the cover slip to the slide with rubber cement. Place slides in a pre-warmed, humidified container. Place the container in a biological oven heated to a temperature between 37-42°C for six hours.
5. Following the six hour incubation period, the slides are removed from the oven and placed into a 0.4X SSC/0.05% Tween 20 solution bath (pre-warmed to ~73°C in a Coplin jar). The slides should be agitated for 1–3 seconds while soaking in the solution and then left to incubate for 2 minutes.
6. The slides are removed after two minutes and immersed in a 2x SSC bath for one minute at room temperature. The slides should be agitated slightly during this wash. Following the completion of this step, the slides should be removed from the Coplin jar and left to air dry in darkness.
7. Before applying stain, briefly rinse the sample slides in dH2O to remove residual buffer salts from the slide to reduce nonspecific background DAPI staining.
8. Pipet 100 µl of the DAPI staining solution directly onto the specimen. Use a plastic coverslip to distribute the dye evenly on the slide. Incubate the specimen in the dark for 30 minutes at room temperature.
9. Carefully remove the coverslip and rinse the specimen briefly with 1X PBS or dH2O to remove unbound dues. Remove excess liquid from the slide by gently blotting around the sample with an absorbent tissue.
10. Dehydrate the slides with the graded ethanol and xylene protocol.
11. Once the slides have dried, view the samples using a fluorescence equipped LM microscope with appropriate filters. All three fluorophores may be visualized using a fluorescence capable microscope with DAPI, FITC, and TRITC filters.

Visualization, Collection, and Amplification of FISH Treated Epithelial Cells

Interphase FISH processing has been tested on both epithelial and white blood cell sample types. Once target cells were located and identified, laser microdissections were performed using the PixCell® II and PALM® instruments. Laser captures of 5, 10, 20, 25, 30, and 40 cells were performed according to the previously discussed standard operating protocols for each LM system.

Identifiler® amplifications were initially performed at higher cellular counts of FISH probed cells. QIAamp® Micro extractions and Microcon® concentrations followed. Identifiler® amplifications were set up based on a scaled down protocol from manufacturer's recommendations, and 6 µl, 32 cycle amplifications were performed. For the purposes of MiniFiler™ processing, probe hybridized epithelial cells were captured in groupings of 5, 10, and 20 cells. These cells were extracted with QIAamp® Micro, concentrated via Microcon® filters, and then amplified with 6 µl, 32 cycle MiniFiler™ amplifications followed by subsequent analysis with the ABI 3100® genetic analyzer.

FISH Processing of Aged Forensic Samples

Interphase FISH processing was also tested on varying aged forensic sample types which were stored for a minimum of 12 months. FISH processing and collections were performed on aged epithelial and white blood cell samples. White blood cell samples were applied to glass substrates and stored for 16 months in the dark at room temperature. Epithelial cells were spotted on cotton fabric and aged for 12 months at room temperature in the dark. Samples originating from two year old aged post-coital swabs were also included in this evaluation. The biological samples were removed from substrates by swabbing with sterile cotton swabs which were then subjected to centrifugation in 1x PBS. Once eluted, cells were applied to glass slides and probe hybridized by the methods previously discussed. Target cells were located, identified, and separated using the PAL M® instrument. Laser captures of 20 cells were performed according to the previously discussed standard operating protocols for each PALM® system. A total of 10 collections were performed for these experiments. These cells were extracted with QIAamp® Micro,

concentrated via Microcon® filters, and then amplified with 6 µl, 32 cycle MiniFiler™ amplifications followed by subsequent analysis with the ABI 3100® genetic analyzer.

III. RESULTS

A. Cell Collection Studies and the Use of Cellulase to Increase Collection Efficiency

Different techniques of cellular collection were investigated to determine the best collection method for the recovery of cells from evidentiary substrates. The three methods of collection evaluated included direct transfer of evidence to slides, the cutting of substrate target areas followed by elution in buffer, and the swabbing of interest areas followed by elution in buffer. In conjunction with these studies, research was also conducted on increasing the efficiency of cellular elutions from cotton swabs by use of cellulase. Blood, sperm, and epithelial sample cell collections were each evaluated from a variety of substrates.

Elution Evaluations of Evidence Swabs Using Various Cellulases

For the initial studies involving mock evidence and cellulases, 400 cells (epithelial and sperm) were each separately spotted on photocopy paper. The low number of recovered cells, which simulated touch evidence, was below the limit of reliable quantification with a hemocytomer. Additionally, residual cotton and paper fibers prevented the accurate enumeration of cells using a hemocytomer. Therefore, DNA extractions and quantifications were used to determine the optimum method to recover cells.

As seen in Table 1 below, *A niger* cellulase was superior to the other cellulases based on the amount of DNA detected from the collected cells. These results are based upon triplicate sample testing. Cellulase from *T reesei* also seemed to increase the amount of DNA recovered as compared to the control swab. (The control swab was immersed in PBS without any cellulase.) The initial experiment revealed that *T. viride* produced less DNA than the control swab. Based upon observations taken during microscopic examinations, it can be inferred that the *T. viride* cellulase may disrupt the structural integrity of the epithelial cells prior to elution, which would result in decrease recovery of DNA.

Table 1. Enzymatic Enhanced Cellular Recovery of 400 Cells Spotted on Paper Based on DNA Extraction Averages

Cellulase Source	Average Epithelial Cell DNA Recovery (pg)	Average Spermatocyte DNA Recovery (pg)
A. niger (7.02 U)	1369	251
T. reesei (16.32 U)	928	192
T. viride (37.4 U)	22	123
Control	673	94
A. niger (7.02U, One month)	998	227
Control (One month)	579	298

Based upon our initial results, a second study was run with only *A niger* compared to non-cellulase swabs on one month old samples. The results show an overall drop in DNA recovery but the same correlation as the first study with cellulase recoveries (998 pg average) higher than control swabs (579 pg average). The sperm results however showed close to the same quantifications of the cellulase and non-cellulase samples. Additional tests were conducted with sperm and white blood cell samples spotted on substrates such as metal, cotton, and leather. The results from these studies also

suggested that the addition of *A niger* cellulase during elution increases the overall DNA yield received from swabbed samples. In summary, our findings indicate that cellulase does increase the total number of cells eluted from collection swabs following the swabbing of various evidence substrates.

Mock Evidence Elution Evaluations of Epithelial and Sperm Cells

Mock evidentiary samples were created by spotting 4,000 epithelial cells and spermatozoa separately on cotton, paper, steel, and brass surfaces. Time point of zero (1-2 days after spotting and allowed to dry), one month, and in some cases, six months were eluted. Collection techniques of wet swabbing and cutting were both used for the cotton and paper mock evidence samples. Only the swabbing technique was utilized for the metal surfaces. Because our cellulase examinations demonstrated improved cellular elution, the *A niger* cellulase at 7.02 U per reaction enzyme was used for the swabbing and cutting techniques. A set of control swab elutions without cellulase was also performed in conjunction with the other tests. Each collection technique was performed in triplicate and elutions were performed in 300 µl of 1X PBS (20 µl cellulase added when applicable) at 37°C with 400 rpm agitation for one hour. Two sets of counts were taken from each replicate to determine the amount of material recovered. Percent recovery of the cells applied to the substrate was then calculated from average of the three replicates. Tables 2, 3, and 4 summarize the results.

Table 2: Epithelial Cell Percent Recovery of 4,000 Spotted Cells Based on Cellular Counts

Substrate	Collection Method	Number of Cells Spotted (100%)	Time Zero (%)	One Month (%)	Six Months (%)
Cotton	Cellulase Swab	4,000	18.91	9.28	11.69
	Cutting	4,000	11.34	1.72	0
	Control Swab	4,000	10.31	3.78	5.84
Paper	Cellulase Swab	4,000	81.47	13.06	15.13
	Cutting	4,000	31.63	2.06	0
	Control Swab	4,000	55.00	8.94	15.47
Brass	Cellulase Swab	4,000	44.00	1.03	2.41
Steel	Cellulase Swab	4,000	56.38	71.16	33.34

Table 3: Epithelial Cell Percent Recovery of 4,000 Spotted Cells Based on Quantification

Substrate	Collection Method	Number of Cells Spotted (100%)	Time Zero (%)	Six Months (%)
Cotton	Cellulase Swab	4,000	14.20	12.36
	Cutting	4,000	12.98	*Below Threshold*
	Control Swab	4,000	7.38	17.13
Paper	Cellulase Swab	4,000	40.82	28.82
	Cutting	4,000	10.90	*Below Threshold*
	Control Swab	4,000	59.25	9.55
Brass	Cellulase Swab	4,000	11.49	0.82
Steel	Cellulase Swab	4,000	33.91	54.44

Table 4: Spermatozoa Percent Recovery of 4,000 Spotted Cells Based on Cellular Counts and Quantification

Substrate	Collection Method	Number of Cells Spotted (100%)	Time Zero (counts) (%)	Time Zero (quant) (%)	One Month (counts) (%)
Cotton	Cellulase Swab	4,000	7.22	8.45	12.38
	Cutting	4,000	2.06	5.97	7.91
	Control Swab	4,000	3.09	14.66	5.50
Paper	Cellulase Swab	4,000	15.81	100.0	10.31
	Cutting	4,000	11.69	3.70	5.16
	Control Swab	4,000	11.69	41.74	5.84
Brass	Cellulase Swab	4,000	36.44	64.17	0
Steel	Cellulase Swab	4,000	51.56	100.0	32.31

Mock Evidence Elution Evaluations of Blood Samples

Mock evidentiary samples were created by spotting 20 µl of whole blood onto cotton, paper, steel, and brass surfaces. The spots were allowed to dry overnight and were then collected by wet swabbing with cellulase aided elution. The biological material was lightly pelleted, and the white blood cell isolation protocol was performed using a hypotonic solution followed by the multiple additions of *Carnoy's Fixative*. The cell suspensions were then centrifuged, re-suspended in low volume solution, stained with hematoxylin, and then spread on glass slides and prepared for LM according to protocol. The cells were collected by LM, DNA was extracted, and amplified using the MiniFiler™ system. Full 9 loci STR profiles were obtained from 15 white blood cells taken from the brass surface (Figure 6). Results from the other surfaces have been inconsistent, and additional testing is currently in progress.

Figure 6: Complete Minifiler™ Profile Generated From 15 White Blood Cells Collected from a Brass Surface and Captured with the PixCell® System

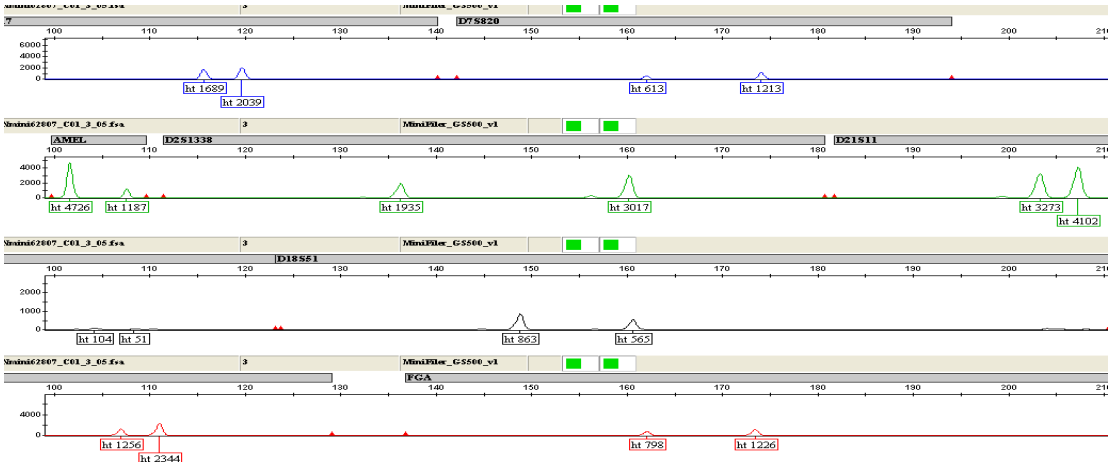
B. Extraction and Concentration of LM Processed Cells

Multiple methods for DNA extraction and amplification were evaluated in order to select a protocol that was consistently compatible with PixCell® II laser capture functions and PALM® LMPC operations. The QIAamp® Micro Extraction kit and PicoPure® DNA extraction kit extraction were evaluated to determine the best system for achieving robust DNA yields and quality downstream profile generation when using the ABI AmpF*l*STR Identifiler® kit, the ABI AmpF*l*STR Yfiler® kit, and the ABI AmpF*l*STR MiniFiler™ kit. Epithelial, blood, and sperm samples were tested during these examinations. At the conclusion of this evaluation process, the QIAamp® Micro Extraction kit was found most efficient and reliable for processing low level (5-10 cells) LM samples.

Qiagen QIAamp® Micro Kit Evaluation

Evaluations of the QIAamp® Micro Kit were performed using cells collected via the PixCell® II instrument. Duplicate sets of 1, 2, 5, and 10 cells were collected using the PixCell® II laser microdissection system. The cells were collected with the LCM ExtracSure® device and QIAamp cell lysis buffer (15 µl ATL cell lysis buffer and 10 µl of Proteinase-K) was added to the cells. The 5 and 10 cell captures yielded full profiles (Figure 7) and the 1 and 2 cell captures yielded low partial profiles of 4 loci or less.

Figure 7: Full MiniFiler® Profile Generated From Ten Epithelial Cells Collected Using the PixCell® System and Extracted Using the Qiagen QIAamp® Micro Kit



PicoPure® DNA Extraction Evaluation

The first set of extraction evaluations were performed on higher cellular amounts of 55, 110, 219, and 438 cells to ensure that the kit would work at levels higher than those typically used in the laser captured samples. There was a single failed amplification, but the rest yielded full profiles (Figure 8). The electropherograms associated with the higher cellular amounts of 219 and 438 cells were overloaded and needed to be diluted and re-injected on the ABI 3100® Genetic Analyzer for proper STR analysis.

For LCN testing of PicoPure®, PixCell® II captures were performed as previously noted. Captures of 5, 10, and 20 cells were performed in duplicate. One amplification from each of the three cellular amounts resulted in a poor DNA profile (zero or one allele). The remaining 20 cell captures yielded a full profile, the 10 cell and 5 cell captures yielded a low-partial profile of 6 alleles (Figure 9). The results indicate that the PicoPure® technique did not perform as well as the QIAamp® Micro kit when processing LCN DNA samples. Table 5 summarizes the PicoPure® testing results.

Table 5: Minifiler™ Amplification Results Extracted Using the PicoPure® DNA Extraction Kit

Number of Cells Extracted	Number of Trials	Full Profile (9 loci)	Partial Profile (2-8 loci)	No Profile (0-1 loci)
5^	2	-	2	-
10^	2	-	2	-
20^	2	1	-	1
55*	3	3	-	-
109*	3	2	-	1
219*	3	3	-	-
438*	3	3	-	-

^ Cell total determined by PixCell® system captures
* Cell total determined by counts and dilutions

Figure 8: Complete Minifiler™ Profile Generated From 55 Epithelial Cells Collected Using the PixCell® System and Extracted Using the PicoPure® DNA Extraction Kit

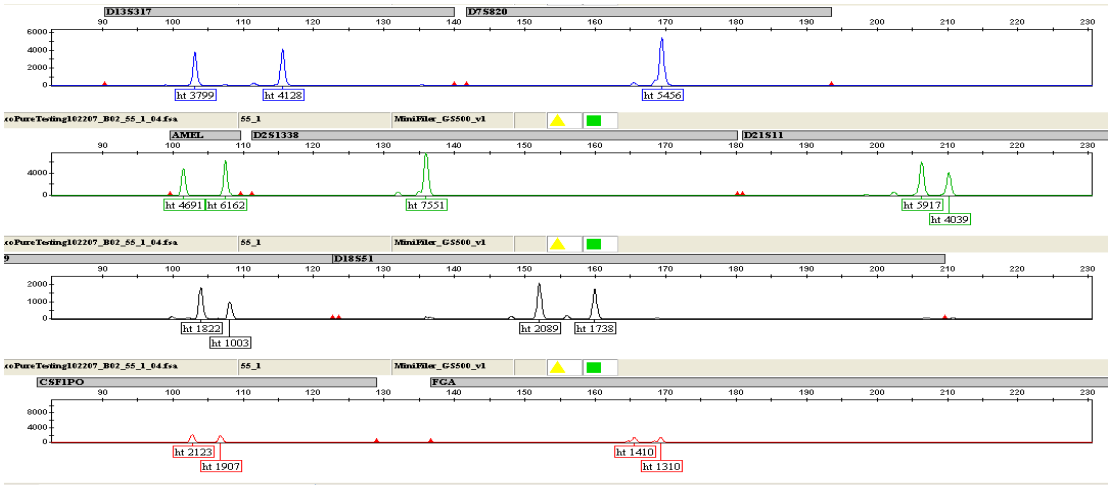


Figure 9: Partial MiniFiler® Profile Generated From Ten Epithelial Cells Collected Using the PixCell® System and Extracted Using the PicoPure® DNA Extraction Kit

C. Comparison of STR Amplification Systems

There were several amplification kits evaluated during this study to test the effectiveness of generating STR profiles from laser captured biological materials at low copy number levels. The Identifiler® kit, Yfiler® kit and the Minifiler™ kit were all individually examined for performance output. These kits have been tested on different types of cells with extractions performed with QIAamp® Micro kits.

Identifiler® Testing

Collections of 5 epithelial cells were performed using the PixCell® II followed by QIAamp® Micro DNA extraction and Microcon® concentration. Extracts were amplified using full volume, 25µl, Identifiler® reactions. These triplicate samples yielded zero alleles. It was suspected that the high volumes of the amplification reactions may have reduced sensitivity to such low DNA amounts. The next round of five cell amplifications were performed with enzyme volumes scaled down from the manufacturer's recommendations. This round of amplifications yielded low-mid partial profiles with the

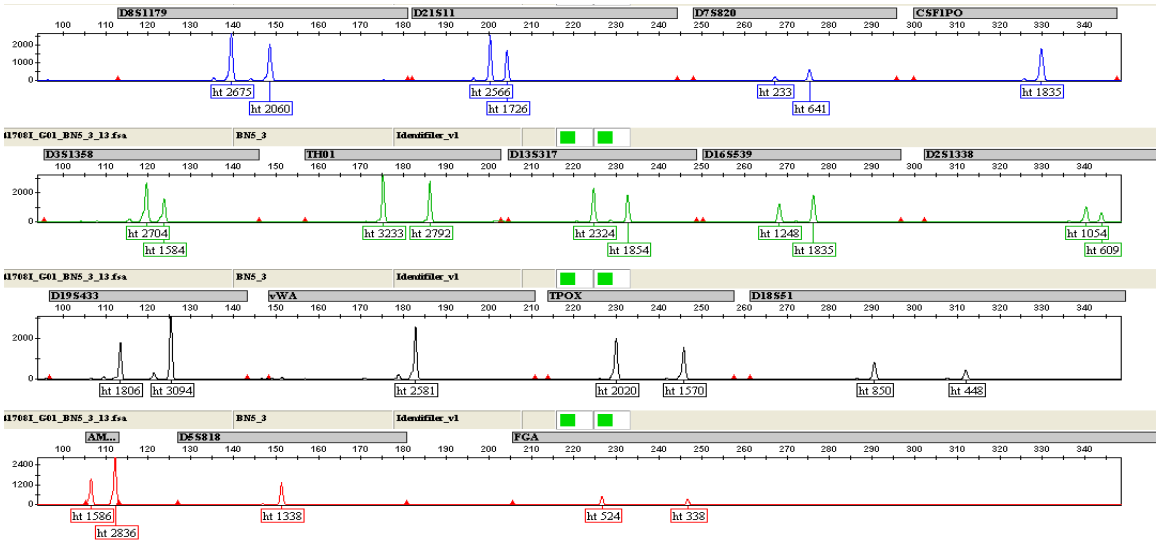
best sample producing 17 out of 32 alleles (Figure 10). At lower levels of epithelial cells, it appears that full profiles are achieved in higher numbers with Minifiler™ as compared with Identifiler®. However, with the difference in loci present in kits of 9 with Minifiler™ and 16 with Identifiler®, it is still possible to achieve more alleles in Identifiler® as compared to Minifiler™. A subsequent study was performed with three donors and four replicates utilizing the scaled down 6 µl 32 cycle Identifiler® reactions. Five cell captures were performed using the PixCell® II system and extracted/concentrated using the QIAamp® Micro Kit and Microcon® YM-100 columns. Of the 12 samples, full 16 loci profiles were achieved in four replicates (Figure 11), partial profiles in six replicates, and failed/low amplifications in two of the samples. Additional testing was performed at the 10 spermatozoa and epithelial cell level as well as 75 pg of control DNA. This data is displayed in Table 6 below.

Table 6: Identifiler® 6 µl, 32 Cycle Testing with Epithelial Cells and Spermatozoa

DNA Source	Number of Trials	Full Profile (16 loci)	Mid-High Partial (8-15 loci)	Low-No Profile (0-7 loci)
5 epithelial	33	8	11	16
10 epithelial	5	2	3	-
10 spermatozoa	7	1	4	2
75pg 9947	15	11	4	-

Figure 10: Partial Identifiler® Profile Generated From Five PixCell® Captures Epithelial Cells Collected From Hematoxylin Stained Cells

Figure 11: Complete Identifiler® 16 Loci Profile Generated From Five Epithelial Cells Collected Using the PixCell® System



Yfiler® Testing

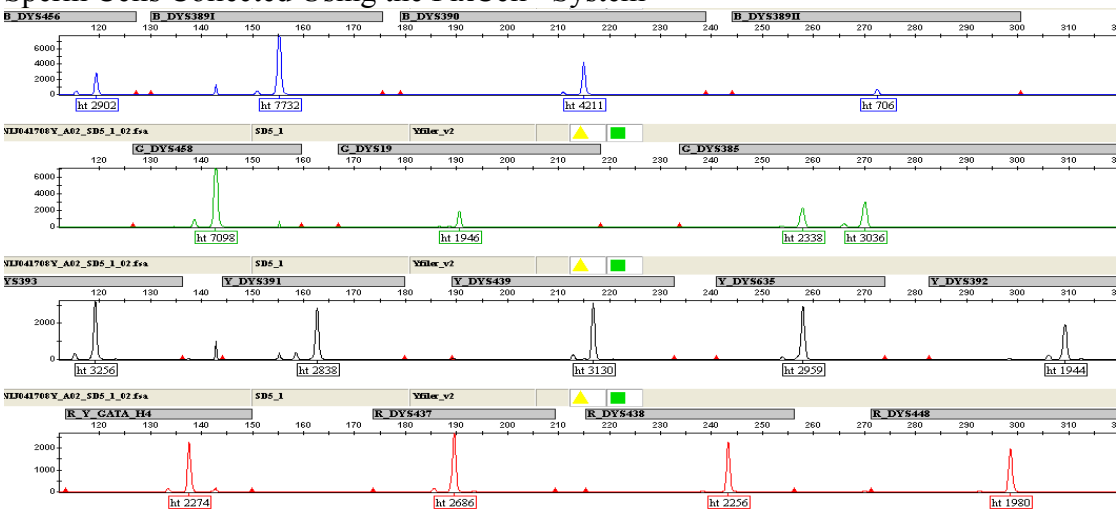
Captures at five, 10, and 30 epithelial cells were performed on the PixCell® II followed by QIAamp® Micro DNA extraction and Microcon® concentration. The 5 cell amplifications yielded low partial profiles with the 10 and 30 cell captures yielding high partial or full 17 allele Yfiler® profiles. Subsequent amplifications of 5 and 10 epithelial

cells as well as 5, 10, and 20 spermatozoa generated full 17 allele Yfiler® profiles (Figure 12). The results of Yfiler® amplification testing thus far are summarized in Table 7 with additional testing currently in progress.

Table 7: Yfiler® 6 µl, 32 Cycle Testing with Epithelial Cells and Spermatozoa

DNA Source	Number of Trials	Full Profile (17 loci)	Mid-High Partial (8-16 loci)	Low-No Profile (0-7 loci)
5 epithelial	8	2	1	5
10 epithelial	9	4	4	1
30 epithelial	2	1	1	-
5 spermatozoa	2	2	-	-
10 spermatozoa	7	3	2	2
20 spermatozoa	5	3	2	-

Figure 12: Complete Yfiler® 16 Loci Profile Generated From Five Hematoxylin Stained Sperm Cells Collected Using the PixCell® System



MiniFiler™ Testing

Our results indicate that the MiniFiler™ amplification kit is the best amplification system for yielding full STR profiles from low levels of cells collected via LM. Examinations demonstrated that the MiniFiler™ amplification kit can consistently produce a full 9 locus STR profile from 5 buccal cells.

For the initial testing of MiniFiler™ paired with LM, an abundant number of cells were collected to ensure proper amplifications. Trials of 10, 20, 50, 100, and 200 epithelial cells and 100 and 200 sperm cells were captured. For the amplification in this initial run, a 13 µl, 30 cycle amplification was run using 5.5 µl of template. Upon viewing the results, most samples showed useful partial profiles that had only observable drop-out at the D16 locus.

Additional sets of captured cells utilized several modifications to the standard amplification procedure. The samples were amplified in a 6 µl reaction with a 32 cycle amplification. Cell captures of 1, 2, 5, and 10 cells were used for this trial. Along with these amplification, 9947A control samples were prepared at concentrations of 3, 6, 12, and 25 pg (Table 8).

Table 8: Minifiler™ Low Copy Number and 9947 Study

Sample	DNA Amplified (pg)	Alleles Present - Minifiler™
1 cell	8.4	3/18
2 cells	12.5	4/18
5 cells	15.3	18/18
10 cells	20.7	18/18
3 picograms	3.3	4/18
6 picograms	6.6	7/18
12 picograms	12.5	15/18
25 picograms	25.0	18/18

Following 3100® analysis, the remaining ~5 µl amplification product was dried down to 1 µl, and the whole sample was re-run on the 3100® in an attempt to generate more alleles in the lower samples. Larger peak heights were seen in some alleles, but no additional peaks were visible.

With the threshold of five cells needed to get a complete profile, cell capture numbers lower than that were investigated more in depth. Increasing amplifications to 34 cycles was attempted in order to generate more alleles in low cell capture extractions. Captures of one cell, two cells, and three cells were performed, extracted, and concentrated as previously described. Six microliter amplifications were run at 34 cycles and 3100® analysis followed. In these samples, quite a bit of allelic drop-in was seen and clean DNA profiles were not typically observed. All four of the samples exhibited allelic drop-in. The sample that worked the best was one of the one cell extractions that exhibited 9/17 alleles with only one allelic drop-in. These results indicate the use of strong caution when employing the use of 34 cycle amplifications.

An additional study was conducted at a five epithelial cell level with Minifiler™ to ensure full 9 loci profiles could be obtained over multiple trials. Three separate, five cell captures, each from a single donor were performed with the PixCell® II. Six microliter, 32 cycle amplifications were ran; two of the samples displayed full 9 loci Minifiler™ profiles and the third displaying 17 out of 18 alleles. This data indicates that full 9 locus profiles can be obtained with MiniFiler™ at the 5 epithelial cell level. The MiniFiler™ amplification kit seems to outperform Identifiler® at the 5 epithelial cell level when compared to the Identifiler® amplifications results.

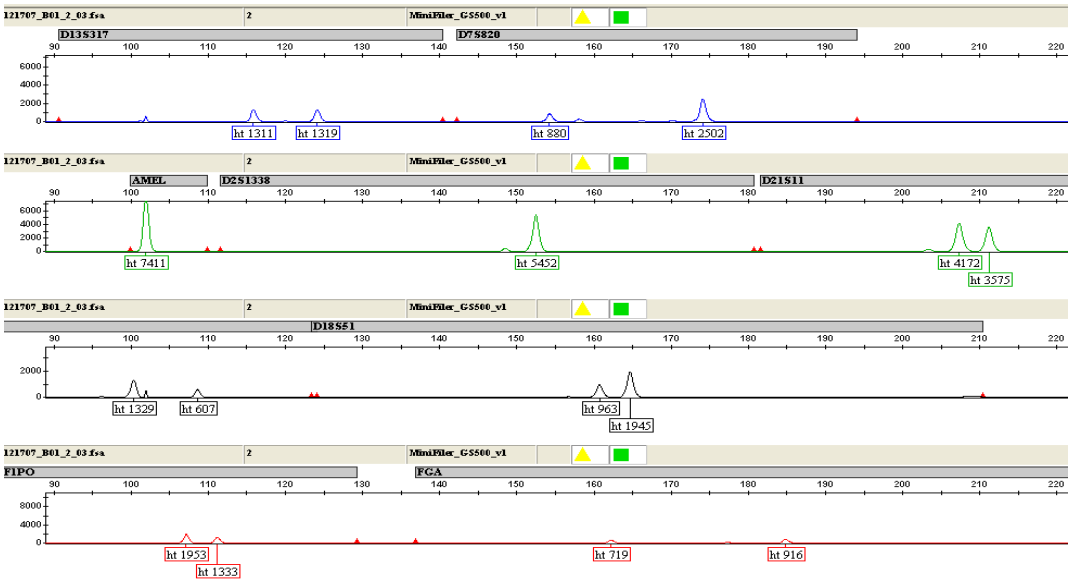
In a multiple person study at the five buccal cell level, three individuals were sampled at four separate trials each. Of the 12 trials, six samples displayed full 18 allele profiles (Figure 13), four had very high partial profiles (16 or 17 alleles) and two of the samples had failed amplifications. All three donors had samples display full STR profiles. Additional testing was various levels of spermatozoa and epithelial cell level as

well as 75 pg of control DNA. Minifiler™ in conjunction with various extraction techniques as well as cellular treatments has been optimal in amplifying LCN samples. The results of all Minifiler™ amplification testing are summarized in Table 9.

Table 9: Minifiler™ 6 µl, 32 Cycle Testing with Epithelial Cells and Spermatozoa

DNA Source	Number of Trials	Full Profile (9 loci)	Mid-High Partial (5-8 loci)	Low-No Profile (0-4 loci)
1 epithelial	4	-	3	1
5 epithelial	20	11	8	1
10 epithelial	5	3	2	-
5 spermatozoa	1	1	-	-
10 spermatozoa	2	1	1	-
20 spermatozoa	1	1	-	-
75pg 9947	15	10	4	1

Figure 13: Complete Minifiler™ 9 Loci Profile Generated From 5 Epithelial Cells Collected Using the PixCell® System

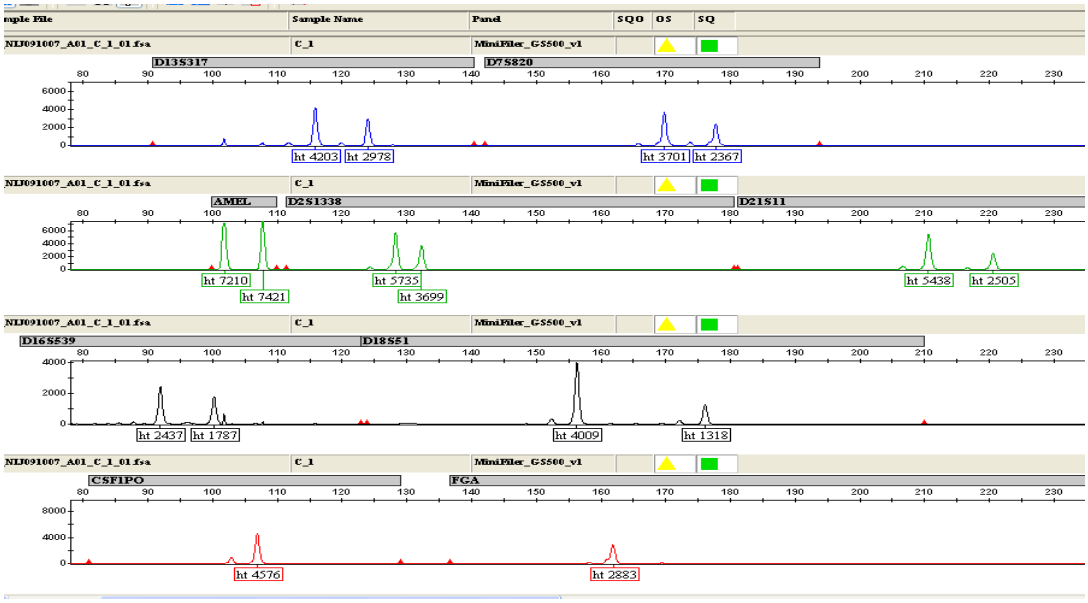


Evaluation of the Effect of Cellulase on Laser Capture Samples

The incorporation of cellulase during our elution studies demonstrated a greater release of cells from cotton swabs than PBS elution alone. To ensure that the *A niger*

cellulase would not affect LCN sample extractions and amplifications, LM needed to be performed on cellulase manipulated samples. Using the MiniFiler™ amplification kit, both cellulase treated and non-treated samples yielded full STR profiles, thus revealing that cellulase at the concentration used has no detrimental effects on yielding full STR profiles (Figure 14).

Figure 14: Complete Minifiler™ 9 Locus Profile Generated From Five Cellulase Treated Hematoxylin Stained Epithelial Cells Collected Using the PixCell® System



Microdissection of Epithelial and White Blood Cells with the Zeiss PALM® MicroBeam

Varying numbers of epithelial and white blood cells were separated and collected using the PALM® MicroBeam. Using the MiniFiler™ kit, STR amplifications of 5, 10, 15, 20, and 25 LM collected cells using the PALM® MicroBeam were performed using both epithelial and blood samples. Sample sets of nine were collected for each cell count. Full profiles were consistently seen from the 15, 20, and 25 cell count collections. Approximately 77% of all the 10 cell count samples produced full or high partial profiles

(one or two loci drop-out). The two electropherograms displayed below (Figures 15 and 16) demonstrate our success with the laser induced catapult collection of these low cell counts. Greater than 50% of the five cell count samples returned partial profiles (~50% drop-out). In a few instances, high partial profiles were generated. The results generated from the collection of samples with the PALM® MicroBeam system is summarized in Table 10.

Table 10: Profile Generation from Epithelial Cells, White Blood Cells, and Spermatozoa collected with the PALM® MicroBeam system.

DNA Source	Number of Trials	Full Profile (9 loci)	Mid-High Partial (5-8 loci)	Low-No Profile (0-4 loci)
25 epithelial	9	9	-	-
20 epithelial	9	9	-	-
15 white blood cells	9	8	1	-
10 white blood cells	9	7	1	1
5 white blood cells	9	-	3	6

Figure 15: Complete Minifiler™ Profile Generated From 10 Microdissected Hematoxylin Stained White Blood Cells Collected With the PALM® MicroBeam

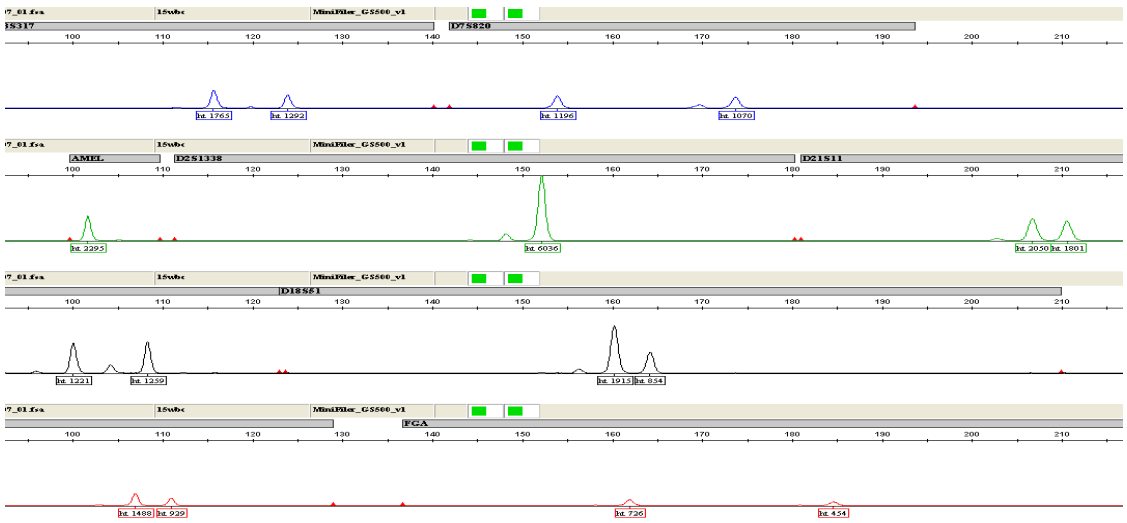
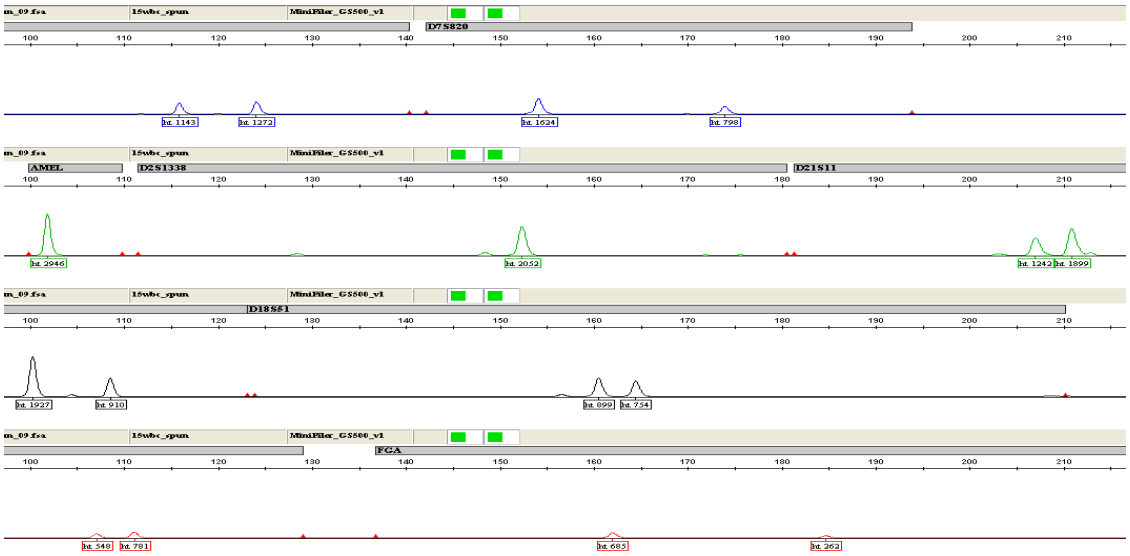


Figure 16: Complete Minifiler™ Profile Generated From 10 Microdissected Hematoxylin Stained Epithelial Cells Collected With the Palm® MicroBeam



Laser Captures of White Blood Cells, Spermatocytes, and Sperm/Vaginal Epithelial Cell Mixtures Using the PixCell® II System

Captures of 5 and 10 white blood cells were performed and then extracted using QIAamp® Micro followed by Microcon® concentration. Amplifications with 6 µl, 32 cycles MiniFiler™ amplifications were then performed. The five cell captures yielded high partial profiles (11-14 alleles) while the 10 cell captures yielded full profiles.

The collection of spermatocytes using LM with the PixCell® II system, extracted using the QIAamp® micro kits, concentrated with Microcon® filters, and amplified with the MiniFiler™ system was further evaluated to determine the limit of sensitivity (minimum number of cells producing an 8 locus STR profile). PixCell® II captures were performed at spermatozoa count levels of five and 20. The five spermatozoa captures displayed a full STR profile. The 20 spermatozoa samples were overloaded and needed to be diluted and re-injected on the 3100® Genetic Analyzer. The re-injection provided clearer data and had a full STR profile as well.

Mixtures of vaginal epithelial cells and semen were prepared and subsequently applied to slides to further evaluate the ability of LM to resolve mixtures and produce single STR profiles. PixCell® II captures were performed for cell counts of 5 vaginal cells and 5, 10, and 20 spermatozoa. The vaginal five cell extractions yielded full STR profiles with no visible contamination from the male contributor. All the spermatozoa extractions generated full profiles or strong partial profiles showing 17 out of 18 alleles. Of the 10 male replicates, only one of the replicates displayed a weak profile containing a female component. The other nine male replicates exhibited only indications typical of a male profile.

MiniFiler™ and LM Testing Summary

The final results of all Minifiler™ amplification testing is summarized in Table 11. This chart includes data from FISH testing which will be discussed in the following sections of the report.

Table 11: Minifiler™ 6 µl, 32 Cycle Testing with Epithelial Cells, White Blood Cells, and Spermatozoa

DNA Source	Number of Trials	Full Profile (9 loci)	Mid-High Partial (5-8 loci)	Low-No Profile (0-4 loci)
1 epithelial	4	-	3	1
5 epithelial	20	11	8	1
10 epithelial	5	3	2	-
20 epithelial	9	9	-	-
25 epithelial	9	9	-	-
5 white blood cells	12	-	6	6
10 white blood cells	10	8	1	1
15 white blood cells	9	8	1	-
20 white blood cells	1	1	-	-
5 spermatozoa	1	1	-	-
10 spermatozoa	2	1	1	-
20 spermatozoa	1	1	-	-

75pg 9947	15	10	4	1
5 epithelial FISH	5	-	3	2
10 epithelial FISH	5	4	1	-
20 epithelial FISH	5	5	-	-

D. The Use of Fluorescent In Situ Hybridization Techniques to Resolve Male and Female Epithelial Cell Mixtures

Fluorescent In Situ Hybridization (FISH) is a traditional cytogenetic technique used to detect the presence or absence of specific chromosomes and/or sequences of an individual's genome. This method utilizes fluorescent probes which are designed to bind to the targeted conserved sequences of individual chromosomes. Fluorescence compatible microscopes are typically employed to visualize the multicolor probes used in these hybridizations.

Visualization of FISH Treated Epithelial and White Blood Cells

Interphase FISH processing was tested on both epithelial and white blood cell sample types. Sex chromosome labeling has been successful with both cell varieties. Processing samples with this technique has allowed us to visually identify the male and female contribution to each sample mixture. Sex chromosomes were easily identified from male/female mixtures of epithelial/epithelial, white blood cell/white blood cell, and epithelial/white blood cell mixtures. An X chromosome is visually identified by the presence of a green fluorescent marker while a Y chromosome can be readily detected by the presence of an orange fluorescent marker. The images below (Figures 17-20) demonstrate the success that has been observed when using this technique to identify the male and female contributors to sample mixtures of various cell types.

Figure 17: CEP-Y® Labeled Male Epithelial Cells with DAPI Counterstain Visualized at 630X Magnification As Viewed Through DAPI/FITC/TRITC filter

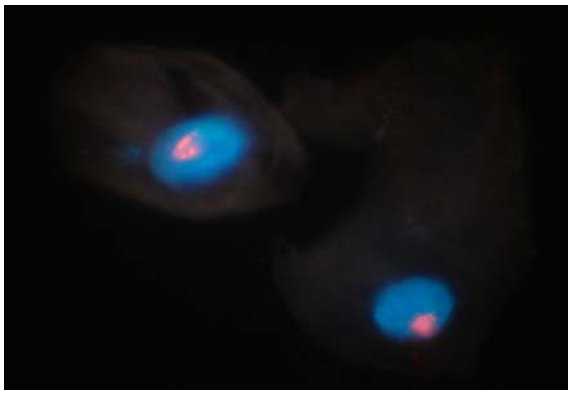


Figure 18: CEP-X® Labeled Female Epithelial Cells With DAPI Counterstain Visualized At 630X Magnification As Viewed Through DAPI/FITC/TRITC Filter (Left) and FITC Filter (Right)

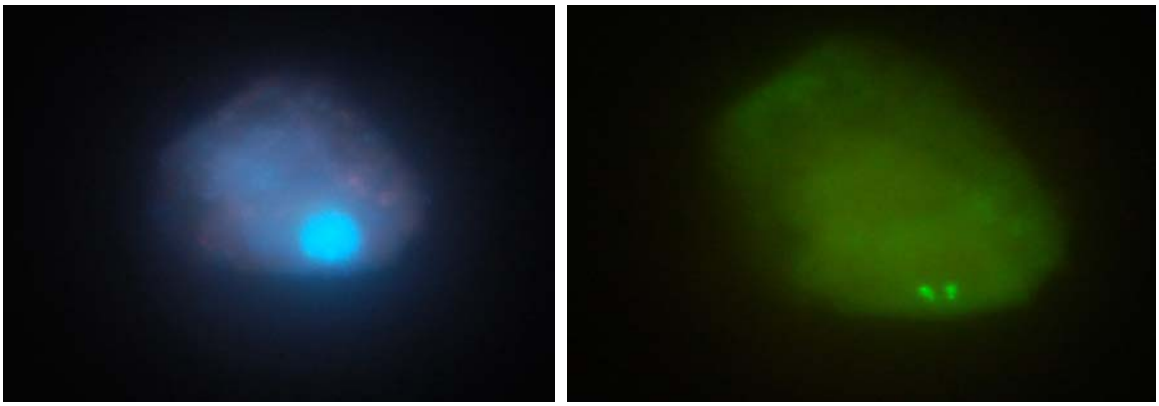


Figure 19: CEP-X® and CEP-Y® Labeled Male and Female Epithelial Cell Mixture with DAPI Counterstain Visualized at 630X Magnification As Viewed Through DAPI/TRITC/FITC Filter (Left) and FITC Filter (Right)

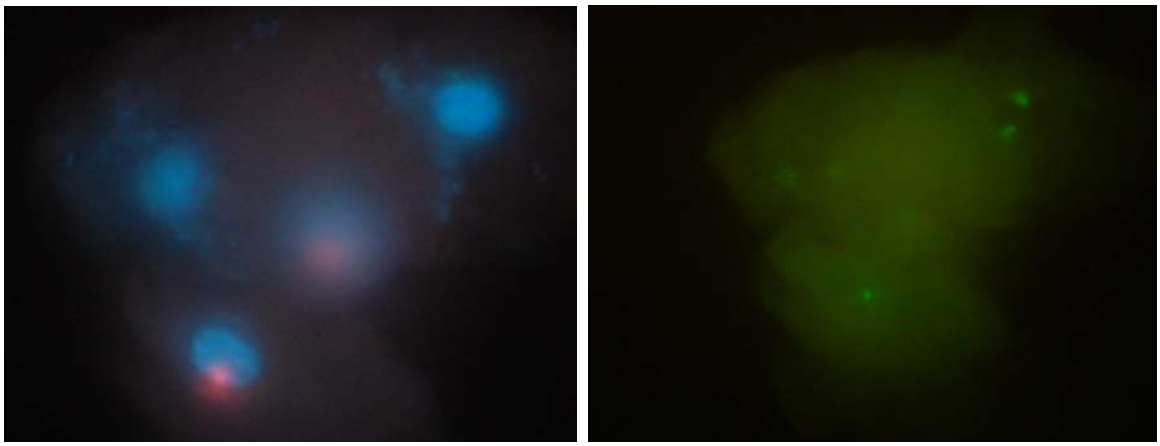
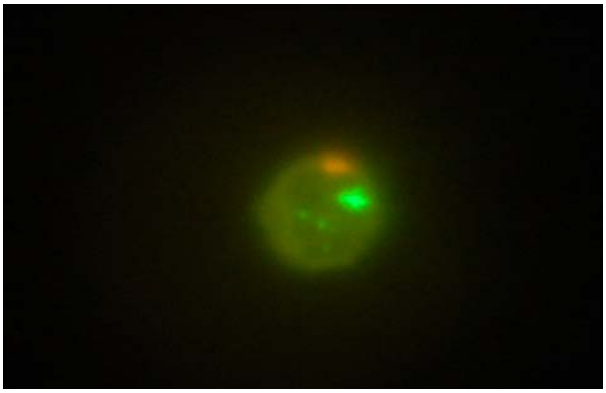


Figure 20: CEP-X® and CEP-Y® Labeled Male White Blood Cell Visualized at 630X Magnification as Viewed Through FITC Filter



Amplification of FISH Treated Epithelial and White Blood Cells

Once target cells were located and identified, laser microdissections were performed using the PixCell® II and PALM® instruments. Laser captures of 5, 10, 20, 25, 30, and 40 cells were performed according to the previously discussed standard operating protocols for each LM system.

Identifiler® amplifications were initially performed at higher cellular counts of FISH probed cells. The 20 cell captures displayed full to high partial STR profiles (16-25 alleles) with the higher cellular amounts displaying full STR profiles or profiles missing only one or two alleles. The profiles displayed below (Figures 21 and 22) were generated from the separation of approximately 25 FISH treated male and 25 FISH treated female epithelial cells from a 1:1 mixture and were amplified for 30 cycles in a 6 µl Identifiler® reaction. These electropherograms demonstrate the possibility of clean separations of male and female components when utilizing FISH techniques. In each of the profiles generated, no carryover alleles were observed from either the male or female fraction of the mixture. The results of Identifiler® amplification testing with FISH treated epithelial cells is summarized in Table 12.

Table 12: Identifiler® Amplifications of FISH Treated Epithelial Cells

DNA Source	Number of Trials	Full Profile (9 loci)	Mid-High Partial (5-8 loci)	Low-No Profile (0-4 loci)
20 epithelial FISH	6	-	5	1
25 epithelial FISH	4	2	1	1
30 epithelial FISH	4	3	1	-
40 epithelial FISH	4	3	1	-

Figure 21: Electropherogram Displaying a Full STR Profile Generated From the Extraction of 25 FISH Processed Male Epithelial Cells

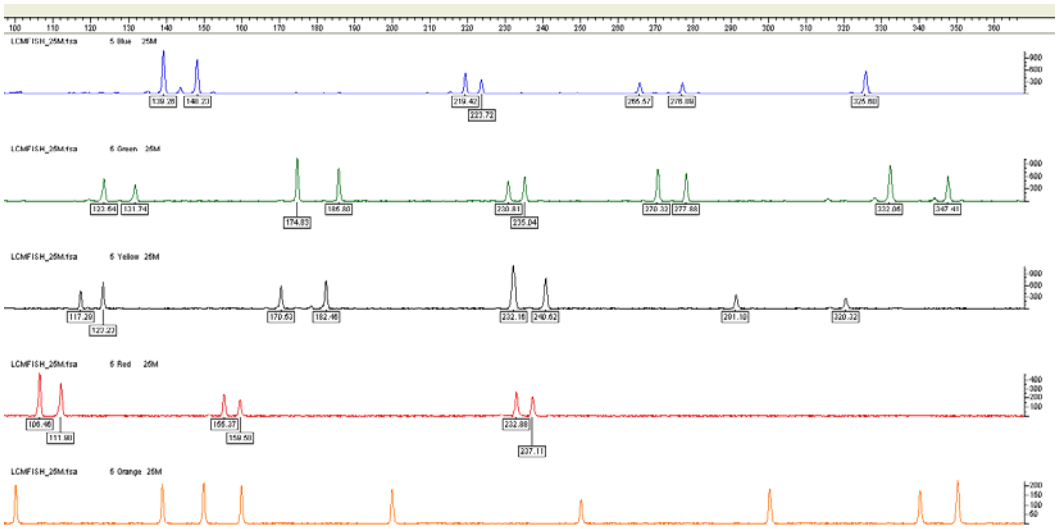
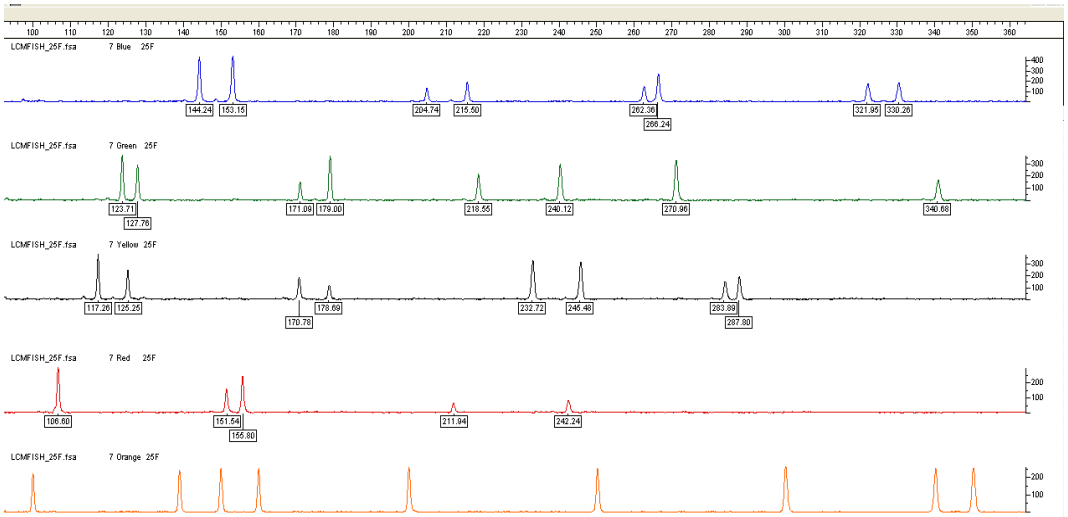


Figure 22: Electropherogram Displaying a Full STR Profile Generated From the Extraction of 25 FISH Processed Female Epithelial Cells



For the purposes of MiniFiler™ processing, probe hybridized epithelial cells were captured in groupings of 5, 10, and 20 cells. The 5 cell captures yielded high partial profile and the 10 and 20 cell captures displayed full and high partial MiniFiler™ STR profiles. Figures 23, 24, and 25 show 10 and 15 FISH stained male and female epithelial and white blood cells and the resulting 9 locus profile using the ABI MiniFiler™ kit.

Figure 23: Full 9 loci MiniFiler™ Profile From Ten FISH Stained Male Epithelial Cells Captured Using the PixCell® System

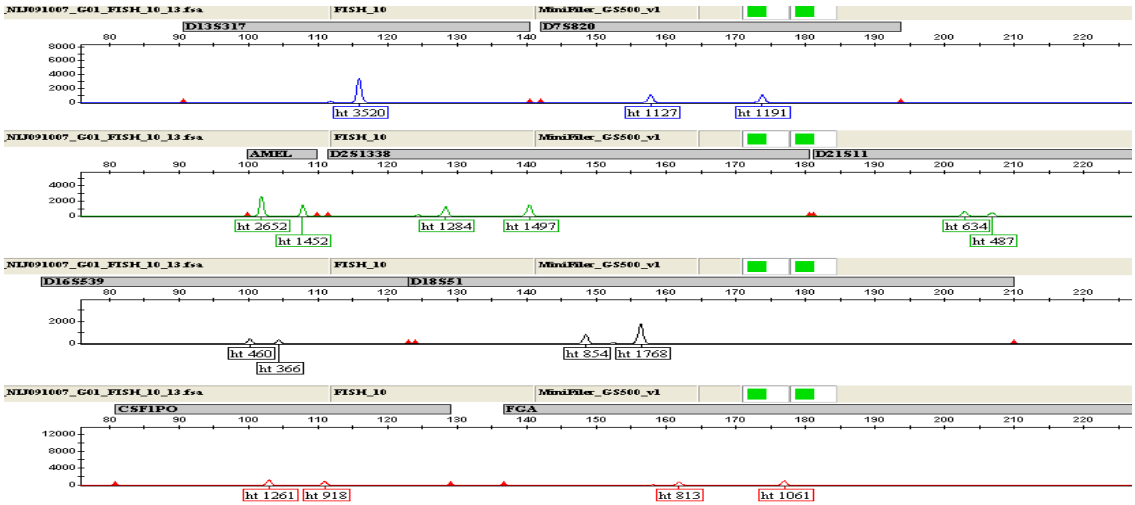


Figure 24: Full 9 loci MiniFiler™ Profile from 15 FISH Stained Male White Blood Cells Separated From a Male/Female White Blood Cell Mixture Using the PALM® MicroBeam

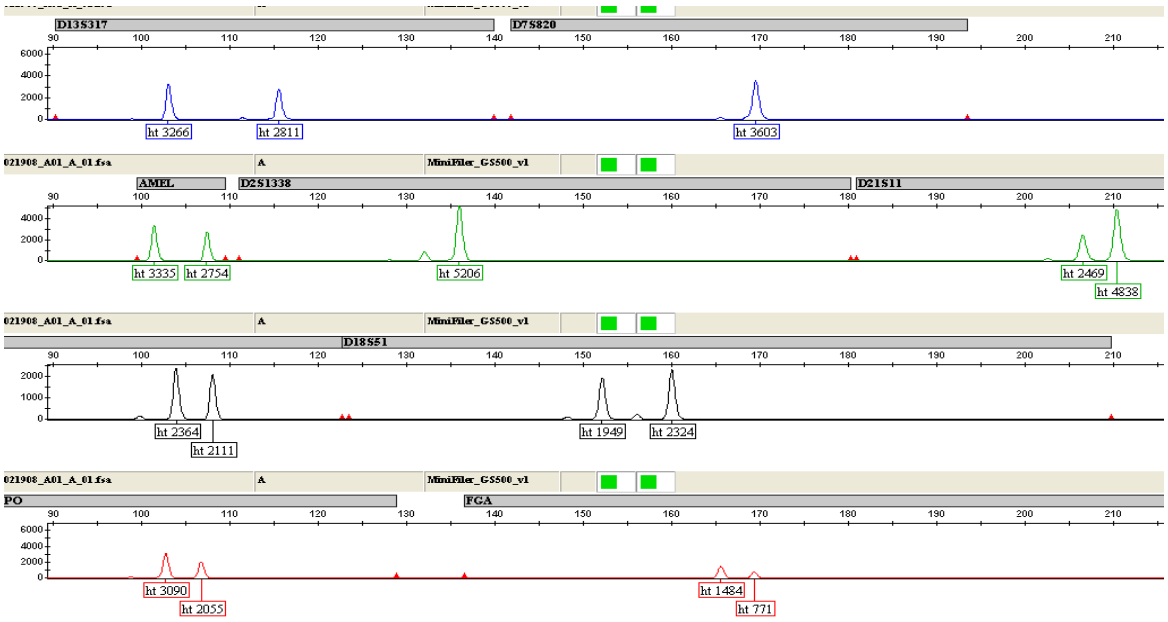
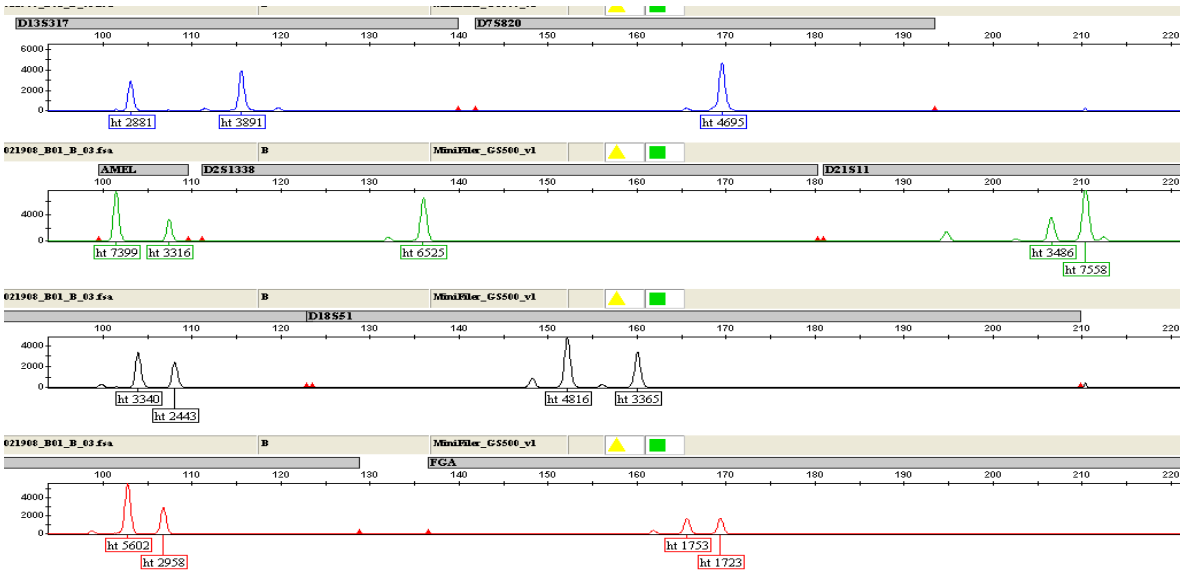


Figure 25: Full 9 loci MiniFiler™ Profile From 15 FISH Stained Male Epithelial Cells Separated From a Male Epithelial/Female White Blood Cell Mixture Using the PALM® MicroBeam



FISH Processing of Aged Forensic Samples

Interphase FISH processing was also tested on varying aged forensic sample types which were stored for a minimum of twelve months. FISH processing and collections were performed on aged epithelial and white blood cell samples. White blood cell samples were applied to glass substrates and stored for 16 months. Epithelial cells were spotted on cotton fabric and aged for 12 months. Samples originating from two year old aged post-coital swabs were also included in this evaluation. Target cells were located, identified, and collected using the PALM® instrument. Probe hybridization was successful on all of these samples. It appears that age of sample does not negatively affect the responsiveness of samples to interphase FISH processing. Ten laser collections of 20 cells were performed for these samples according to the previously discussed standard operating protocol for the PALM® system.

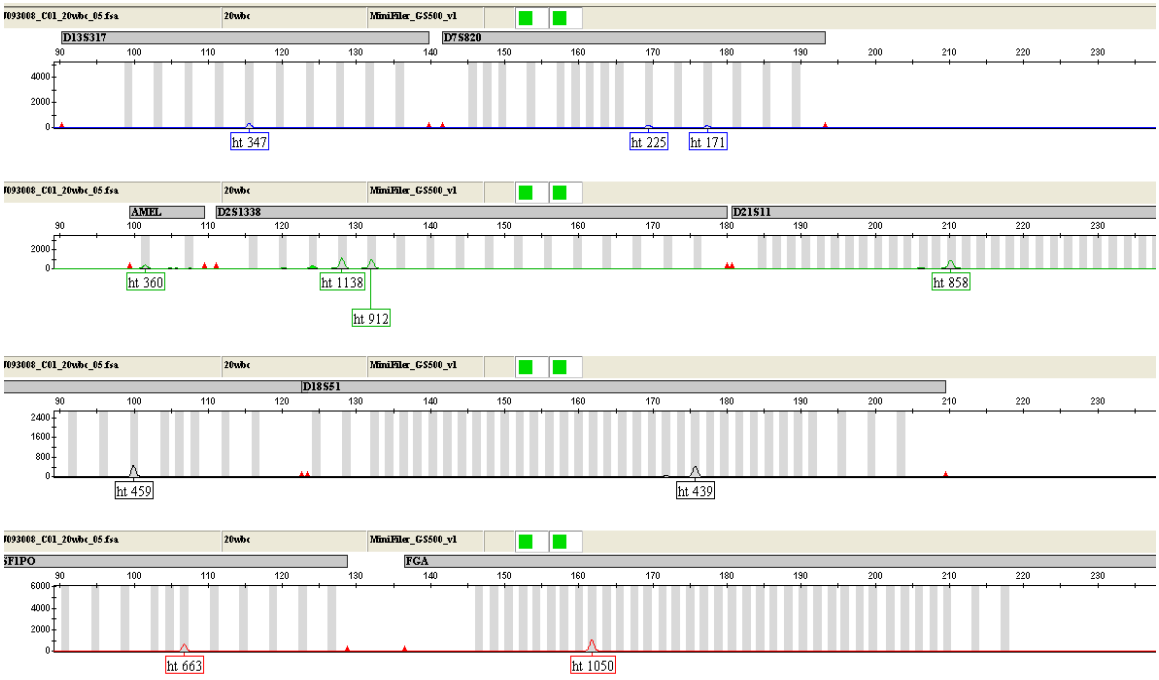
Minifiler™ amplifications were performed on all collections of FISH probed cells. No full profiles were observed from these collections. The 20 cell captures of 12 month old epithelial cells applied to cotton substrates generated one low (0-4 loci), three mid-partial STR profiles (5-8 loci), and one sample did not generate any profile. The 20 cell captures of 16 month old white blood cells applied to glass substrates also generated two low (1-4 loci) and two mid-partial STR profiles (5-8 loci). The partial profile displayed below (Figure 26) was generated from the collection of approximately 20 FISH processed white blood cells. This electropherogram demonstrates the possibility of clean collections of cellular components originating from aged samples when utilizing FISH techniques and LM instruments. The 20 cell captures of two year old aged epithelial cells originating from post-coital swabs generated two low (1-4 loci) profiles. It should be

noted that moderate evaporation of samples was observed following amplification. Evaporation is always a concern when amplifying samples in lower reaction volumes. The evaporation witnessed may have adversely affected the results of this particular study. The evaluation results of FISH treated aged samples are summarized in Table 13.

Table 13: Minifiler™ Amplifications of FISH Treated Aged Samples

DNA Source	Number of Trials	Full Profile (9 loci)	Mid-High Partial (5-8 loci)	Low-No Profile (0-4 loci)
20 Epithelial Cells from Cotton (12 month)	4	-	2	2
20 White Blood Cells from Glass (16 month)	4	-	2	2
20 Post-Coital Epithelial (24 months)	2	-	-	2

Figure 26: Electropherogram Displaying a Partial STR Profile Generated From the Extraction of 20 FISH Processed Male White Blood Cells



E. Summary of Results

The following list highlights the results of our work on NIJ Grant # 2006-DN-BX-K032:

1. Established a standard method to prepare and capture different cellular types using the Arcturus PixCell® II and PALM® Microbeam systems.
2. Obtained single source STR profiles from different morphological mixtures (i.e. sperm/epithelial cells) using Laser Microdissection techniques.
3. The use of low concentration cellulase increases yields from cotton swabs taken from mock evidentiary samples. The enzyme *A. niger* appears to have no negative effect on the laser capture, DNA extraction, and STR typing processes.
4. Methods to efficiently adhere cells to slides were evaluated. The optimal slide preparation for laser microdissection microscopy involves pipetting 5-15 µl fluid containing biological cells and hematoxylin stain followed by a 30 second to one minute air incubation. Preparing the slides in 75%, 95%, 100% ethanol and xylene solutions is the optimum method to affix cells to the slides so that the cells can be captured using LM techniques. This method allows for successful staining applications, as well as downstream STR analysis. Implementing the fast evaporating acetic acid and methanol solution used in white blood cell isolating may prove to enhance cellular binding for LM.
5. The Qiagen QIAamp® Micro kit seems to be the best extraction technique for isolating DNA from laser captured cells. The supplementary protocol entitled "Isolation of Genomic DNA from Laser-Microdissected Tissues" combined with double elutions and Microcon® sample concentration provided the best results.
6. Applied Biosystems Identifiler®, Yfiler®, and MiniFiler™ amplification kits have been evaluated. All systems are compatible with laser microdissection techniques. The MiniFiler™ system is the most sensitive amplification system, and reliably amplifies full STR profiles from 10 FISH prepared cells collected with the Arcturus PixCell® II system.
7. Implemented FISH X/Y probes to differentiate male and female cells of similar morphological mixtures.
8. Successfully resolved various LCN sample mixtures (i.e. sperm/epithelial, epithelial/white blood cell, epithelial/epithelial, and white blood cell/white blood cell) using FISH, LM, and optimized extraction/amplification methods.

IV. CONCLUSIONS

Discussion of Findings

Successful resolutions of various LCN sample mixtures can now be accomplished using the materials and methods outlined in this report. Many new and/or improved techniques for the processing of LCN samples were examined and developed during our sixteen months of work with LM instruments and evidence mixtures. Methods for the collection of cells from paper and cotton substrates using cellulose were evaluated and found to be highly efficient in comparison to other methods. The use of low concentration cellulase increases yields from cotton swabs taken from mock evidentiary samples. The enzyme, *A niger,* appears to have no negative effect on LM processing, DNA extraction, and STR typing processes.

Basic protocols for collecting cells using LM with the PixCell® II instrument and the Zeiss PALM® MicroBeam system were utilized and improved upon. The establishment of a standard method to prepare and collect different cellular types using these two systems allowed for the generation of single source STR profiles from different morphological mixtures using LM. During the development of these protocols, methods to efficiently affix sample cells to microscope slides were also evaluated and optimized. The optimal slide preparation for laser microdissection microscopy involves pipetting 5-15 µl fluid containing biological cells and hematoxylin stain followed by a 30 second to one minute air incubation. Preparing the slides in 75%, 95%, 100% ethanol and xylene solutions is the optimum method to affix cells to the slides so that the cells can be captured using LM techniques. This method allows for successful staining applications,

as well as downstream STR analysis. Implementing the fast evaporating acetic acid and methanol solution used in white blood cell isolating may prove to enhance cellular binding for LM.

A standard method to extract DNA from laser captured cells using the QIAamp® Micro kit was established. The QIAamp® Micro kit consistently performed as the best extraction technique for isolating DNA from laser captured cells. The supplementary protocol entitled "Isolation of Genomic DNA from Laser-Microdissected Tissues" combined with double elutions and Microcon® sample concentration provided the best results for LCN samples. Full profiles were obtained from multiple collections of 5 and 10 cells using this extraction method. The PicoPure® DNA extraction kit was also tested and performed well at higher cellular counts (>20) but failed to consistently produce complete interpretable profiles at the 5-10 cell count range.

The Identifiler® kit, Yfiler® kit, and the MiniFiler™ kit were each assessed and found to be suitable for the amplification of the DNA extracted from LM collected cells. All kits are compatible with each laser microdissection setup. The MiniFiler™ system was found to be most sensitive and reliably amplifies full STR profiles from 10 hematoxylin stained and/or FISH prepared cells collected with the PixCell® II or PALM® Microbeam system.

A method to differentiate male from female cells using Fluorescent In Situ Hybridization (FISH) methods via Vysis CEP X® and CEP Y® probes was developed. Protocols and protocol enhancements were examined and implemented for the successful collection of FISH stained cells using the PixCell® II and the PALM® MicroBeam instruments. Interphase FISH processing was tested on both epithelial and white blood

cell sample types. Sex chromosome labeling has been successful with both cell varieties. Processing samples with this technique has allowed us to visually identify the male and female contribution to each sample mixture. Sex chromosomes were easily identified from epithelial/epithelial, white blood cell/white blood cell, and epithelial/white blood cell mixtures. Aged forensic samples were also tested with some success. The aged forensic samples could be differentiated as male and female, however only partial profiles were obtained from these LM collected cells. An X chromosome is visually identified by the presence of a green fluorescent marker while a Y chromosome can be readily detected by the presence of an orange fluorescent marker. The results from this study demonstrate that low copy male/female cellular mixtures of similar morphology can be successfully separated and profiled using FISH and LM with the PixCell® system. FISH probes can be hybridized to the nuclear DNA of various cell types without inhibiting further downstream genetic analysis.

By using what was learned during the work on NIJ Grant # 2006-DN-BX-K032, forensic labs can now successfully and efficiently resolve various LCN sample mixtures by incorporating FISH, LM, and optimized elution/extraction/amplification methods into their standard operating protocols.

Implications for Policy and Practice

This proposal outlines LM as a technique for physically separating mixtures of cells as well as enhancing our ability to analyze touch evidence from a minimal amount of cells. With respect to sexual assault evidence, current methods often result in a mixture profile of male and female contributor. LM provides a technique capable of physically separating sperm cells from epithelial cells. LM has the potential to reduce the

amount of time to process a sample (as compared to differential lysis methods) but also makes available samples that would have typically yielded poor results, e.g. samples with a limited number of sperm or multiple male contributors. Methods for the differentiation of male from female cells using FISH methods via X and Y probe screening methods will also provide means for the separation of mixture evidence not containing spermatozoa. This can help law enforcement and legal professionals convict sexual predators as well as reduce the current backlog of assault cases.

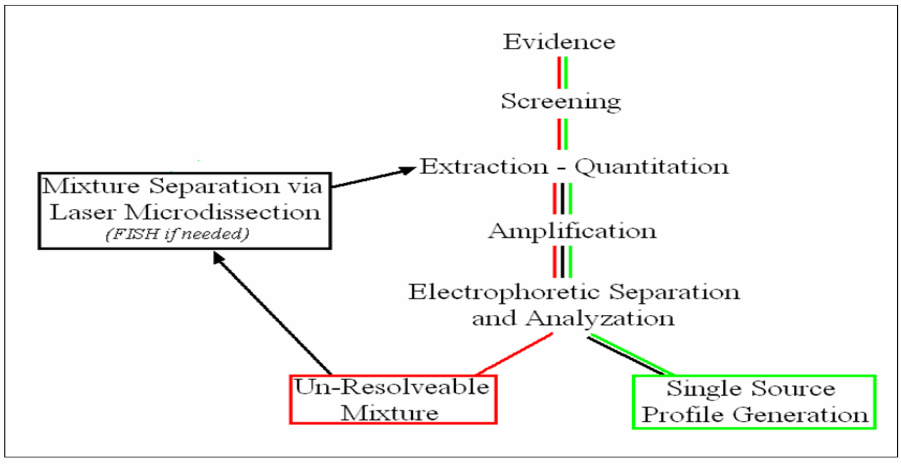
Concerning touch and assault evidence, although handled objects can be a source of cells, they are often low in number which presents problems for both extraction and amplification. Additionally, objects handled by several individuals typically produce a mixture of DNA profiles. However, by using LM and possibly FISH techniques to isolate epithelial and/or white blood cells, along with enhanced elution, extraction and amplification techniques, we can optimize the analysis and produce more consistent results. The ability to use extremely low amounts of cells for STR analysis will provide a solid means of handling mixture evidence. This would create an additional source of evidence available to the criminal investigator.

Of great importance to any crime laboratory is the accessibility of new techniques, both financially and scientifically. The skills necessary for LM are basic microscopy and other techniques already established in a DNA analysis lab, while the financial requirements of this technology are within range of many state laboratories. A high throughput laboratory could purchase an automated system (the cost ranges from $150,000 to $300,000 for the PALM® Microbeam or Arcturus Veritas™ Systems, depending on the accessories added) which automates the cell search function, while

smaller labs that process fewer samples could obtain a non-automated PixCell® LM instrument, for approximately $100,000. Additionally, some companies (i.e. Arcturus Engineering) are selling refurbished manual LM systems for less than $70,000.

Laser microdissection provides a method to physically separate mixtures of cells that have similar and/or different morphologies as well as enhancing the ability to analyze touch evidence from a single cell. An LM instrument may be employed in typical forensic laboratory for multiple processing scenarios. LM systems may be used only after sample evidence has been processed and found to contain mixtures. If evidence is known to contain a sample mixture prior to processing, then LM can be used to initially separate cells and provide for clean, single donor profiles. This could save laboratories precious money and time. The multiple pathways of utilizing LM in the laboratory are exhibited in Figure 27. Regardless of the manner of implementation, LM can and has provided a tool for the law enforcement community to process difficult samples and generate consistently efficient data.

Figure 27: Flow Chart Diagram Visually Depicting the Functional Pathway of an LM Instrument Implemented in a Forensic Laboratory



Implications for Further Practice

Laser microdissection has proven to be an effective method for cell mixture separations in the forensic laboratory. While sperm and epithelial cell sexual assault mixtures can easily be separated based upon morphological differences, mixtures of the same cell type are more difficult to separate. During our work on NIJ Grant # 2006-DN-BX-K032, we have developed the capability to successfully separate male/female cellular mixtures of similar morphology using chromosome X/Y FISH probing. In the cases of cellular mixtures of the same gender, we theorize that developing fluorescent probes based upon the genetic polymorphisms of various human blood typing groups or other polymorphisms to create additional FISH screening methods could provide a basis for separation of these samples with LM instruments.

Improvements in LM front-end operations could be developed to enable direct lysing of cells on slides as an alternative option to commercial DNA extraction kits. This would permit the direct collection of nuclei, reduce the number of cells required for DNA profiling, and eliminate the DNA extraction step of the overall process. Incorporation of procedures of this type would provide alternative methods of sample processing for those labs utilizing LM technologies. Techniques of this nature would also be ideal for labs attempting to process difficult evidence containing low copy cellular mixtures.

Interpretation of STR data and especially LCN data can be as important as all the procedures that lead to the data. When done properly and with multiple cells, LM can provide clear STR profiles of a male donor with the absence of any additional alleles from a female donor [6, 10, and 12]. However, when dealing with extremely low

amounts of cells (i.e. 1-5), multiple alleles will tend to drop out due to the minimum amount of DNA that was originally amplified. When allele dropout occurs during single cell analysis a "consensus profile" can be obtained from separate single cell PCR experiments matched with the actual profile of the cell donor [20]. Therefore pooling of results from multiple independently amplified cells should greatly improve the analysis of diallelic loci, and the misdiagnosis rate of diallelic loci should decrease exponentially with the number of cells analyzed [23]. Although this technique is not ideal, with the combination of FISH and the knowledge of a single donor, it could be an invaluable tool if evaluated thoroughly.

V. REFERENCES

1. Benoyahu, D., et al., *Gene expression in skeletal tissues: application of laser capture microdissection.* J Microsc, 2005. 220(Pt 1): p. 1-8.

2. Bohm, C., D. Newrzella, and O. Sorgenfrei, *Laser microdissection in CNS research.* Drug Discov Today, 2005. 10(17): p. 1167-74.

3. Chimenti, C., et al., *Laser microdissection in clinical cardiovascular research.* Chest, 2005. 128(4): p. 2876-81.

4. Yang, F., et al., *Laser microdissection and microarray analysis of breast tumors reveal ER-alpha related genes and pathways.* Oncogene, 2005.

5. Di Martino, D., et al., *Single sperm cell isolation by laser microdissection.* Forensic Sci Int, 2004. 146 Suppl: p. S151-3.

6. Elliott, K., et al., *Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides.* Forensic Sci Int, 2003. 137(1): p. 28-36.

7. Budimlija, Z.M., et al., *Forensic applications of laser capture microdissection: use in DNA-based parentage testing and platform validation.* Croat Med J, 2005. 46(4): p. 549-55.

8. Di Martino, D., et al., *Laser microdissection and DNA typing of cells from single hair follicles.* Forensic Sci Int, 2004. 146 Suppl: p. S155-7.

9. Robino, C., et al*., Incestuous Paternity Detected by STR-typing of Chorionic Villi Isolated from Archival Formalin-fixed Paraffin Embedded Abortion Material using Laser Microdissection*. J Forensic Sci, 2006. 51(1): p. 90-2.

10. Seidl, S., et al., *Contact-Free Isolation of Sperm and Epithelial cells by Laser Microdissection and Pressure Catapulting*. Forensic Science, Medicine, and Pathology, 2005. 1(2): p. 153-7.

11. Gill P., et al. *Forensic application of DNA "fingerprints"*. Nature, 1985. 318 (6046):577-79.

12. Sanders, C. T., et al. *Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis.* J Forensic Sci, 2006. 51(4): 748-57.

13. Anslinger, K., et al. *Digoxigenin labeling and laser capture microdissection of male cells.* Int J Legal Med, 2005. 119: 374-7

14. Collins, K., et al. *Identification of females cells in postcoital penile swabs using fluorescence in situ hybridization.* Arch Pathol Lab Med, 2000. 124: 1080-2.

15. Findlay, I., et al. *DNA fingerprinting from single cells.* Nature, 1997. 389: p. 555-6.

16. Voorhees, J.C., et al. *Enhanced elution of sperm from cotton swabs via enzymatic digestion for rape kit analysis.* J Forensic Sci, 2006. 51(3): 574-9.

17. Rook, S. R., et al. *Whole genome amplification of DNA from laser capture microdissected tissue for high-throughput single nucleotide polymorphism and short tandem repeat genotyping.* Amer Soc Invest Path, 2004. 164(1): 23-33.

18. Gill, P., et al. *An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA.* For Sci Int, 2000. 112: 17-40.

19. de Leeuw, W.J.F., et al., *Detection and isolation of male cells in oligo and azoospermic sexual assault cases using interphase X-, Y FISH and laser microdissection.* Proceedings of the 17th International Symposium on Human Identification, 2006. (*www.promega.com/GENETICIDPROC/ussymp17proc/abstracts/bstract33deLeeuw.pdf)*

20. Kloosterman, A.D. and P. Fersbergen. *Efficacy and limits of genotyping low copy number (LCN) DNA samples and multiplex PCR of STR loci.* J Soc Bio, 2003. 197(4): 351-9.

21. Gaines, M.L., et al., *Reduced Volume PCR amplification reactions using the AmpFlSTR® Profiler Plus® kit*. J Forensic Sci, 2002. 47(6): p. 1224-37.

22. Eisenberg, A.J. et al. *Evaluation of the AmpFlSTR® MiniFiler™ PCR Amplification Kit for Use with Compromised DNA Samples.* **Forensic News** 2006.

23. Garvin, A.M., et al. *Highly accurate analysis of heterozygous loci by single cell PCR.* Nucleic Acid Res, 1998. 26(15): 3468-72.

24. Barch, M., et al. *The AGT Cytogenetics Laboratory Manual*. 1997. Philadelphia: Lippincott-Raven.

25. *The Arcturus PixCell® II Laser Capture Microdissection Instruction Manual.* Mountain View, CA; Arcturus Engineering Inc.

VI. DISSEMINATION OF RESEARCH FINDINGS

A journal article that will be submitted for publication in *The Journal of Forensic Sciences* (JFS) is in current development. This article will discuss much of the research and findings witnessed during our work on this grant. In particular, this paper will focus upon the use of FISH techniques and LM for the successful separation and STR analysis of forensic mixtures.

In addition to the impending article submission, we have also presented our research in lecture and poster form at the following meetings:

A. The NIJ Conference: Forensic DNA: Tools, Technology, and Policy – Washington, D.C., July 23, 2007:

Laser Microdissection as a technique to isolate sperm cells and improve the analysis of touch evidence. (Oral Presentation) Presented by Robert Bever, Ph.D.

B. 18th Annual Symposium on Human Identification – Hollywood, CA, October 2007:

Identification and Separation of Male/Female Mixtures of Various Cell Types Using Interphase FISH Techniques and Laser Capture Microdissection Methods. (Poster Format) Presented by Robert Driscoll.

C. 60th AAFS Annual Meeting – Washington DC, February 22, 2008:

Physical Separation and STR Analysis of Male/Female Epithelial Cell Mixtures and Male/Female White Blood Cell Mixtures Using Interphase FISH Techniques and Laser Capture Microdissection. (Oral Presentation) Presented by Robert Bever, Ph.D.

D. Fifth Annual Advanced DNA Technical Workshop West – San Diego, CA, March 12, 2008.

Physical Separation and STR Analysis of Forensic Mixtures using Laser Microdissection and Fluorescent In Situ Hybridization. (Oral Presentation) Presented by Dane Plaza.

E. The First Annual Technological Advances in Human Identification Conference – Hampton, VA, April 10, 2008.

Physical Separation and STR Analysis of Forensic Mixtures using Laser Microdissection and Fluorescent In Situ Hybridization. (Oral Presentation) Presented by Rob Driscoll.

F. Seventh Annual Advanced DNA Technical Workshop East – Captiva Island, FL, May 21, 2008.

Physical Separation and STR Analysis of Forensic Mixtures using Laser Microdissection and Fluorescent In Situ Hybridization. (Oral Presentation) Presented by Dane Plaza.