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NIJ final technical report

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ABSTRACT

A significant portion of all forensic casework samples handled by US crime laboratories pertains to sexual assault. In order to successfully carry out DNA analysis of sexual assault forensic cases the separation of sperm from epithelial cells is critical. Current practice in most crime laboratories involves a two-step differential extraction process, a chemical process which differentially disrupts sperm and epithelial cells and hence releases their DNA for downstream STR analysis. This process is time-consuming, labor-intensive and commonly results in female DNA carryover into the male fraction which interferes with DNA analysis and criminal identification. To address these drawbacks we investigated a powerful technology called holographic optical trapping (HOT) for separation of sperm from epithelial cells in sexual assault samples prior to DNA analysis. Optical trapping is a well established scientific technique which has been widely applied in cell biology to manipulate cells. It is a non-destructive method where trapped cells can live and reproduce while being held in the traps for extended periods of time. Our company, Arryx, Inc has developed a robust commercial research instrument that can simultaneously deploy and move multiple optical traps independently of each other in three dimensions, employing the unique and patented Holographic Optical Trapping (HOT) technique. This technology could provide an automated means for separating spermatozoa from epithelial cells in sexual assault samples offering single cell resolution. Our research, presented here, focuses on various aspects of the technique and its use in handling sexual assault samples. Using mock forensic swabs we have demonstrated the compatibility of optical trapping with PCR and STR analysis. We have shown that HOT can operate on both unlabeled as well as fluorescently labeled samples. HOT is also compatible with the use of microfluidic chambers and it is feasible to process samples in an automated fashion within reasonable processing time. Although, the

automation component was not the focus of this study but rather intended for future development, we have shown that HOT offers a powerful alternative approach in separating sperm from epithelial cells thereby reducing the DNA carryover problem. Even without automation of HOT for sperm separation, the time taken for separating sperm from epithelial cells is less than 5 hours and therefore amenable for adoption by forensic crime laboratories to analyze sexual assault forensic cases.

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EXECUTIVE SUMMARY

(A) Synopsis of the Problem

Forensic DNA analysis for human identification relies upon the ability of STR analysis to accurately and uniquely distinguish and identify different individuals. STR analysis has become a powerful and widely used method for forensic DNA analysis. However, the application of STR in a common lab workflow suffers from several shortcomings. In a typical forensic workflow employing differential extraction, one of the most significant problems is the carryover of female DNA to the male DNA fraction. A recent survey by Applied Biosystems estimates that such carryover of female DNA into the male DNA fraction occurs in 19% of sexual assault forensic samples. This generally occurs because differential extraction (DE) does not fully isolate the DNA of the assailant (typically from sperm) from those of the victim (typically from vaginal or other epithelial cells). The DNA to be analyzed by STR for sexual assault evidence should ideally come from the sperm of the assailant. However, the sperm sample is frequently contaminated with (1) epithelial cells lining the vagina and occasionally with (2) epithelial cells from the mouth and (3) cells from the skin. Thus the sensitivity and reliability of STR analysis would be improved significantly if sperm cells were separated from all other cells prior to DNA extraction and STR analysis. The availability of a practical technique for precise fractionation of cells by type would eliminate DNA carryover in most cases. In addition, it was recently reported that 41% of sexual assault forensic cases have remained unsolved due to limitations in currently adopted methods used in forensic laboratories. Thus there is a strong need for developing better alternatives to address these issues. While laser capture microdissection (LCM) promises to achieve a similar end, it involves extra sample

processing steps and requires significant manual effort. These, along with other concerns such as the poor workflow integration and up-front cost of instrumentation, has prevented its adoption in forensic DNA analysis. Another strategy which showed promise to separate perpetrator's sperm cells from those of the victim's epithelial cells employs magnetic beads coated with sperm specific antibodies. The sperm specific antibodies are meant to capture sperms in a mixed cell sample by binding to sperm specific cell surface antigens. The unwanted epithelial cells are removed by a simple wash. Since this technique relies on the specificity of the reaction of the antibody on the bead and the antigen(receptor) on the sperm, the purity of the antibody used might pose problem with cross-reactivity in the sperm binding and sorting process. This approach also suffers from the pitfall that sperm specific antibodies can be expensive to generate and the success of the approach relies on sperm being intact. The third approach of using Y-STR analysis looking at 17 loci on the Y chromosome, eliminates the female DNA carryover problem, however this technique does not uniquely identify the individual and does not associate the DNA profile with sperm (as opposed to male epithelial cells). Y-STR is generally employed when the female DNA content of the sample dwarfs the male DNA content, creating a particularly high probability of DNA carryover. It can be useful in cases where a suspect is available and CODIS is not required. Because Y-STR is not unique (related males have identical Y-STR profiles), the technique does not address the need for a robust cell isolation solution. In recent years, improvements have been made on the commonly used differential extraction technique for separating sperm DNA from non-sperm DNA. Promega's DifferexTM system is one such example which has increased throughput in the analysis of sexual assault forensic samples using STR analysis but the problem of female

DNA carry-over still remains. More recently, chip based DEP (Dielectrophoresis) where cell separation relies on differences in the motion of cells when placed in a non-uniform electric field have also been attempted but with little or no success. DEP forces acting on a cell rely on the precise redistribution of electrical charges on a cell in the presence of an applied field. Since sperm and epithelial cells are different in cellular sizes, chemical composition, and membrane structure, they respond differently to non-uniform electric field resulting in DEP mobilities that forms the basis for cell separation. However, DEP offers poor recovery, incomplete separation in most cases (NIJ final report for 2005-DA-BX-K001 by Martin R Buoncristiani and Mark D Timken), besides being a slow process (2-5hours) which cannot be parallelized. More recently, acoustic differential extraction (ADE) (Norris et al, Anal.Chem.2009) has been proposed and attempted for cell separation. The acoustic trapping microdevice consists of piezoceramic microtransducers imbedded into a printed circuit board layer which serves as the bottom of a microchannel and a glass fluidic layer containing the microchannel structure. Applying an alternating voltage to the microtransducers creates standing ultrasonic acoustic waves within the microchannel generating pressure minima in the center. Cells or other objects are drawn towards these minima where the magnitude and direction of the forces are dependent on the physical properties of the cells. However, ADE suffers from problems such as lack of single cell isolation capabilities and incomplete separation of the male fraction. (NIJ final report 2006-DN-BX-K021 mentions that ADE achieved 55% or less purity). Thus there exists a strong need to develop a robust method for isolating sperm cells from other cells or free DNA which would overcome many of the problems discussed above, offering the needed purity in genetic material for STR analysis.

Optical trapping offers a highly promising technique which can isolate sperm from epithelial or other cell types and is compatible with downstream STR analysis. It offers scope for automation, can work with cells in suspension without requiring need for additional or alternative media support, and is relatively very fast when compared to commonly used DE and other techniques. It also promises greatly increased purity of the extracted sperm fraction DNA, providing optimal results even for cases where the number of sperm cells is relatively very low. Optical trapping also eliminates major concerns about cell damage since the cells are not physically touched (we have trapped yeast cells which have continued to divide while held in traps, thus demonstrating that they were alive). Holographic optical trapping (HOT) is an improved and advanced variant of optical trapping which offers scope for complete automation. Such automation of HOT will provide rapid turnover and less labor intervention, eliminating bottlenecks often experienced in the current approaches used in STR analysis. The report presented here highlights the capabilities of HOT and its applications in forensic DNA analysis focusing on sexual assault forensic cases.

(B) Purpose of study

The general goal and purpose of our research was to explore the use of HOT in separating sperm from epithelial cells and assess its adaptability in forensic DNA analysis of sexual assault samples. The instrument we have used for the physical separation of cells of forensic interest from other contaminants, creates a hologram to alter a single laser beam's wavefront and then focuses this light to form traps. The wavefront is altered

so that downstream, the laser beam forms a large number of individual laser beams with relative positions and directions of travel fixed by the exact nature of the hologram. The hologram can be calculated from a user-specified pattern of desired trap positions and movements. Currently our commercial instrument BioRyx200 has the demonstrated ability to trap multiple (up to 200) particles in any chosen pattern. Using our HOT technology one can hold, move, rotate, join, separate, stretch, and otherwise manipulate hundreds of microscopic and nanoscopic objects, ranging from the size of a human cell down to less than 1/1000th the diameter of a human hair. It uses low power to hold an object and is therefore ideal for handling cells such as those in forensic samples.

Optical Trapping History and Background

Optical trapping was developed by Arthur Ashkin at Bell Labs in 1970. It uses focused laser beams to form traps that can grab and move particles ranging in size from nanometers to tens of microns. Most biological cells fall conveniently into this size range. Hence, optical trapping has proven to have great utility in cellular biology, where adoption has slowly grown over the last 15 years. A variety of cell types ranging from bacteria to yeast to mammalian cells have been successfully trapped using this technique. Trapped cells can be moved quickly, easily, and non-invasively with light, and with high precision without the intrusive and cumbersome probing of a micropipette. Both visible and near-IR laser light commonly used in optical trapping can readily pass through most glass, making manipulation possible within optically clear, open or sealed chambers. Sealed chambers allow samples to be manipulated without contamination, damage, drying, or requiring special environmental chambers. This powerful capability has led to

applications for optical trapping in artificial insemination where sperm is trapped and steered using laser. Other biological applications of optical trapping include studies on a variety of motor proteins such as various classes of myosin, kinesin, polymerases as well as in the studies of DNA structure and conformation.

Despite the success of non-holographic optical trapping, there were limitations that restricted further deployment of the technique. These included (a) the inability to create large numbers of optical traps for sophisticated manipulations involving many particles, (b) the lack of methods to increase throughput by parallel processing, (c) the inability to manipulate objects in 3D, (d) a lack of flexibility in shaping the light to form optimized optical traps which perform better than simple point traps, and (e) the lack of a committed entity to provide a robust, turnkey commercial optical trapping subsystem with a software library enabling end-user automation.

Holographic optical trapping (HOT), invented in 1997, was developed to overcome these limitations. HOT employs a phase mask (hologram) to shape a laser beam's wave-front, splitting the single beam into many optical traps, each computer-controlled and positionable in a three dimensional (3D) sample volume. For computer-controlled dynamic holographic optical trapping, the phase mask is generated using a spatial light modulator (SLM) which controls the orientation of a bi-refractive liquid crystal over each pixel on the 512x512 pixel surface. With recent advances, SLM's are now available with refresh rates of up to 100 frames per second and can withstand several Watts of laser light power. Advances in algorithm implementation at Arryx have

improved the speed of hologram calculation from minutes, several years ago, to milliseconds or tens of milliseconds today. These speed improvements enable real-time use of holographic optical trapping and higher throughput operations under automated computer control. The laser beam which is reflected from the SLM is imaged onto the back focal plane of the objective lens using a telescope and forms the pattern of traps in and around the objective focal plane, inside the microscopic sample. The computer-generated pattern can be quickly updated to play an animated sequence of trap positions, guiding particle movement along specified trajectories. The net result is a complete micro- or nano-manipulation system, providing the user with the ability to move large numbers of cells or other particles, simultaneously, along arbitrary paths in three dimensions. The addition of a CCD camera and image processing provides a machine-vision guided system that can automatically execute sophisticated manipulations and isolations. Arryx' unique Holographic optical trapping technology therefore can simultaneously trap many objects in arbitrary positions for sorting and isolation. It also has the important capability to steer each object in 3D, allowing each trap to easily be lifted above the bottom of the cartridge/cell separation chamber and moved over other cells and debris which would otherwise be in the cell's path. Arryx' BioRyx® 200 research instrument, commercially sold since 2002, allows multiple cells to be manipulated in 3D and in real-time by clicking and dragging a computer's mouse. Trapping of human sperm with this system has been demonstrated on numerous occasions. This provided a strong foundation for developing HOT for forensic cell separation.

In addition, Arrayx has recently developed an enclosed optical trapping device which employs machine vision for completely automated sample processing for the analysis of human blood type (A,B, Rh). This initial demonstration system can scan, autofocus, image, and manipulate cells in a disposable cartridge for the dedicated purpose of detecting the presence or absence of red blood cell (RBC) antigens indicative of blood type. Using optical traps to detect the binding of RBC's to each of several antibody patches on a substrate, the blood type is inferred. This system is under very active development as Arrayx develops its first medical diagnostic device. The instrument, software, disposable microfluidic cartridge) and anti-sticking surface coatings developed at Arrayx provided additional and credible foundation for development of HOT for forensic cell isolation.

(C) Research Design

The project was aimed to address the following: (a) Develop the core components required for forensic processing (b) Assess risk factors (if any) associated with using this technology in forensic DNA analysis (c) Compare quality of DNA profile of laser-manipulated versus control samples and finally (d) Develop the knowledge to build a prototype system for automated isolation of sperm from a mixed sample containing sperm, epithelial cells, and other materials based on the results from (a)-(c). Objectives (a) - (c) were related to the experimental objective of illustrating that a sample which is similar to that from a rape kit can be eluted from evidence samples such as cotton swabs, separated from contaminants using HOT and then subjected to PCR-based STR analysis for identification. These objectives were intended to confirm that HOT-based sperm

separation is feasible after sample elution from a swab and can be used with existing rape kit swabs and STR analysis. While objective (d) was not the main focus of this study and has been reserved for phase 2 of the work, the goal was still kept alive passively in this work so that a good sense of potential risk factors could be obtained. This in turn would allow us to gauge the viability and efficacy of a prototype system as a widely deployable device for analysis of rape kits for other forensic applications. This meant that the results from the work done in this phase based on goals (a)-(c) and presented here should provide a strong foundation towards achieving the ultimate goal of developing an automated sperm separation device based on HOT. Hence some work on developing microfluidic cartridges for cell sorting and the basis for developing automation software such as automated sperm recognition may be referred in this report but will not be discussed in any detail.

Initially, the short term goals of the proposed plan was to test the feasibility of such HOT-based sperm isolation for Forensics. The immediate questions for testing such feasibility were outlined as follows. (1) Can sperm be clearly identified (morphologically) under a microscope from other contaminating cells post elution without staining? Can the sperm be trapped and separated from other cells? (2) If dyes are needed to identify sperm cells from other contaminating cells, do those dyes obstruct standard PCR and STR methods? (3) How many sperms cells can be simultaneously trapped and moved at a given speed using HOT? (4) Does optical trapping cause DNA degradation and therefore hinder PCR and STR analysis? While these questions mostly pertained to experimental and analytical objectives, they were also tied down to design

objectives for the more complex long-term goal of building a prototype device which can be automated. The design objective also involved incorporating an efficient sample handling system. Full-fledged automation will be possible with the results and knowledge acquired during the project span.

To summarize, the core of our HOT-based forensics DNA research and development proposal and research comprised of four components: (1) elution of sperm from samples, (2) imaging and sperm identification (3) isolation of sperm from other contaminants with HOT using appropriate microfluidic chambers (4) assimilating knowledge obtained from (1)-(3) towards developing automation algorithms for the separation process and delivery of the purified sperm to distinct containers for downstream PCR based STR analysis. Our research was conducted on a variety of mock forensic samples as deemed appropriate. The choice of such samples for testing was decided upon consultation with our collaborator Orchid Cellmark who provided us with the mock samples and also carried out the downstream STR analysis on the trapped samples. Typically 5-10 μ l of cell mixture eluted from mock forensic swab was used for trapping and sperm separation. Trapping and recovery of trapped samples was evaluated by a variety of means primarily microscopic visualization in real time during the trapping process. The trapped samples were recovered via pipeting and were sent to Orchid for DNA extraction and STR analysis. For all trapped samples analyzed for STR, control samples from the same batch that were not trapped were also STR analyzed for comparison of STR profile quality and completeness. Typical number of trapped cells sent for STR analysis was ~400.

(D) Results and Conclusion

Our data demonstrate that we are capable of successfully eluting sperm from mock forensic swabs, trapping and separating them from unwanted cells to obtain a complete and unambiguous STR profile, staining sperm with a number of different fluorescent dyes at various confirmatory steps and finally recovering the trapped sperm for downstream DNA analysis. While we tested a variety of mock forensic swabs, the bulk of our samples included sperm only swabs to establish the range of utilizable trapping power for separation. In our study, we dealt with samples with enough sperm count so that at least 400 sperm could be trapped, and separated for STR analysis. Since automation including auto-detection of sperm is the focus for the next stage of research, the samples that were trapped and tested in this study were manually identified in a bright field image and trapped using a mouse click. Therefore the results presented here were based on a semi-automated trapping process which required significant manual intervention. This made the trapping part tedious, although the whole process was significantly shorter (~3-4 hours to trap 400 sperm) than a typical differential extraction process. However, based on our study, we are confident that we would be able to achieve the automated separation of ~400 sperm well below 1 hour without any manual intervention. We were also able to successfully trap fluorescently tagged sperm without causing any DNA damage as revealed by STR analysis. While fluorescent staining is not a prerequisite for automated trapping, we realized that for automated sperm detection to be fast and reliable for trapping, we may need to fluorescently label the sperm. Hence we did a study where we labeled the eluted sperm from mock forensic swabs with a number of different fluorescent dyes such as Christmas tree stain, Propidium Iodide, Sybr 14,

Hyliter, DAPI and Alexa 546-NHS ester and compared the labeling efficiency and specificity, photostability, labeling time, relative staining pattern of sperm versus epithelial cells and finally their suitability for use with HOT. All of the trapped samples containing labeled sperm gave complete STR profiles. Based on the labeling efficiency, photostability and ease of use of the dyes for labeling we found that PI and DAPI are ideal for use in labeling eluted cell samples for automated sperm detection and trapping. These results are illustrated in a number of figures shown and discussed later in the detailed report section. Also, in the bulk of our study reported here, we used home-built passive microfluidic cartridges with either glass or COC (cyclic olefin co-polymer) bottom which were compatible with HOT to carry out sperm separation. The recovery of sperm from these devices post trapping was greater than 95%. We also designed and tested a couple active microfluidic cartridges for use with trapping to speed up the separation process and also to be able to deliver the separated sperm in closed chambers avoiding cross-contamination. However, the separation process was less than effective and allowed the eluted cell mixture containing sperm to travel to unwanted regions such as the output chamber. This was an unforeseeable challenge and we hope to address that with better design and valving mechanism used on the microfluidic chip. Thus overall we have assessed the potential risk factors and identified areas of improvement so that we can work effectively towards the goal of developing a prototype automated sperm trapping and separation system. We not only addressed the key objectives and goals as indicated in our research design but went beyond to bridge the results of this study with the needs of the next phase of research which is to build an automated sperm sorting device.

(E) Implications for Policy and Practice

Our research and studies have demonstrated that the principle of HOT can be successfully used to separate sperm and epithelial cells using mock forensic swabs. However in its current state we do not recommend the preferential use of HOT over existing chemical differential extraction for forensic casework handling because HOT based sperm separation is not yet automated and consequently requires significant manual intervention and time. In its current form HOT based sperm sorting has few merits to replace well established differential extraction method. Further testing is definitely required to establish HOT as a robust and reliable method for handling sexual assault casework samples. While HOT still offers real time visual monitoring of the separated fraction which commonly used differential extraction does not offer, the ability to parallel process multiple samples with DE may remain an advantage over the current state of HOT based sperm separation.

However, with further development in the areas of active microfluidic cartridge development and automated sperm detection and trapping, we can not only improve the speed of cell separation but we can also significantly reduce manual labor and provide with pure fractions of sperm and epithelial cells. With those improvements in place, we believe that HOT would become a viable alternative to standard differential extraction offering advantages that no other cell separation techniques have offered till date.

Implications for future research

There are two primary areas that will remain the focus for future research related to the work presented here. One is the development of active and efficient microfluidic cartridges for cell separation and the other is the development and implementation of automation algorithms for sperm recognition, trapping and movement and the integration of the different hardware and software components. Both of these will improve the time needed for sperm separation using HOT and minimize the extent of manual labor involved. As new microfluidic cartridges are designed and developed for HOT based sperm separation, the recovery of the trapped sperm will be kept in mind since often the choice of material for microfluidic device fabrication could interfere with cell recovery process. Each new material and design tested for microfluidic chip, we will need to ensure that trapping efficacy is not hampered and neither is the STR analysis.

Another extension of our research would be to integrate the sperm separation process with DNA extraction and amplification process using PCR on a single microfluidic chip. While a few labs have demonstrated capabilities for on-chip PCR, its use in the context of forensic research is still being evaluated. The success of on-chip HOT based sperm separation with on-chip DNA extraction and PCR could hopefully give birth to on-chip total forensic analysis. Such total on-chip forensic analysis would make the overall process faster and cheaper by saving on reagent costs.

DETAILED DESCRIPTION & TECHNICAL REPORT

I. Introduction

According to the U.S. Department of Justice there are currently more than 225,000 reported cases of sexual assault in the United States. A report published last year stated that of those reported cases, only 41 percent of the cases have resulted in an arrest or have been solved. One of the key problems contributing to slow progress in solving sexual assault crimes still remains the admixture of DNA (from both the victim and the assailant) which leads to ambiguous STR (Short Tandem Repeat) based human identification. Another factor contributing to such backlog is the slow and laborious process of differential extraction which is currently the adopted method for sexual assault evidence analysis.

Various research efforts have focused on reducing such backlog in solving sexual assault forensic cases by addressing the drawbacks of current DE method. Alternate methods such as LCM (laser capture microdissection), ADE (acoustic differential extraction), differential filtration techniques, microfluidic device based separation of sperm and epithelial cells (Landers et al), antibody based separation techniques, etc have been tried to separate sperm from epithelial cells. However, none of the methods were perfect for use and were fraught with problems that prevented their adoption in a crime laboratory for analysis of sexual assault forensic samples.

Differential extraction is the most common method currently used in the separation of male and female DNA fractions in a sexual assault evidence sample. First,

the cells are eluted from the sample swab or other material, which generally produces a mixed cell solution, with cells from both the assailant and the victim. The suspended cells are exposed to mild lysing agents, so as to exploit the difference in stability of spermatozoa membranes relative to those of epithelial cells. Under ideal conditions, this leaves sperm cells primarily intact (the sperm tails are degraded but the nuclear material is not released) while the epithelial cells are lysed. Washing is used to separate the released nuclear material from the epithelial cells from the intact sperm nuclei. The sperm are then lysed to release their DNA. Under optimal conditions, differential extraction aims to achieve two isolated and pure fractions of DNA. This method is less than ideal however, due to the significant frequency of producing mixed DNA results and the amount of labor involved in sample processing. It also does not perform well with samples with very low sperm count, relative to epithelial cell count.

Hence various groups have investigated alternatives to differential cell extraction. For example, Elliott, et al. suggested using laser capture micro-dissection (LCM) to selectively capture sperm cells from slides prepared from swabs. While this method is capable of isolating sperm, it is expensive, labor-intensive, and incompatible with high-throughput applications. The major challenge in LCM is differentiating male and female cells. Hematoxylin/eosin staining is used for morphologically differentiating the two fractions. This stain has minimal effect on downstream DNA analysis but it is less than ideal for morphological differentiation. Other stains such as Christmas tree stain (nuclear fast red/pico indigo carmine) differentiate sperm cells from epithelial cells but inhibit PCR. Sanders, et al., 2006, have shown that acridine orange inhibits PCR reactions as

well. In another approach, Chen, et al., used a nylon mesh membrane (8 micron pore size) to demonstrate separation of sperm cells from epithelial cells. This method, too, is less than ideal and causes clogging of membranes with epithelial cells and therefore incomplete separation of free DNA from sperm cells is commonly encountered. Recently, an antibody-based recognition of sperm cells has been proposed which works with dried sperm and pre-made forensic slides and not necessarily with eluted sperm in solution. Its feasibility with automation has not been tested either. Such sperm-capture antibody approach would allow selective removal of sperm from a background of epithelial cells and therefore allow the separation of the DNA from the sperm and epithelial fractions avoiding DNA carryover (Eisenberg 2002). While such method can be automated but the success and adoption of this method has suffered from the setback that the method causes significant sperm loss during the wash steps. The antibody-antigen binding process has been found to be unreliable and inefficient since the sperm cell surface antigens are unstable especially after the drying step. Horsman et al have also recently reported the use of a microfluidic device for the separation of sperm from epithelial cells. It is based on differences in the physico-chemical properties of sperm and epithelial cell such as cell morphology, cell density and preferential adsorption of one cell type over the other which in turn affect the relative mobilities of the epithelial versus sperm in a microfluidic device. Another approach that has also been attempted is the use of Y-STR based analysis. Although this method works well with samples having limited number of sperm, it is not a preferred choice over standard STR typing using autosomal chromosomes which offers much larger discrimination in human identification. More recently DEP (dielectrophoresis) and ADE(acoustic differential trapping and extraction)

have been investigated as alternative choices for sperm separation in sexual assault forensic samples but ran into problems. While DEP can be automated, it suffers from 30-50% sperm loss and hence requires significant improvements before it can be adopted in forensic practice. Also, in the non-automated form it is a rather slow process, cannot be parallelized in operation and does not offer complete separation of sperm in most cases. ADE, which uses acoustic forces to trap sperm suffers from the pitfall that it has a significant female lysate carryover. Hence better fluidic controls need to be adopted when using ADE so that lysate trapped in the acoustic standing wave does not get released upon termination of the ultrasound contaminating the male fraction. In addition, ADE has been demonstrated only on samples containing at least 2000-4000 cells and not on samples where the sperm count is significantly lower. It also requires that the epithelial cells are completely lysed before ADE is employed. Hence complete epithelial cell lysis is a prerequisite for ADE to work efficiently in the sperm separation process in order to avoid female DNA carryover. Unlike optical trapping ADE does not offer single cell resolution in the trapping process which can be of great advantage when handling samples with few sperm cells.

The report presented here proposes the use of optical trapping as a rational and effective alternative method to separate sperm from epithelial cells with significant reduction in time and labor involved in sample processing prior to STR analysis. This method and technique can be completely automated, can be carried out in microfluidic chips or cartridges and is fast and efficient.

Optical trapping is a well established technique for trapping microscopic objects including cells. Arryx's patented Holographic Optical trapping can simultaneously deploy and move multiple optical traps. The movement of each trap can be controlled independently of each other in 3D using software control. This technology could not only provide a means for separation of spermatozoa from epithelial cells in sexual assault samples but also allows scope for automation. The advantages of HOT technology over standard techniques such as differential extraction is significant offering improved speed and efficiency, sample purity in genetic material content and reliability. By separating sperm from other cells using HOT prior to DNA extraction, HOT can dramatically reduce or eliminate the carryover of unwanted DNA, which do not arise from sperm and interferes with downstream STR. This is a problem that exists with current methods such as differential extraction. This report focuses on the development of HOT and its characterization for forensic DNA analysis particularly handling sexual assault cases.

The research presented here aims to directly address an improved front end analysis where sperm can be separated from contaminants and unwanted cells via optical trapping and ultimately translating that research in building an automated device to do the same in future. Specifically this report describes in detail progress made till date since January 2009 targeting the objectives mentioned in our proposal such as

- (a) developing the core components required for forensic processing
- (b) assessing the risk factors associated with using this technology in forensic DNA analysis

© confirming that HOT based manipulation of samples does not impair subsequent genetic analysis

(d) Optimizing trapping parameters such as power, time of trapping, etc for best throughput and STR results and

(e) develop microfluidic devices for the introduction of forensic sample and the isolation and subsequent withdrawal of the sorted cell species using small volume handling.

Our research and development efforts matched the goals outlined in the GANTT chart submitted with the grant and in some cases went beyond those targets. They are discussed in detail below according to each objective stated above.

II. Methods

Development of the core components required for forensic processing

The core components associated with forensic processing include (a) sample (sperm,etc) elution from mock forensic swabs, (b) sperm staining to confirm presence of sperm, (c) sperm sorting using HOT and performing STR analysis on the sorted sperm. It must be noted that objectives (a), (b) and (c) are closely tied and therefore these sections may include overlapping information in the methods as well as in the results and discussion section.

1. Sperm elution and visualization

We used mock forensic swabs for all our study. The mock swabs were provided to us by our collaborator, Orchid. In order to prepare sperm-only mock swabs, a given volume (1-10 μ l) of semen from a donor male was put on a cotton swab and the

swabs were left to dry in controlled sterile environment before being shipped to us for elution, visualization and trapping. For preparing mixed swabs containing sperm and epithelial cells, buccal swabs from donors (in order to obtain epithelial cells) were collected first before adding a known volume of semen to the same swab. Once again, the swabs were dried overnight before they were shipped to us. We used swabs containing sperm only as well as those with both sperm and epithelial cells and occasionally swabs with only epithelial cells on them. The eluted samples from sperm only and epithelial only swabs were used as controls. The mixed mock swabs were prepared containing varying number of sperm and epithelial cells. For swabs containing epithelial cells, buccal epithelial cells from a healthy female donor was obtained on a cotton swab. Then a fixed volume of diluted semen from a healthy male donor was added to it to obtain a mixed swab. The swabs were dried for over 2 days and elution was carried out on the swabs within a year from its preparation date. The STR profiles from the healthy donors were known beforehand for comparison study.

Sperm elution protocol from forensic swabs was tested and perfected to reduce the time required for sample elution and for improvement of yield. Orchid Cellmark, our collaborator in this project, provided us with a modified proprietary protocol which we have been using throughout the project span.

In situations where there are very few cells on a sample swab, optimization of cell elution protocol plays a big role in the ultimate downstream STR processing. The optimized protocol was carried out on multiple mock sample swabs. Using mock

swabs with a given number of sperm on them, cell counting post elution was compared to the original input of cells and efficiency of elution was estimated to be as high as 70%. Post-elution, the sperm lose their tails which is well documented in literature. The eluted samples were subsequently used for all the trapping and labeling studies.

The eluted samples were visualized under a Nikon microscope. Objectives with magnification ranging from 10x and 40X were used. Images were acquired using a cooled CCD camera (QImaging, Canada).

2. Sperm staining and microscopy:

Staining of eluted samples is often a standard practice in forensics laboratories. In order to show that we are capable of following standard forensic practice on cell staining at our Arrayx facility, we pursued staining of eluted cells. Christmas tree stain (from Serological Research Institute) was used for such staining purposes. It is a differential biological stain that assists in scanning slides to show the presence of sperm. It is a two stain method comprising of a green dye (Kernecktrot solution) and a red dye (picoindigocarmine solution). This allows sperm heads to be colored red, tails green and contaminating epithelial cells as green or blue with red nuclei. Staining was carried out following manufacturer's protocol. Stained samples were either used for trapping experiments or to facilitate development of auto-detection software for automating sperm recognition and trapping (primary focus for next phase of the project).

In addition to Christmas tree stain, we also tested a variety of fluorescent dyes for labeling the eluted samples. The dyes tested were either nuclear dyes or cell periphery dyes such as DAPI, Hoechst, Propidium Iodide (PI), Sybr 14. All dyes were purchased from Life Technologies, Carlsbad, CA and staining of samples was carried out following manufacturer's protocol. The stained samples in the presence or absence of trapping were subjected to STR analysis to demonstrate the compatibility of their use with HOT and STR analysis.

A typical staining protocol with one of the dyes we have used namely Hoechst 33342 is described here. 100mg Hoechst 33342 was dissolved in PBS buffer to obtain 250mM stock and 2 μ l of that stock was used to label 15 μ l of eluted sperm. Labeling was carried out for 15min at room temperature. Post labeling, the samples were washed twice with PBS buffer by adding ~500 μ l PBS buffer, spun at room temperature for 5min on table top centrifuge at 13k rpm. The supernatant was discarded and pellet was resuspended in PBS buffer. The spin and wash steps were repeated. The labeled sperm were either directly sent to Orchid (our Collaborator) for STR analysis or trapped and then sent for STR analysis.

In addition to organic dye based fluorescent labeling, we also tested labeling of the eluted sperm using the sperm specific antibody based label, Hyliter from IFI, Hillside, IL. Manufacturer's protocol was followed but required sample fixing. It was

subsequently modified to label non-fixed sperm sample but the efficiency in labeling was very poor.

3. Trapping and Sorting of sperm using HOT

We used Arrayx's patented HOT technique on samples eluted from mock swabs using our commercial BioRyx 200 platform. An IR fiber laser (IPG Photonics, Oxford, MA) was used for trapping using 1064nm wavelength and a 40x air objective, NA=0.9 (Nikon Instruments). Trapping was tested on both unlabeled and fluorescently labeled sperm which were eluted from the mock swabs. Sperms were trapped over a range of trapping power to test for low power (50-200 mW/trap) and high power trapping (200-450 mW/trap) compatibility with downstream DNA analysis. The trapped samples were subsequently sent to Orchid for DNA extraction and STR analysis. Typically for moving and separating the trapped sperms in a given sample, 4-6 sperm were moved at any given time. For trapping fluorescently labeled sperm, a triple filter cube (DAPI/Fluorescein/PI cube from Chroma Technology) was used with white light (lamp) illumination in wide field mode for fluorescence excitation in conjunction with 1064nm laser illumination for trapping.

To address the topic of maximum achievable and desirable speeds for the movement of trapped objects, speed tests were carried out using various sized silica beads as well as eluted sperm. As expected, the speed was dependent on the strength of the traps used but we limited ourselves to a maximum trap power of 450mW/trap for these tests. Depending on the substrates used for trapping (glass, COC, etc) the

speed of movement ranged from 65 $\mu\text{m}/\text{sec}$ to 100 $\mu\text{m}/\text{sec}$. The speed was lower on COC substrate than on glass substrate since each substrate interacts with the excitation laser light in a different way and affects the strength of the trap.

In HOT, the objects were manually identified, trapped with the mouse-click, moved over a short distance by clicking and dragging the mouse over a short distance within a field of view ($\sim 200 \mu\text{m}$ for a 40x air objective) or moved long distance over several fields of view by moving the microscope stage but keeping the traps fixed. The movement of trapped objects is amenable for complete automation and will be developed in the next phase of the research such that an automated sperm separation prototype device can be made in the near future based on HOT technology.

4. DNA extraction, quantitation and STR analysis

DNA extraction from the trapped sperm samples was accomplished using Qiagen's EZ-one Investigator kit (Qiagen Corp, Valencia, CA). Real time PCR (qPCR) with human specific primers was used to determine the quantity of human genomic DNA using ABI's Quantifiler Kit (Life Technologies, CA). The DNA was amplified (10 μl was used for PCR) using Applied BioSystem's 9700 system. Finally 1 μl of the amplified product was used in STR analysis employing capillary electrophoresis. Specifically 3100 Genetic Analyzer and the Identifiler kit from Applied Biosystem, now part of Life Technologies was used for STR processing. The STR results of trapped or labeled samples were compared against unlabeled and untrapped control samples from the same batch of eluted sperm samples. All STR profiles were obtained from samples having at

least 400 sperm so that enough DNA could be extracted from these samples as per FBI guidelines. DNA extraction, quantitation and STR analysis were all carried out by our collaborators at Orchid.

5. Fabrication of microfluidic chips

We tested trapping on a number of different microfluidic chips, passive and active, containing sealed or open chambers. Microfluidic chambers and channels were created using either laser etching or standard milling and heat embossing techniques. The length, depth and geometry of the channels and chambers were varied to arrive at best trapping and movement criteria. This is an area of continued development and will extend into the next phase of our research efforts.

We started out with the simple design and least complex passive chip which was used in most of our experiments. It was assembled from 3 layers (an acrylic layer, an adhesive layer and either a glass bottom or COC bottom layer). While the adhesive layer attached first to the acrylic layer was laser etched, the bottom layer was simply adhered to the laser etched acrylic and adhesive combo layer to complete the chip. The input and output chambers were either circles (1.5mm diameter) or squares (1-2mm x1-2mm) which were connected via sealed channel (500 μm -1500 μm in length). Laser etched chips led to rough edges which were subsequently improved to obtain clean and sharp edges when milling and embossing techniques were employed. Typical sample volumes used in the chip were 5-8 μl .

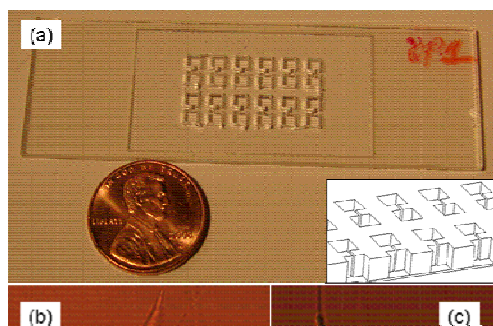


Figure 1

Figure 1 shows a picture of a test chip for preliminary test. The overall size of the chip is 1x3 inch, which is the same as a regular microscope slide so that the chip could be mounted on our existing microscope stage associated with the BioRyx 200 system. As shown in the inset, the chip is made up of two layers: the top layer is a 2-mm thick plastic that was cut by laser to include several sets of chambers connected by a narrow channel. The bottom layer was a number 1 coverslip. The two were joined via an intermediate layer of glue. Each set contains a pair of identical chambers (2x2 mm cross section), connected by a 1-mm long, 0.2-mm wide channel.

In designing and testing microfluidic devices for sperm separation via optical trapping we started our work using different materials keeping in mind minimum sperm damage and the compatibility of the materials with laser trapping. We came up with several designs of the microfluidic devices that differed in their dimensions, number of channels and the number of independent operations (uni- or bi-direction flow) with or without valves for control. The ease of the fabrication process was also kept in mind.

Glass, Poly (methyl methacrylate) (PMMA) and COC (cyclic olefin copolymer) are all good fits for optical trapping application and hence used in our chip fabrication. These materials are optically transparent particularly in the visible and IR which is compatible to the existing laser trapping system, as strong traps can be formed inside the chambers. All the materials were biocompatible and allowed sperms to stay afloat (without drastic non-specific binding to the substrates) and no visible damage to sperm was noticed post-trapping.

To demonstrate trapping of sperm using such a chip, PBS buffer (without sperms) were loaded into both chambers connected via a narrow channel (fig 1(a)), and then sperms are added into one chamber (called source chamber or input chamber). After the initial loading, laser traps were created to pick up target sperms in the input chamber, and subsequently move them towards the output chamber, where no sperm was initially present. The insets show the images of sperms (bovine) crowded in the input chamber (fig 1(b)) and trapped and moved to the output chamber (the image shows two sperms being trapped by the laser) (fig 1(c)). No backflow of sperm from output to input chamber was noted over the course of several minutes. Another interesting feature which plays to our advantage of sperm separation is the limited Brownian motion of sperm in the chambers.

Based on the successful results from the initial test, we designed a second chip, as shown in figures 2 (a-c) below. Again, the overall size of the chip is 1x3 inch. Each chip contains two identical microfluidic systems, whose schematic is illustrated in figure 2(c).

The fluidic system includes a chamber, six ports, and channels connecting each port to the center chamber. Sample solution, containing mostly sperm, is introduced into the chamber through the sample port. Laser traps are formed inside the chamber, so that target cells can be transferred from chamber to the side channels over a short distance. Once the cells are moved to the side channel, buffer solution was driven from one port (either Epi-buffer or Sp-buffer), and the cells were carried to the other port (Epi-out or Sp-out).

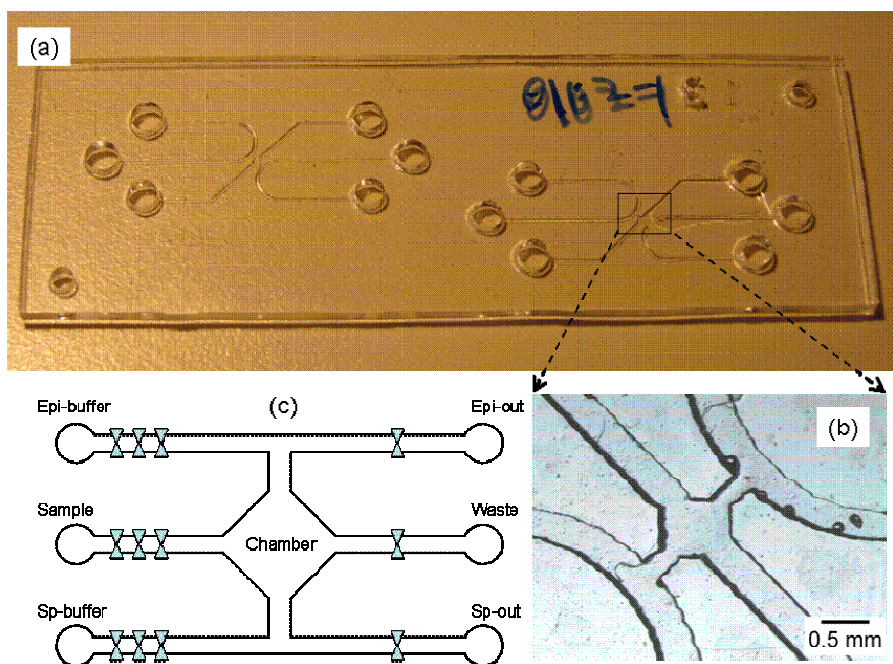


Figure 2

III. RESULTS AND DISCUSSION

(a) Sperm handling, labeling and STR analysis

The most important step in accomplishing any of the goals as outlined in our research design was to successfully obtain samples for testing. This meant effective elution of samples from the mock forensic swabs. The eluted sperm cells after

withstanding rigorous chemical treatment were clearly identified morphologically when viewed under a microscope. The results shown below document the characteristic tear-drop shape of eluted tail-less sperm. The brightfield image of 4 sperms eluted from a mock swab shown below in figure 3 was recorded using a 40x air objective, NA=0.9. This also demonstrated that staining with fluorescent dyes is not needed for identifying eluted sperm cells before they are trapped. This reduces an extra sample processing step often required in other techniques. These cells were clearly distinct in morphology from epithelial cells or contaminants such as bacteria or yeast which may be found in certain forensic samples.

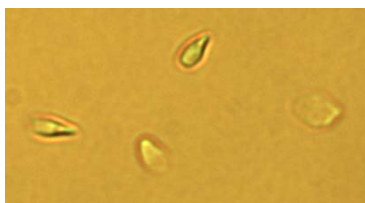


Figure 3

Next we obtained a number of different images with the different dyes (organic or antibody based) of the eluted sperm samples. We compared the efficacy of the labeling (how many of the sperm got labeled), the ease of the labeling process (how long did it take to complete the labeling reaction) and finally the compatibility of the labeled samples with trapping and downstream STR analysis. Images from such experiments are shown below starting with the most commonly used dye (Christmas tree stain). Figures 4(a) & 4(b) depict successful labeling of eluted sperm using Christmas tree stain. Christmas tree stain is a two stain method comprising of a green dye (Kernechtrot solution) and a red dye (picoindigocarmine solution). This allows sperm heads to be colored red, tails green and contaminating epithelial cells as green or blue with red nuclei.

As is expected the sperm tails were lost in the elution process and hence we only see the red stained sperm heads. It is clear that not much epithelial cells are seen in these samples since the samples tested here were from swabs with mostly sperm cells on them.

The figure 4(a) on the left is one where the sperm cells were not heated but were spray fixed. The figure 4(b) on the right is one where the samples were heated on a hot plate and spray fixed. The samples were from two different swabs. These images were acquired using a Hamamatsu Camera attached to a Nikon TE 200 fluorescent scope using a low magnification (20X) air objective.

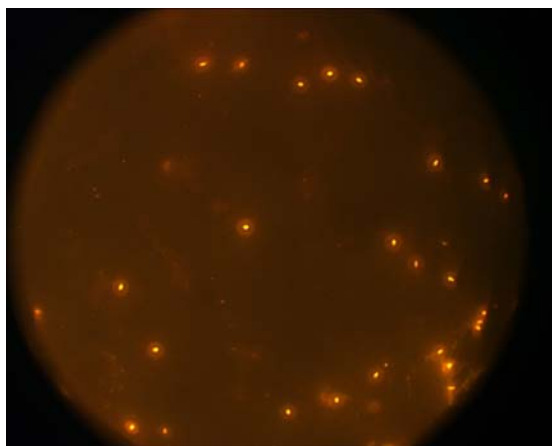


Figure 4(a)

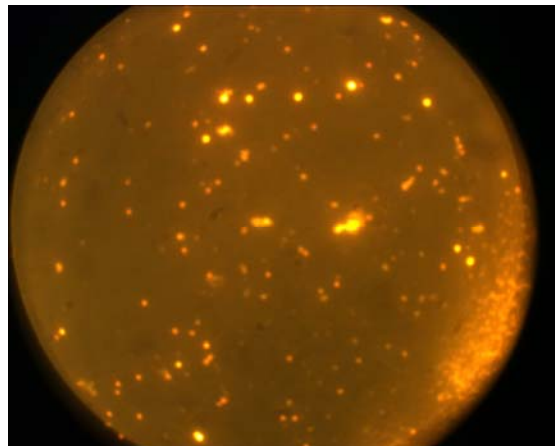


Figure 4(b)

Besides demonstrating our ability to carry out standard forensic practice of labeling sperm with Christmas tree stain, we tested other dyes for sperm staining. We are aware that the need for staining might arise when we implement automation. While automatic sperm recognition and trapping certainly works on unstained samples, but better contrast using fluorescently labeled sperm samples will improve and expedite

pattern recognition in auto-detection of sperm. In an automated sperm sorting device, machine vision will be the first step to identify a sperm before it is trapped and moved from one area (input chamber) to another (output chamber) inside a microfluidic chip. Even though such fluorescence labeling incorporates an extra step in sample handling, it could be a small price to pay when speed of sperm separation can be improved many-fold. Perhaps such labeled sperm would be extremely valuable where only a limited number of sperm is present amidst an overwhelming number of non-sperm entities in some forensic casework samples.

Other disadvantage of sperm staining includes extra cost and if organic nuclear or cell periphery dyes are used they may stain both sperm and non-sperm cells equally efficiently. Whether such fluorescent dye based sperm staining is going to add additional value to automate sperm separation remains to be seen and will be tested in the next phase of the research. Nevertheless, the following results have contributed to our understanding for adopting the best approaches for Phase II of our research. Shown in figure 5 is a side by side comparison of brightfield versus fluorescent imaging of a given sample labeled with Propidium Iodide.

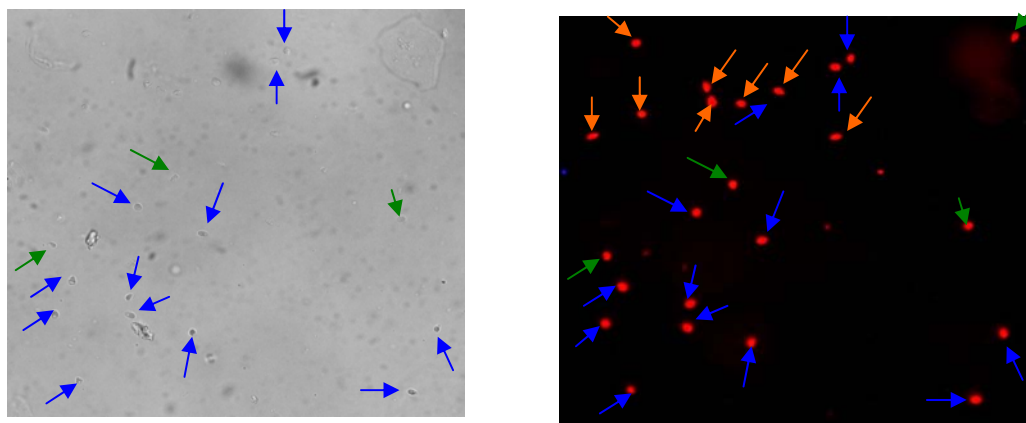


Figure 5. Blue arrow: shows the easily identifiable sperm in both images; Green arrow: shows the ones easily identified in the fluorescent image but ambiguous in the brightfield image; orange arrow: shows the ones that did not show up in the brightfield but easily identified in the fluorescent image. Note that the junk and epithelial cell did not get fluorescently labeled with PI although this is a sperm only swab.

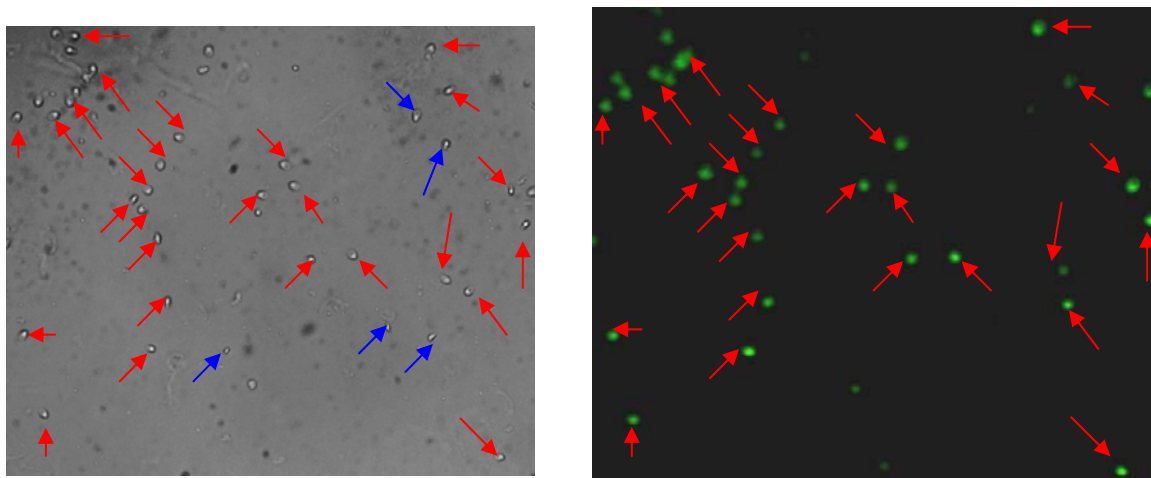


Figure 6. In this particular staining example it appears that some of the sperm that were clearly identified in the brightfield image (marked in blue) did not get stained with Sybr14 and did not appear in the fluorescent image. The other possibility is that those few sperm (indicated by blue arrow) floated away between the time the brightfield and the fluorescent images were taken since these samples are not fixed.

Figure 6 depicts staining of eluted sperm with Sybr 14. While figures 5 & 6 show labeling of samples eluted from sperm only swabs, the following are examples where the samples eluted were from a mixed (sperm and epithelial) swab. Since the organic dyes were not sperm specific, they labeled both sperm and epithelial cells but the staining

signature in the two different cell types are quite different such that machine vision can distinguish a sperm from an epithelial cell.

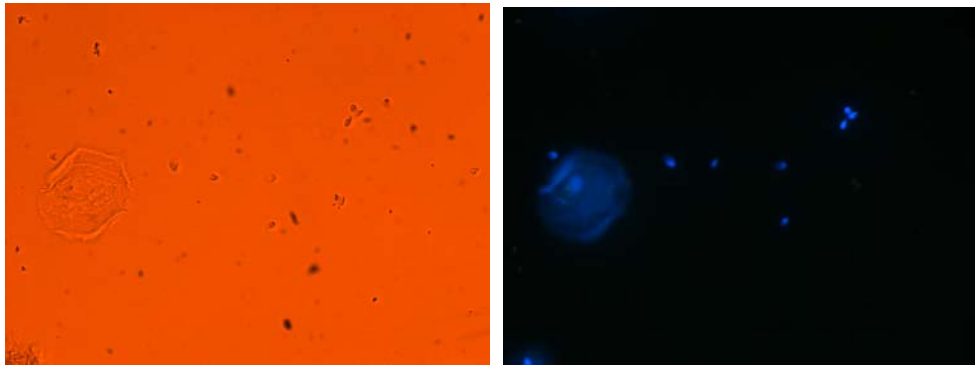


Figure 7

Figure 7 shows DAPI stained sperm and epithelial cells (eluted from a mixed swab) in brightfield and fluorescence modes once again showing high efficiency(essentially 100%) in labeling the sperm which is comparable to that obtained with PI (red stain) and Sybr 14 (green stain) (shown below in figure 8 & 9). Note that none of the debris got labeled with any of these dyes and arrows point at sperm.

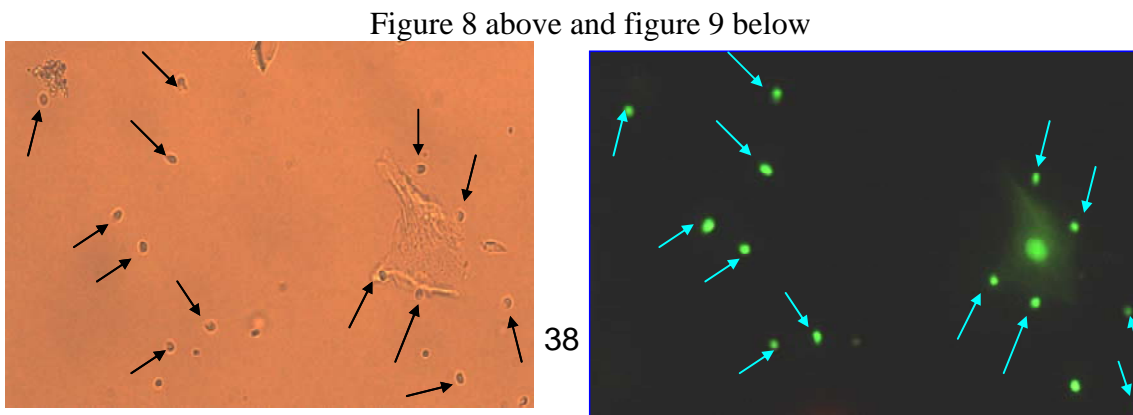
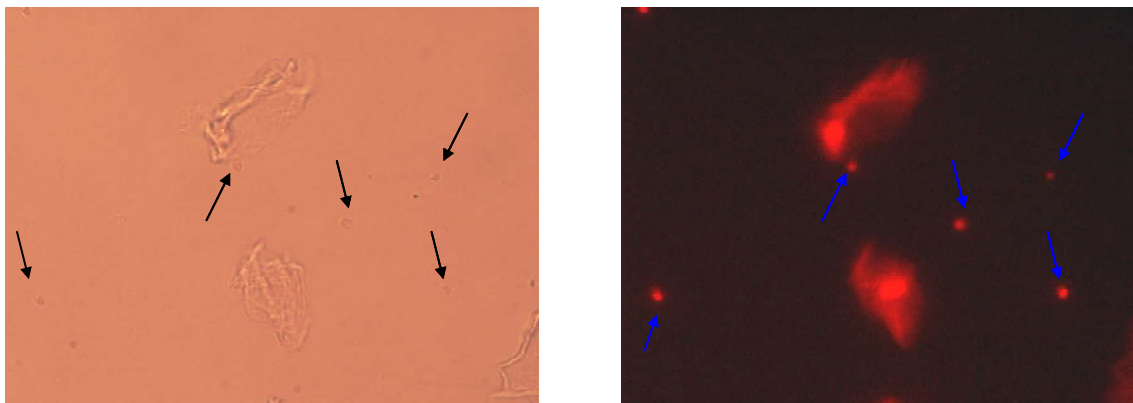


Figure 8 above and figure 9 below

In choosing these dyes for cell staining, attention was also paid to their quantum yield and photostability, and the excitation-emission wavelength of the dyes that is compatible with our IR-laser based optical trapping setup. STR analysis was subsequently performed on the stained sperm to ensure downstream compatibility with the final forensic processing step.

Besides using organic dyes such as DAPI, Sybr 14 or propidium iodide to label sperm, one can also use sperm specific staining using labeled antibodies that target sperm-only proteins (typically cell surface antigens). Antibody based labeling is more expensive and laborious than organic dye based labeling but is generally more specific.

Recently, researchers have developed a family of antibody based dyes which have been shown to be very specific to human sperms. We tested one such dye, the HyLiter dye from IFI for suitability of its use with our HOT technique. The standard labeling protocol with HyLiter required cells to be fixed and we were able to reproduce good labeling. Imaging of sperm with HyLiter on fixed samples is shown in figure 10. Surprisingly it was noticed that HyLiter does occasionally label epithelial cells as shown in the inset figure below since specificity of antibodies is never absolute. This confirms

some of the impending issues with Hyliter as faced by other forensic laboratories (personal communication).

However, in order to use Hyliter with our trapping experiments we required that the cells be not fixed. We modified the protocol significantly in order to label sperm without fixing. In doing so, the efficiency of labeling dropped significantly (only a small fraction of the sperm got labeled) and Hyliter was soon abandoned as a choice of dye for labeling sperm to be useful with our trapping experiments.

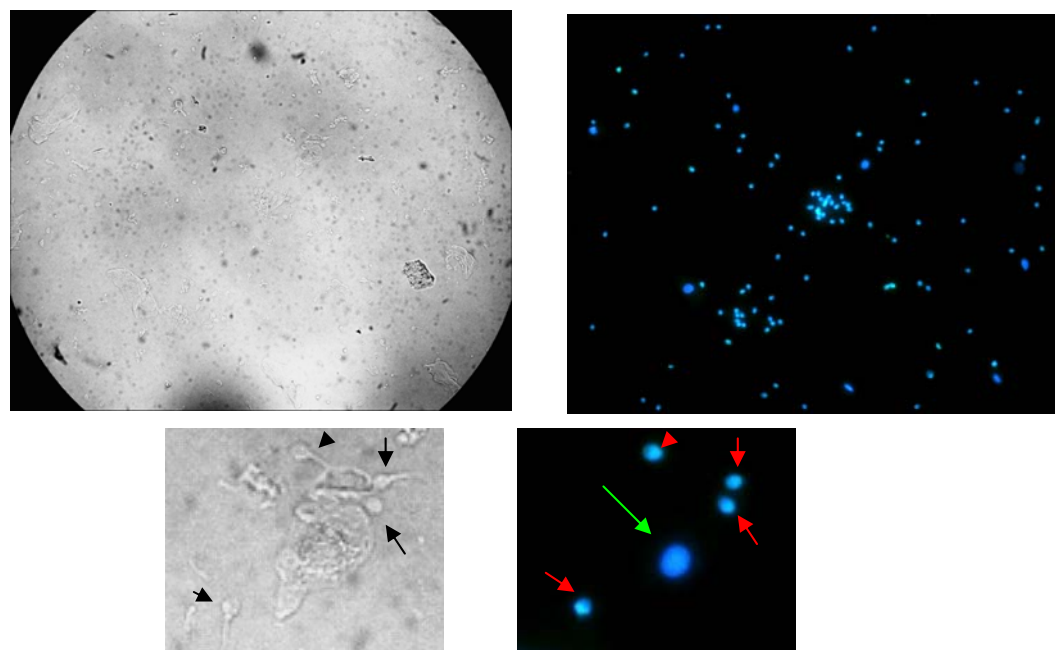


Figure 10

Table I below summarizes the results from our experiments with sperm staining and based on the ease of labeling, photostability, compatibility with HOT and STR analysis (discussed next), propidium iodide(PI) seems to be the most attractive candidate. DAPI and Sybr 14 are also equally attractive alternatives but we chose to focus our

experimental efforts on PI labeled sperm wherever fluorescent staining was used in our study since it was the most photostable dye.

Name of dye	Pros	Cons	Comments
PI (propidium Iodide) (from Invitrogen)	<ul style="list-style-type: none"> • Easy and quick labeling (10-15min) • Photostable • Good fluorescent signal 	<ul style="list-style-type: none"> • Toxic (carcinogenic) 	Compatible with STR analysis in our hands.
Sybr 14 (from Invitrogen; part of live-dead sperm assay)	<ul style="list-style-type: none"> • Easy and quick labeling(10-15min) • Good fluorescent signal 	<ul style="list-style-type: none"> • Likely moderately toxic to user (carcinogen) • Photobleaches quite readily 	Compatible with STR analysis in our hands.
Alexa546-NHS ester	<ul style="list-style-type: none"> • Less toxic to user than PI and Sybr 14 • Less expensive than Hyliter dye 	<ul style="list-style-type: none"> • Long labeling time(~2hrs) • Fluorescent signal not strong 	Long labeling time & weak fluorescent signal makes it unattractive for use.
Hyliter (from IFI)	<ul style="list-style-type: none"> • Good fluorescent signal • Fairly photostable • Sperm specific labeling since it is antibody based 	<ul style="list-style-type: none"> • Expensive • Long labeling time 	Antibody based labeling unlike the other dyes. Labeling works best on fixed samples. Hence unsuitable for our needs with trapping.
DAPI (from Invitrogen)	<ul style="list-style-type: none"> • Easy and quick labeling(10-15min) • Good fluorescent signal 	<ul style="list-style-type: none"> • Likely moderately toxic to user (carcinogen) • Photostable 	Compatible with STR analysis in our hands.

Table I

Once the elution and cell staining procedures were optimized, we tested the compatibility of the dyes with STR analysis. In order to do so, a positive control sample of unlabeled sperm was always used. The STR results from fluorescent tagged sperm

were thus directly compared against unstained sperm eluted from the same mock forensic samples. The dyes tested to date are primarily nuclear dyes such as Hoechst, DAPI, syto-red, propidium iodide, sybr 14 etc as well as the commonly used Christmas tree stain and our results have indicated that none of these dyes interfered with STR since we were able to obtain full profile from all these different fluorescent tagged sperm. A typical STR profile obtained from a sample of stained sperm is shown below (figure 11) (right) and compared to that of an unlabeled sample (positive control) (left).

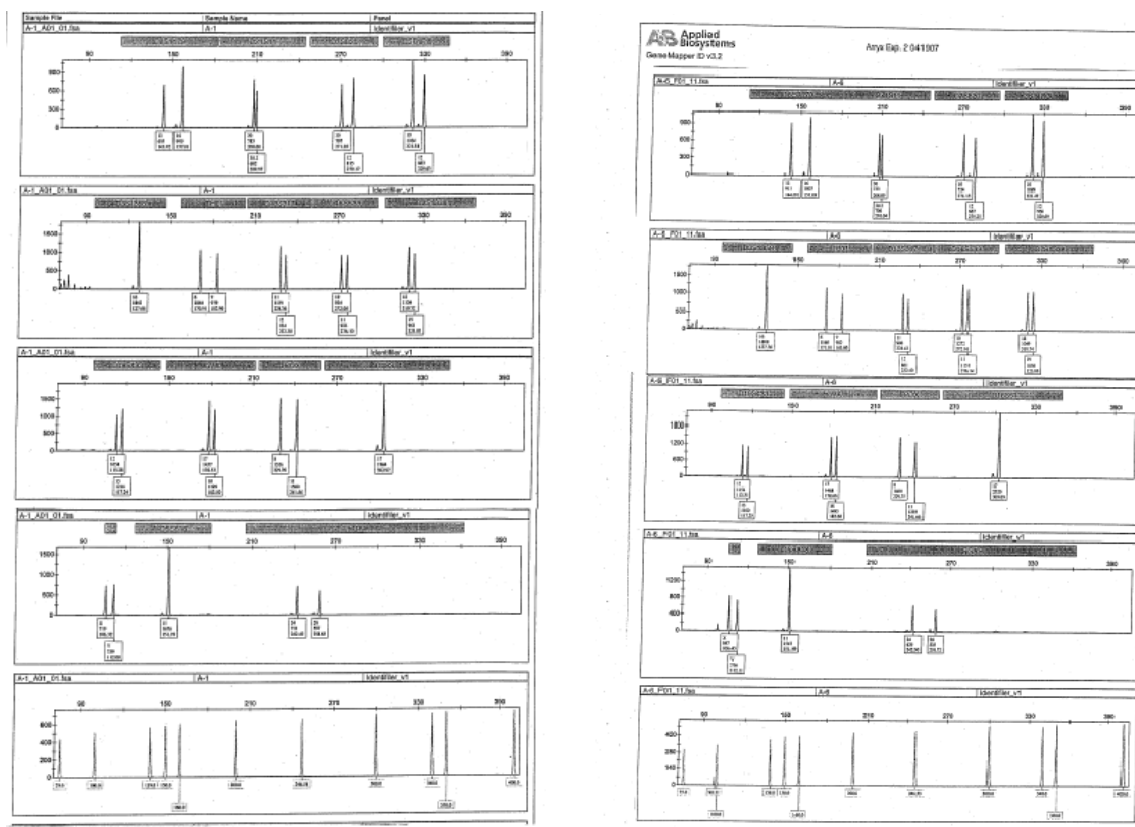


Figure 11

Assessing risk factors associated with using this technology in forensic DNA analysis and confirming that HOT based manipulation of samples does not impair subsequent genetic analysis

Having demonstrated the compatibility of STR analysis with the use of certain organic dyes (used to label the sperm), we next performed STR analysis on these sperm in the presence and absence of traps. Specifically we carried STR analysis on the following class of samples

- (i) Unlabeled and untrapped sperm (always acted as the positive control)
- (ii) Labeled but not trapped sperm
- (iii) Unlabeled but trapped sperm
- (iv) Both labeled and trapped sperm

Our results indicate that we can use PI, Syto-Red, Hoechst, DAPI with HOT based trapping and obtain complete STR profiles. However, due to limitation in the availability of sufficient number of mock forensic swabs and the short duration of the project prevented us from repeating these tests extensively. We will continue to repeat some of STR tests on labeled sperm to obtain statistically significant data in phase II of the research. Dyes which are non-toxic, highly photostable (do not bleach) and have high quantum yields (so that the signal is high and therefore offer high contrast between labeled entities and background) and have fluorescence in the visible were most desirable and considered suitable for machine vision based auto-detection of sperm. Since these labeled sperm would be used for holographic optical trapping using a 1064nm IR laser,

careful attention was paid to the choice of dyes and dichroics available so that the trapping wavelengths are separated from the excitation/emission wavelengths for the dyes used for fluorescence. Figure 12 shows a complete STR profile obtained using trapped but unlabeled sperm.

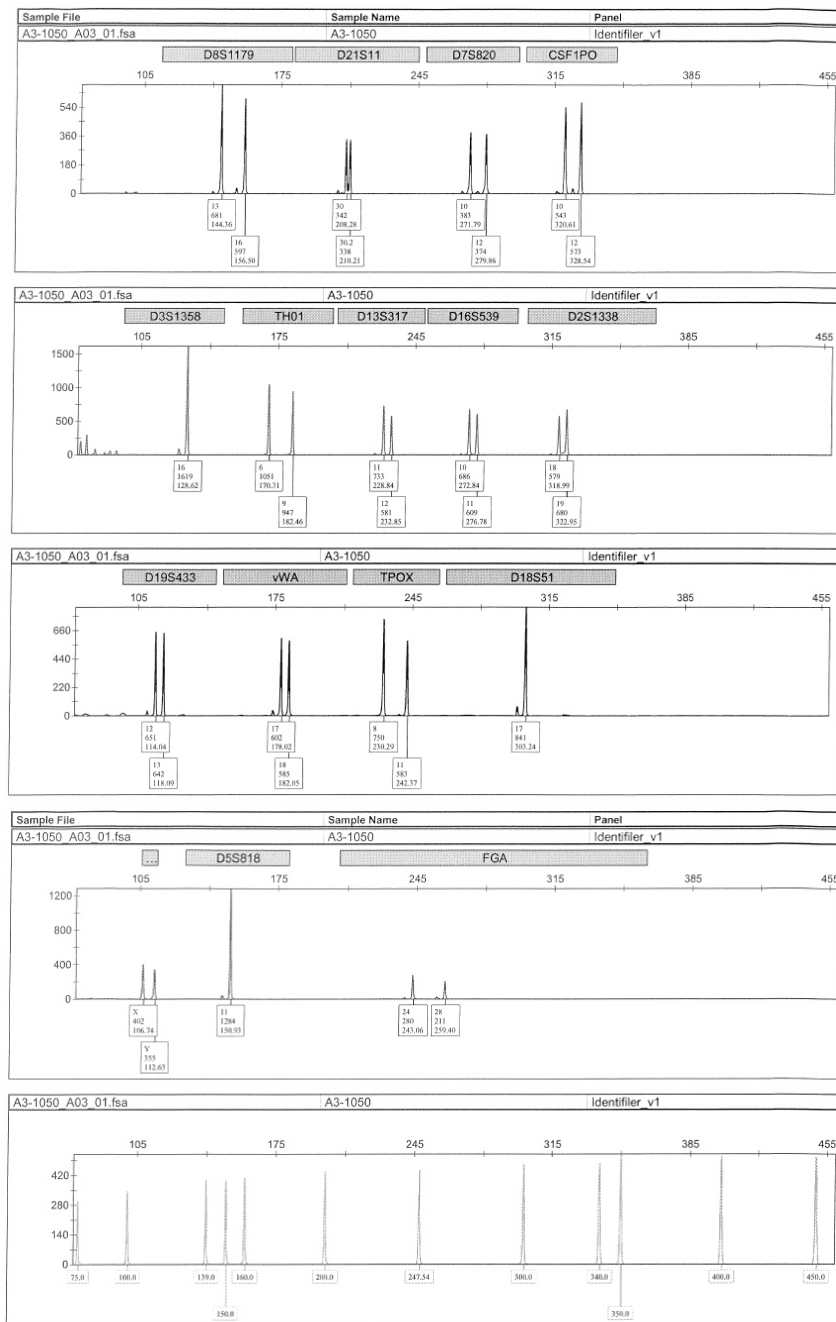


Figure 12

Figure 12 shows an electropherogram of ~400 optically trapped and manipulated sperm using Applied Biosystems' Identifiler STR kit. The electropherogram was found to match that of a control sample which was not optically trapped. (Sample and STR analysis courtesy of Orchid)

Optimize trapping parameters such as laser power, time of trapping, optimum trap speed for movement etc for best throughput and STR results

At the optical trapping stage, we tested the efficacy of trapping and sought answers to the questions: (1) how many sperms could we trap and move at a given power, (2) what was the least and maximum trapping power appropriate for trapping and moving multiple sperms over a given distance which depended on the microfluidic chip design (3) How long did it take to trap a given number (say 400 sperm) at a given power per trap? (4) Which microscope objective is best suited for trapping purposes?

The following figures 13 & 14 show optical trapping of multiple sperm both live sperm from semen and also on sperm eluted from sample swabs (Figure 15).

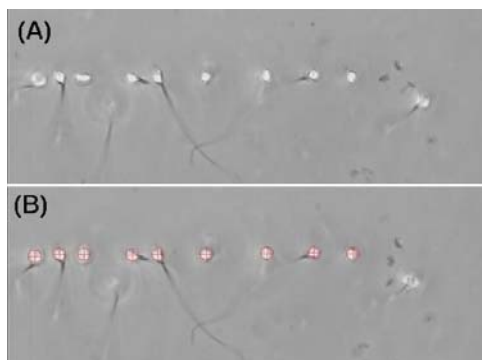


Figure 13

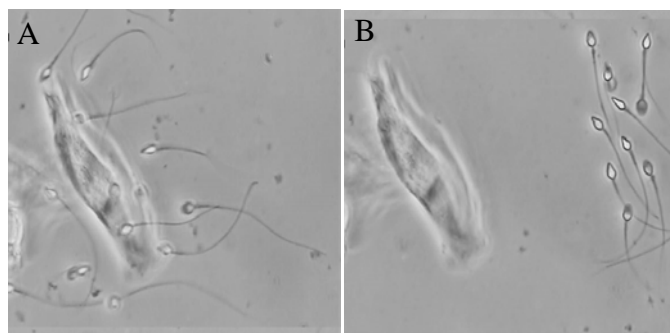


Figure 14

Figures 13 and 14 show the trapping of sperm from semen and the separation of sperm from epithelial cells (buccal) using HOT

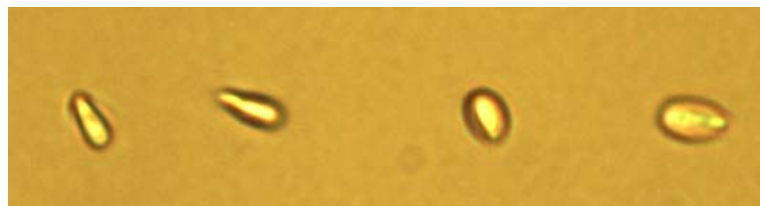


Figure 15

Figure 15 here shows trapping of sperms eluted from mock forensics swabs. Hence unlike the trapped sperm in figures 13 and 14, the sperms in figure 15 are tail-less.

Specifically we tested trapping of eluted sperm (not stained) at different laser powers and performed STR analysis. One class which we call low power trapping used 60mW-200mW/trap and other which we call high power trapping used 250mW-400mW/trap. In both cases ~400-500sperms were trapped (as was required by Orchid) and we were able to obtain full STR profile. These tests were subsequently repeated without change in our conclusion.

So the question that remained to be answered was the range of trapping power required for moving sperms efficiently from one destination to another such as from the sorting region to the completely sorted region and how many of such sperms can be trapped and moved at a given time. While short range movement over 1-2 FOV span(200-400 μ m) has been successfully accomplished using >10 sperms at a given time, for long range movement(over 1-2mm) fewer sperms (no more than 8, typically 4) have been used. The speed of the movement of a trapped object is a function of the power used per trap, the refractive index of the object, the shape (circular objects are easier to trap than non-circular or elongated objects) and orientation of the object (parallel or perpendicular to the trapping plane) and also the wavelength of the laser used for trapping such as the object to be trapped does not absorb too much heat at that wavelength. For biological applications, IR lasers have found reliable use and we used a 1064nm IR fiber laser. Typically, laser power of no more than 250mW/trap was used to trap sperm in order to move them in a group of 4-6 sperm over a distance of 4-5 FOV. Such trapped sperm were subsequently tested for STR analysis and showed no anomalies in their STR profile output. Figures 16(a) & 16(b) show the progressive trapping, movement and final

collection of the separated sperm. The red and green circles show the position of the active traps. While most of these tests have been carried out using passive two chamber chips (connected via a channel) sample chambers and not the ultimate chip design we envision using with an automated system (currently beyond the scope of our grant), some of these tests would therefore need to be repeated in phase II of the project and optimized to get the right range of parameters for trapping power, number of traps to be used and the distances that one could move a certain number of trapped entities inside such a microfluidic chip using an automated sperm sorting device (the multi-year long term goal).

The results from our speed tests on movement of trapped objects showed that the maximum velocity of moving 4 sperm using no more than 250mW/trap was between 65-100 μm /sec and depended on the substrate for trapping (glass versus COC). The speed is reduced when using COC bottom chips when compared to glass. However COC is significantly cheaper than glass and therefore is a preferred material choice when fabricating a large number of disposable microfluidic chips.

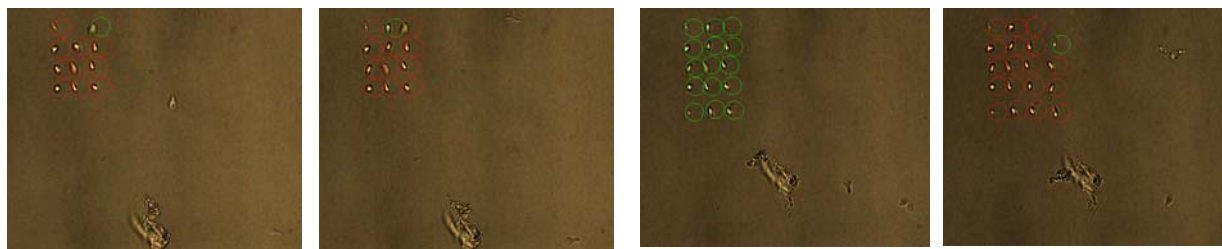


Figure 16(a)

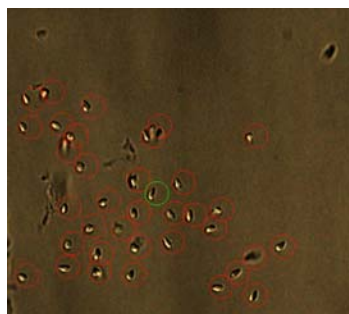


Fig 16(b) shows the collection of a number of trapped sperm from mock forensic swab brought to a desired location on the sample chip

Figure 16(a) above shows the progress in the number of sperm trapped going from 11 sperms to 18 sperms which are then moved.

Post-trapping, the trapped sperm were recovered by washing the wells of the sample chamber a couple times with PBS based Mo-lite buffer (provided by Orchid). The percentage recovery of sperms from the wells was >99%. While figure 16(a) demonstrates progressive trapping and collection of trapped sperm using unlabeled sperm in brightfield, the following results (figure 17) were obtained using PI labeled sperm.

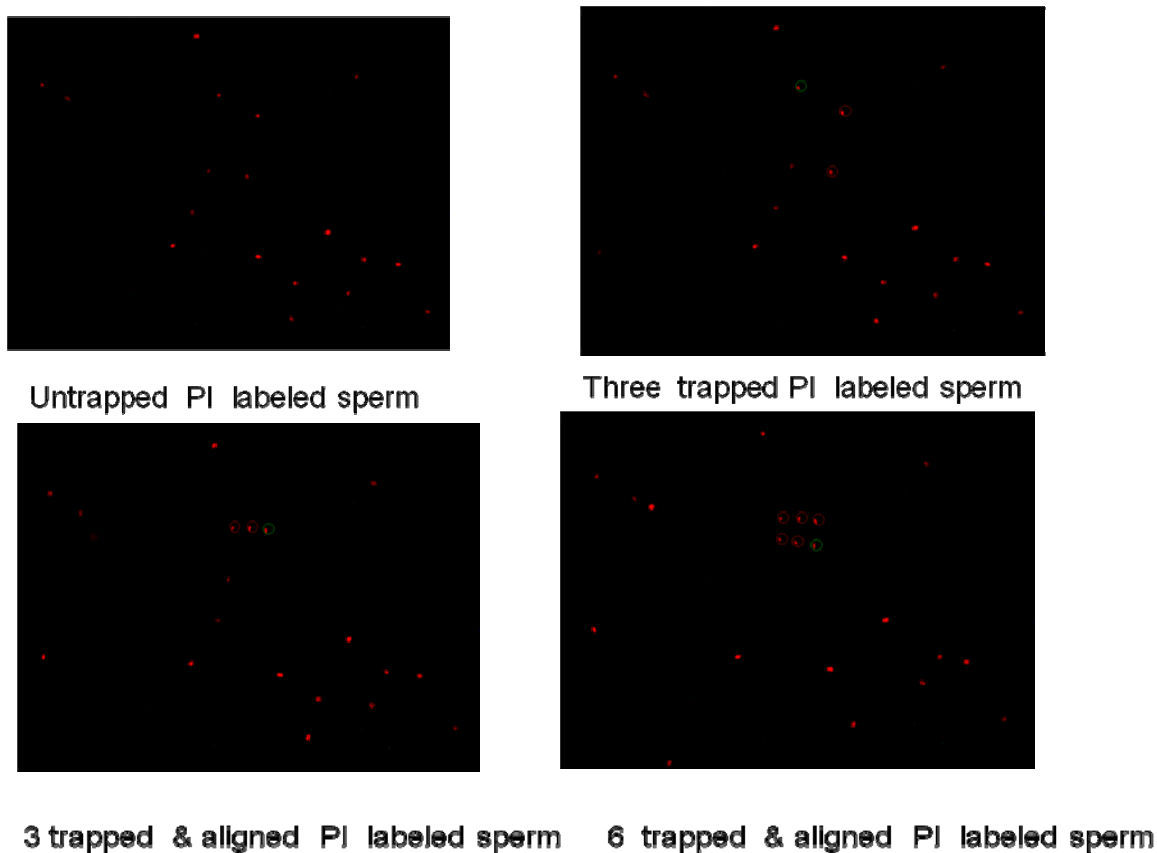


Figure 17

Figure 18 shows the trapping of labeled sperm in both brightfield and fluorescent mode.

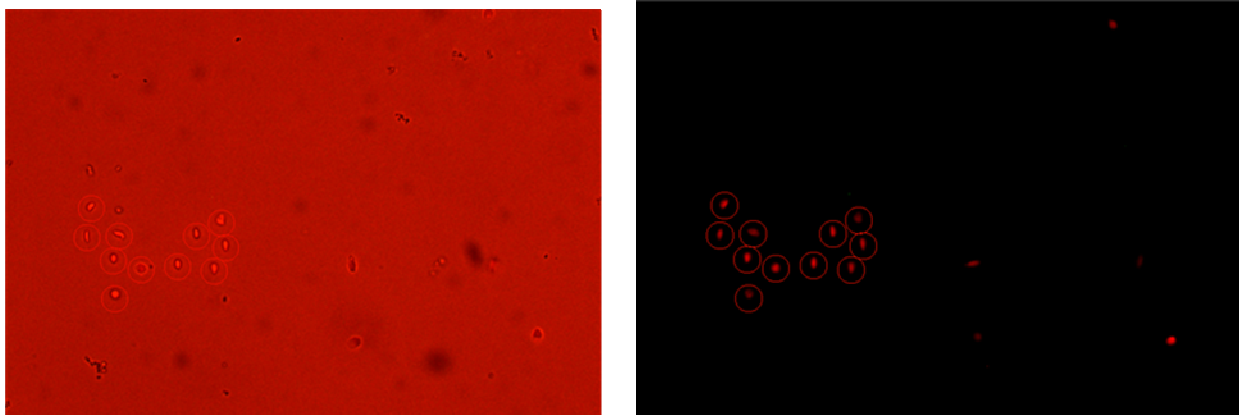


Figure 18

Whether we choose to adopt fluorescent staining of sperm as a standard procedure for automation remains to be seen in phase II of the work. However we have demonstrated that HOT works equally well on both fluorescent tagged as well as unlabeled sperm.

Develop microfluidic devices for the introduction of forensic sample and the isolation and subsequent withdrawal of each cell species using small volume samples

Microfluidics is becoming increasingly popular for various biotechnology applications. Its popularity grew out of the fact that miniaturization of sample chamber and volume, to fit on a chip (often disposable) can speed up reactions, cut down on the amount used for precious materials, reduce reagent cost significantly and hence the overall cost. It offers additional advantages of portability (by creating disposables) and ensures sterile conditions by use of sealed chambers.

In the field of forensics with particular focus on STR based criminal identification obtained from sexual assault evidence samples, the use of microfluidics (whether a modular design or all-on-one-chip) is attractive for several reasons. It offers scope for cell separation (such as the separation of sperm from other unwanted cells and debris) through active pumping and creating a disposable which is compatible with Holographic Optical Trapping (HOT) and amenable to complete automation. Reactions or experiments that can be carried out in such sealed disposables can prevent cross-contamination (eliminating multiple pipetting steps). In cases, where limited sample is available for analysis, the use of microfluidics reduces waste of precious materials (reduced volume handling).

In the following section we present (1) a basic design of an active microfluidic chip intended for sexual assault evidence sample handling (2) fabrication considerations that were taken into account and improvements made in the fabrication process and the (3) overall performance of the chips using a number of test samples.

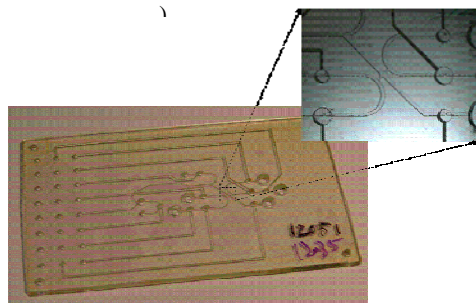
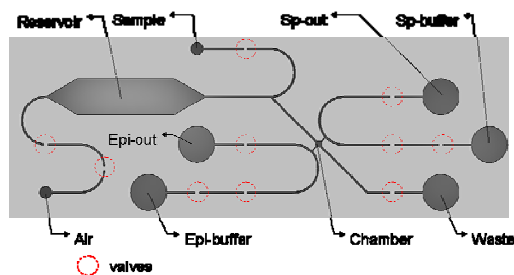
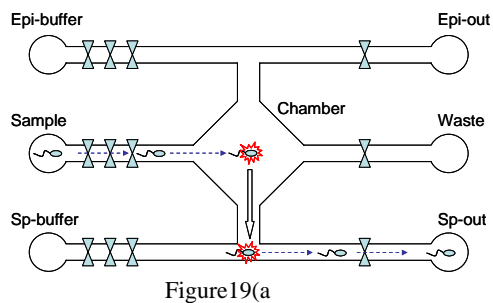


Figure 19(c)

Figure 19(b)

The disposable plastic cartridges developed for isolation of forensic cells by type. Buffer and forensic sample are introduced at the left (Fig 19(a)). Optical traps (red) move selected cells to the upper (epithelial) or lower (sperm) channel where they are pumped to the right and extracted. The disposable cartridge (design, Fig 19(c)) is fabricated at Arryx R&D facilities.

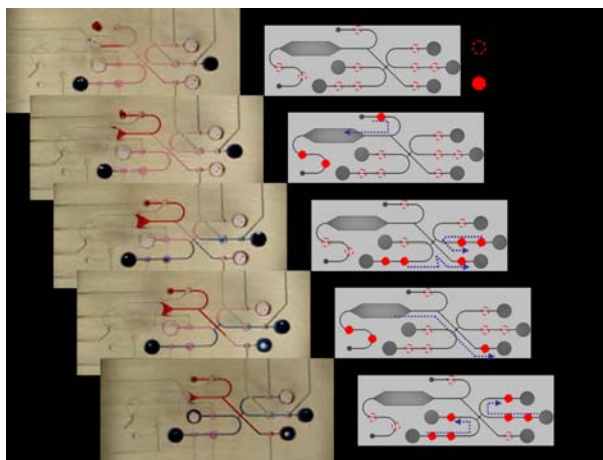


Figure 20

Fig 20 depicts the flow pattern of the fluids and sequence of operation when introduced on such a sample chip using different colored food dye solutions. This figure also shows that the pattern followed by the food dyes is what is expected of the chip as shown in the cartoons on the right.

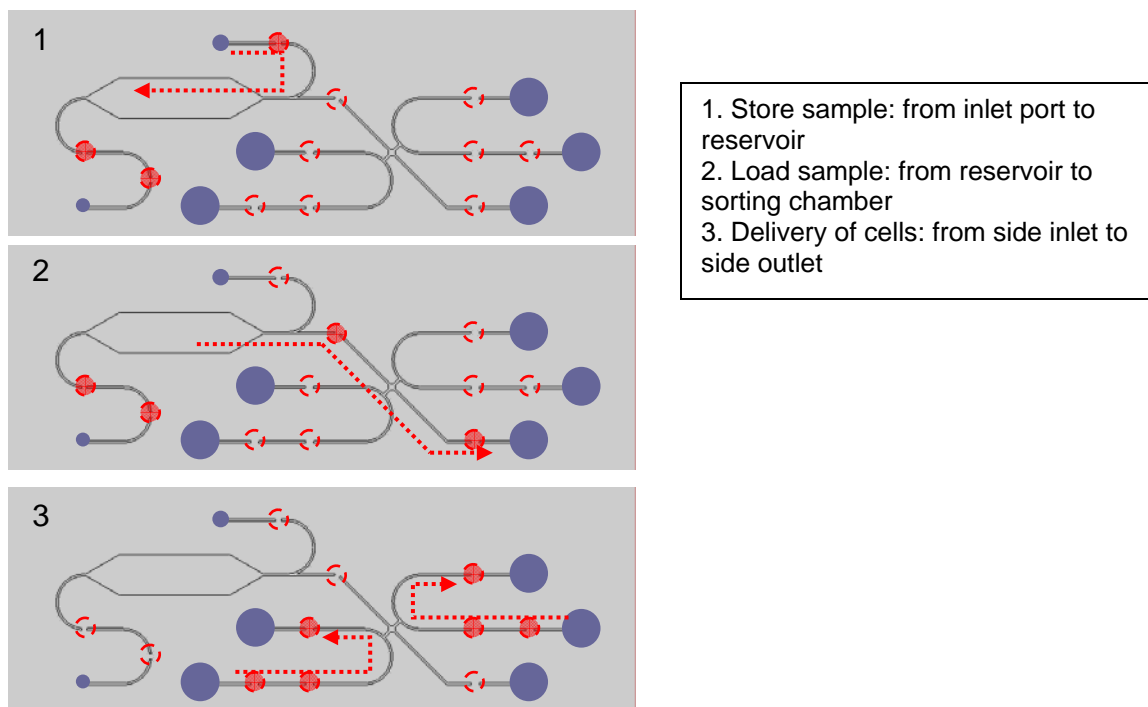


Figure 21

Figures 19 through figure 21 describe in detail the layout of the microfluidic device and its mode of intended operation. The design of the microfluidic chip was intended to meet the following criteria.

- (1) While sample is being driven from inlet port into reservoir no sample should leak into the sorting chamber.
- (2) For sample driven from reservoir into the sorting chamber, sample should not leak into side channels
- (3) Cells in side channels (if any) should not get back into sorting chamber. HOT based sperm sorting was carried out in a small volume sorting chamber.
- (4) Post sorting, liquid driven from side inlet port to side outlet port for carrying target cells downstream, no sample should leak into side channels and

(5) Any cells that remain in the side channels must not get back into the sorting chamber.

Even though the bulk of our proof-of-concept trapping and sorting studies were carried out on passive microfluidic chips (2 chambers connected via a short narrow channel), we tested a few active chips with trapping during the short span of our project. However, we ran into problems related to criteria (2) &(3) which we believe is due to malfunctioning of the pneumatic valves on the active chip. Hence phase II will address refinement and further testing of these active microfluidic chips for forensic sorting and analysis using HOT.

On the other hand, we achieved the following improvements in our chip fabrication during the course of this project

- (1) bending/warping of the chip was minimized
- (2) better channel geometry containing sharper edges were obtained (see figure 22 below)
- (3) higher yield of functional chips (as opposed to chips with defects) were obtained and
- (4) time required to fabricate each chip was significantly reduced.

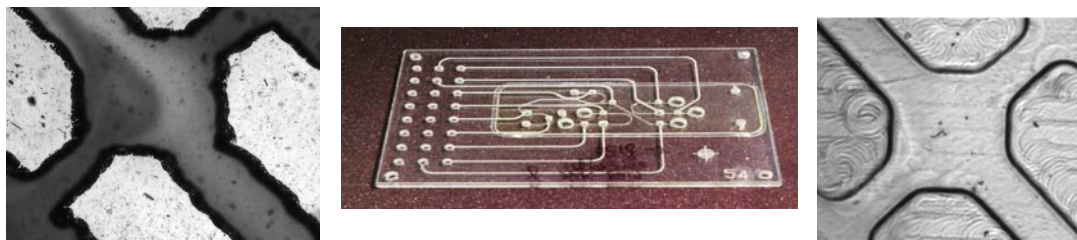


Figure 22

Figure 22 show the improvements in channel geometry: one on the far left used the older version of fabrication and that on the far right used the current improved version.

In brief, the optimized fabrication process aimed to make the fabrication process faster, easier and to yield better results. Instead of using thermal bonding, adhesive film was used to enclose the pneumatic layers. To eliminate the bending of the chip from laser welding process, 2 mm thick Acrylic was used to cut pneumatic layer since it is more sturdy and finally to achieve uniform bonding during the laser welding process, a foam was placed underneath the chip so that uniform pressure during the bonding process is maintained.

While our preliminary data so far indicate decent sperm separation using HOT on a microfluidic chip, further testing is required. Under the manual mode of trapping, movement and sorting, the time taken for sperm separation (at least 400 to obtain complete STR profile) is quite long (3-5hours). However, we strongly believe that this time will be drastically reduced when automation is incorporated. Our results show that HOT based sperm trapping can work within microfluidic chambers without interfering with STR analysis even though the fabrication of such devices needs modification and improvement. The compatibility of optical trapping with microfluidics offers benefits such as minimal reagent use, elimination of sources of cross contamination, minimize pipetting steps thereby avoiding human error associated problems.

IV. CONCLUSIONS

Our results have confirmed the compatibility of HOT for sperm separation for forensic analysis. The results from the different areas of development such as sperm

elution, staining, STR analysis and microfluidics have provided us with a strong foundation to carry out the goal of building an automated sperm sorting device. We have specifically obtained answers to our questions outlined in the research design. While some areas especially the development of microfluidic devices need further improvement to efficiently work with HOT, nevertheless we have obtained data on substrates and materials for such devices that are compatible with HOT and STR analysis. COC is one such material and will allow us to keep cost down when disposable cartridges are mass produced from them.

While a proper assessment on the speed of HOT based sperm separation cannot be made at this time since most of the steps were manually operated, our results are better or at least comparable (4 hours or less) to the time taken by other alternative approaches studied for sperm separation such as DEP and AED etc. HOT based sperm separation is also less lossy compared to the other alternatives. Conclusions drawn from our research and study certainly facilitated in refining our gameplan and workflow in order to achieve the bigger goal of building a prototype device for automated separation of sperm from forensic sample based on the powerful technology of holographic optical trapping.

IMPLICATIONS FOR POLICY AND PRACTICE

In its current form, HOT may be used for sperm separation but it is far less attractive due to the involvement of labor and manual intervention during sperm identification and trapping. However, we strongly believe that as we develop the automation for the various sub-processes such as sperm recognition, sperm trapping and movement, we can cut

down on the separation time to under 1 hour. Under those circumstances we would strongly urge forensic laboratories to try HOT based sperm separation. We hope to partner with different forensic laboratories for such field trials.

IMPLICATIONS FOR FURTHER RESEARCH

Automation and Future direction

Our results clearly indicate a pathway towards building a prototype device for automated sperm detection and sorting. We have shown that Arrayx has all the needed abilities for conducting forensic research. More importantly, we have established HOT as an useful and attractive technique for separating sperm from non-sperm entities in a forensic sample. The use of HOT is compatible with downstream PCR and STR analysis. While work on the automation front is in its infancy and is intended for the next phase of our forensics work, we have initiated steps towards automating various components.

The following diagram (Figure 23) illustrates the various components that would be integral in developing an automated sperm separation system based on HOT.

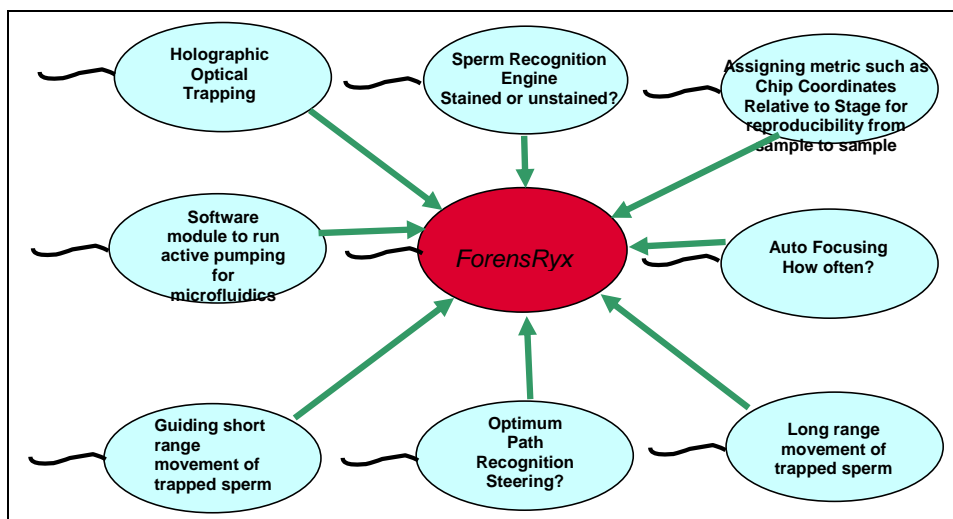


Figure 23

Our proposed software package for the prototype must be capable of handling the hardware for

- (1) Camera Control
- (2) Stage movement so that guided movement is achievable
- (3) Motion feedback (stage with x,y & z-encoders)
- (4) Dynamic auto-focusing
- (5) Trapping capability (SLM control)
- (6) Driving the microfluidic device with the different pump manifolds.

In developing image processing routines for autodetection of sperm, we can use either brightfield images of sperm or fluorescently stained sperm. Various images of sperm with different contrasts, different number densities in a given field of view (crowded versus sparse samples) and images where some sperms are in focus while others are not, were tested with the pattern recognition routines currently under development.

To develop machine vision for auto-detection of sperm we took into account the following scenarios which are likely to be augmented if the sperms are not stained. Such detection will have some error margins. The primary sources of errors in the auto-detection process included the following.

- 1) It is possible to miss some sperms (false-negative error)
- 2) It is possible to accept non-sperm objects as a sperm (false positive)

It is the false positive errors that must be completely avoided for complete separation of sperm from epithelial cells from sexual assault evidence samples.

Since time is a critical factor in the sexual assault sample handling, we need to keep in mind the time needed to collect 400 sperms. Our target is to collect 400 sperms in less than an hour, preferably 30 minutes. For machine vision to function optimally we are also working towards implementing Dynamic Auto-focusing such that the best focus is retained at all time throughout the course of the trapping experiment.

Our objective in developing the prototype instrument should be capable of (1) detecting a sufficient number of sperms on a sample chip (2) Move the detected sperms to a predefined location and finally (3) accomplish the above in a reasonable time using optical trapping.

Progress on the automation front therefore includes automation of stage movement. Our motorized stage (from Prior) now has x, y, and z- encoders to automate stage movement for automated sperm trapping. Basic routines for communication of the

microscope stage with software, specifically LabView are in place. Limited automation is also in place to drive the active microfluidic chip, finding the region on the device where trapping and sorting will take place.

In order to incorporate fluorescence imaging with trapping for the automation work we probed the capabilities of various commercial cameras available that can detect low light signals. Such cameras would be used if we decide to use fluorescent images for pattern recognition of sperm. This is currently reserved for cases where the number of sperm is very limited and the majority of the samples comprise of non-sperm objects such as epithelial cells.

Our progress to date has given us insights into optimizing and streamlining our workflow as well as created the needed foundation for incorporating automation with our existing research. All these efforts combined is highly targeted in order to achieve the bigger goal of building a prototype device for automated separation of sperm from forensic sample based on the powerful technology of holographic optical trapping.

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VI. DISSEMINATION OF RESEARCH FINDINGS

We propose to publish the results of all laboratory work in peer-reviewed journals. We are currently preparing a manuscript for publication summarizing our results and findings from the current study. Additionally, we plan to present the results of our studies at national and regional meetings relevant to Forensics such as those organized by AAFS (American Academy of Forensic Sciences) or the Symposium on Human Identification sponsored by Promega. We have presented a poster on our work at the annual NIJ conference in June 2009. Additionally, our work was also presented by the principal

investigator at the 2nd Annual Green Mountain DNA conference in July, 2009. We have also presented work-in-progress at internal meetings and discussed our work with various forensic scientists at various points of time. We maintain close communication with our collaborators at Orchid and will continue to do so. We have also interacted with local forensic practitioners at the DuPage County Sheriff's office and will expand and build on similar interactions with other forensic labs. Our presentation at both the NIJ annual meeting and at the Green mountain DNA conference was received with great enthusiasm and garnered a lot of interest in our HOT technology and its unique application in forensic research and practice. Since then we have discussed our research and findings with out of state forensic laboratories and medical examiners offices as well that handle sexual assault cases. These include our communication with Dr. Eric Buel at Vermont forensic lab (Department of Public Safety) and with Dr Roger Kahn of the Harris County Medical Examiner's office.

Additionally, we plan to present our work at scientific conferences not specific to forensic science but focused on other aspects of the proposal, such as the optical trapping and microfluidic components. Such meetings will bring wider exposure to our work and may generate broader interest in HOT and forensic science. It will also allow for evaluation of the underlying science by experts from other disciplines.

We anticipate that the ability to rapidly separate sperm from sexual assault evidence samples using HOT will have a positive effect on reducing the current backlog of sexual assault cases and will lead to more successful prosecutions and convictions. We

will continue to seek participation by practitioners such as local, state or federal law enforcement agencies where needed. Arrayx will also support the installation of a HOT-based instrument at Orchid for testing. Arrayx will provide training, support and protocols that can be directly integrated into existing procedures, methods and SOPs, greatly simplifying the day-to-day use of this technology. Arrayx's past experience with local companies with business focus in forensic analysis will also benefit the dissemination of knowledge about this exciting technology.

Any new inventions conceived during the course of the proposed work will be filed in patent applications in accordance with existing guidelines.