Development of an Automated Holographic Optical Trapping Method for Sexual Assault Evidence Kit Analysis

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Abstract

This report describes a technology development effort directed towards creating an automated system for isolating human sperm from sexual assault evidence swabs. After elution, the evidence derived from these swabs usually contains epithelial cells from the victim, as well as sperm from the assailant, and current differential extraction methods often fail to fully isolate the DNA of the victim from that of the assailant. As a result, during the amplification process using PCR, DNA from both the victim and assailant is amplified and STR analysis yields a mixed profile rather than a unique profile to match the assailant. This DNA carryover can produce STR results which are more difficult to interpret, in that the interpretation requires complex mixture-analysis calculations. The availability of a practical technique for precise fractionation of cells by type prior to PCR would eliminate DNA carryover in most cases.

The system under development combines microfluidic and machine-vision technologies with holographic optical trapping (HOT) for separating sperm from epithelial cells and other contaminants to address the DNA carryover problem. HOT is an extension of the well-established scientific technique of optical trapping, which has been widely applied in cell biology. Using HOT, one can simultaneously trap many objects in arbitrary positions and steer each trapped object in three dimensions to user-defined locations for isolation. The addition of a microfluidic device for fluid control, and computer vision for sperm identification, offer the potential to automate a large portion of the process of isolating sperm for analysis, while simultaneously reducing the sample volume consumed in the process.

We have achieved three key milestones in producing such a system. The first is the design, fabrication, and testing of a disposable microfluidic device, with active fluid control, which is compatible with HOT and with handling sperm-containing fluid eluted from mock evidence swabs. The second achievement is developing a strategy for automated computer-based identification of sperm inside these devices. This strategy employs both the use of an STR-compatible fluorescent dye and image analysis software for identifying dyed sperm. Finally, we developed a number of software routines for hardware automation, and a framework for combining routines to create complex processes relevant to automated HOT-based isolation of sperm, based on the identification provided by the image analysis software. This work lays the foundation for building a fully automated HOT-based cell separation device for processing sexual assault evidence which can benefit forensic labs and help reduce the backlog in handling such forensic evidence.

Table of Contents

Executive Summary

Development of an Automated Holographic Optical Trapping Method for Sexual Assault Evidence Kit Analysis

Background: Statement of Problem

The backlog of DNA evidence is a growing issue in American crime labs. At the end of 2009, there were over 110,000 unanalyzed cases, and trends suggest that the problem has only become worse since then. Sexual assault cases account for a significant fraction of the DNA evidence submitted for forensic analysis, and most of this evidence is in the form of semen samples collected on cotton swabs. These forensic evidence swabs typically contain both sperm from a male assailant and epithelial cells from the victim. Separation of the assailant's DNA from the victim's, prior to STR analysis, is an important step in obtaining a clear STR profile of the assailant. Currently, differential extraction is the standard technique used for this purpose. Differential extraction exploits chemical differences between sperm and epithelial cell components, by first lysing the epithelial cells, washing the unlysed sperm nuclei, and finally extracting the sperm DNA for analysis. However, it is not uncommon for this separation process to be incomplete, so that there is carryover of victim DNA into the assailant fraction. This, in turn, results in a mixed STR profile, which may not be interpretable for the purpose of assailant identification.

To address both the evidence analysis backlog and the DNA carryover issue, forensic DNA analysis for sexual assault evidence would greatly benefit from improvements in the following three areas: cell separation techniques, incorporation of microfluidics for higher throughput and reduced reagent cost, and automation to reduce manual handling, minimize costs, and increase throughput.

Background: Purpose of Research

The work presented here builds upon previous work, which was funded under NIJ award 2008-DN-BX-K123. That work established the compatibility of optical trapping and fluorescent staining of sperm with STR analysis, and laid the groundwork for the development of an integrated device for using holographic optical trapping (HOT) for automated isolation of sperm cells from eluted sexual assault evidence swabs. The research covered under the current grant (NIJ award 2009-DN-BX-K260) revolved around developing key components of such a device. In particular, the goals of the research described in this report were:

1. Developing a disposable cartridge for introduction of the mixed cell solution, processing, and withdrawal of the solution containing the sorted sperm fraction
2. Investigating the ability of our system to optically trap sperm through polymers rather than glass, with a view towards reducing the cost and complexity of the microfluidic cartridge

3. Implementing fluorescent staining strategies to simplify machine vision and automation
4. Developing machine vision based automatic sperm recognition, and assessing the efficiency, speed and reliability of automated sperm recognition using brightfield and fluorescent imaging
5. Developing, testing and integrating key software modules important for building an automated prototype device for HOT based sperm separation.

Methods: Holographic Optical Trapping

Optical trapping is a technique for manipulating microscopic particles, such as cells, using strongly-focused laser beams. Holographic optical trapping (HOT) is a variation of optical trapping that uses a phase mask (hologram) to shape a laser beam's wave-front in order to create many optical traps from a single laser beam. One implementation of the technique uses a computer-controlled liquid-crystal spatial light modulator (SLM) to generate the necessary phase masks and update them in real time, thus enabling computer-controlled, simultaneous, three-dimensional manipulation of multiple particles.

The core of this study and development effort is an automated version of Arryx's BioRyx® 200 instrument. This device employs a 512x512 pixel liquid-crystal SLM (Boulder Nonlinear Systems), a 1064 nm continuous wave (CW) laser with at least 3 W of output power (IPG Photonics YLM-3-1064-LP), and a Nikon 40x, 0.95 NA air-immersion objective lens (Nikon MRD00400). Each component is controlled using LabVIEW™ code that was custom-developed for this project.

Methods: Other Key Techniques Used in this Research

In addition to HOT, there are several key laboratory methods that we have used in our development efforts. In particular, these methods are:

- **Microfluidics:** "Microfluidics" is a catch-all term for the study of, and techniques used to manipulate, fluids on very small scales. A microfluidic device, also referred to as a "chip," typically comprises a series of micro-scale fluid channels, along with means of moving fluid through those channels. For an application such as sperm isolation, microfluidic chips provide the link between the separation abilities of optical trapping and the necessary inputs and outputs accessible to pipettes and other sample transport mechanisms.

- **Fluorescent labeling of sperm:** Computer-based image analysis for identifying sperm cells is greatly aided by labeling the sperm with a fluorescent dye and imaging the sample with fluorescence microscopy, rather than by using brightfield imaging of unlabeled sperm. Results of the previous research phase (under grant 2008-DN-BX-K123) indicated that propidium iodide (PI), among others, was compatible with both HOT and downstream STR analysis, and so PI was used as the preferred dye for the work covered by this report.

- **Sperm sample acquisition:** Testing of both HOT and microfluidic manipulation of sperm was performed using samples eluted from mock forensic swabs provided by Orchid Cellmark. Most tests used sperm eluted from swabs containing sperm only, although some of the later work involved material eluted from swabs containing both sperm and epithelial cells. Elution was performed using a proprietary protocol supplied by Orchid Cellmark.

Results: Microfluidic Chip Design

One of the major challenges in this phase of research was the development of a microfluidic chip that contained active fluid control, in which sample flow can be automated and controlled by the computer. Development of this chip involved multiple iterations of channel design, material selection, and sample treatments, before a device that performed acceptably well in all areas was achieved. Although work remains to be done, we have largely succeeded in creating a design that we believe:

1. Is compatible with optical trapping
2. Has adequate and robust fluid manipulation capabilities, which will enable correct sample injection and separation
3. Allows for adequate sample throughput over the course of processing a sample

The microfluidic devices that we have been developing use the technique of on-chip, active valving and pumping developed by Grover and Mathies. These active devices are based on valves incorporated into the microfluidic channels. These valves are controlled by pressure applied through a second "pneumatic" layer which is separated from the fluid layer by an elastomer membrane. Pressure in the pneumatic layer was supplied via a pair of diaphragm pumps and a computer-controlled Lee valve manifold.

To enable easy modification of channel design, the chips created during the development phase comprise five layers of material. At the bottom is a 150 μm thick glass microscope coverslip, which is plasma-bonded to a laser-cut silicone layer that holds the fluidic channels. A second silicone layer separates the fluidic channels from a laser-cut PMMA layer which contains the pneumatic channels. Finally, the chip is topped with a second PMMA layer that seals the pneumatic channels and contains inlets and outlets for test samples, as well as connections for the Lee valve manifold.

Testing of multiple devices under similar conditions indicates that the volume of sample fluid pumped through the device will not vary more than ±13% from the mean value, under normal operating conditions. This range is expected to be sufficient to meet the needs of the next phases of the project. Material pumped through the device's input channel and separation region was not found to contaminate the output channels, nor to penetrate significantly far into the channels connecting the output channels to the separation junction.

Initial tests of the active chip design indicated significant problems with sperm adhering to the channel walls, as well as sedimentation in a reservoir area that was part of the initial design. Both of these effects caused a dramatic decrease in sperm concentration between the sample inlet and the separation area, leaving very few sperm available for trapping and isolation. The sedimentation issue was solved by replacing the initial sample reservoir with a recirculating loop, which keeps the sperm in suspension throughout the course of a measurement. The problem of sperm adhering to the channel walls was initially addressed by adding 2.5 mg/mL bovine serum albumin (BSA) solution to the sample. While this greatly mitigated the sticking problem, it introduced new difficulties. Therefore, we abandoned this approach in favor of coating the channel walls with Pluronic® during the chip manufacturing phase. The Pluronic coating had the desired effect of reducing the degree to which sperm stuck to the channel walls to a tolerable level.

The next phase of chip development will be to move from the five-layer design to a three-layer design that, most importantly, replaces the glass-and-silicone fluidic layer with a single layer, molded in cyclic olefin copolymer (COC). Doing so will improve ease of manufacture and device durability, and may also eliminate the need for coating the channels with Pluronic. As a proof-of-principle for this modification, we tested the ability of optical traps to manipulate sperm through a COC substrate, instead of a glass one. This proved successful, indicating that moving forward with the COC substrate should not be detrimental to the ability of our system to trap and isolate sperm.

Results: Automated Identification and Separation of Sperm

The second major area of effort during the research phase covered by this report was the development of software for machine-vision identification of sperm in the separation junction of a microfluidic chip, followed by trapping and moving the sperm to an output channel. This software interfaced with a hardware system that was previously built by Arryx for internal development of automated holographic optical trapping applications.

Initially, we looked at methods for identifying both fluorescently labeled sperm viewed with fluorescence microscopy, and unlabeled sperm viewed with brightfield microscopy. While both methods appear to be viable, it became clear early on that our routines for identification and separation of fluorescent sperm were more efficient and reliable than those for unlabeled sperm. Because the automation of trapping and isolation is dependent upon the computer first being able to correctly identify sperm to be trapped, most of the subsequent automation work was done using fluorescently-dyed sperm. Propidium iodide (PI) was usually used.

The current version of our software is capable of correctly identifying over 95% of PI-dyed sperm in a (367 μm x 275 μm) field of view.

The software for optical trapping and isolation of sperm inside a microfluidic device is designed around a “recipe” based architecture. The system is controlled by the main progam, named Forensic Automation System (FAS). FAS is capable of running several

diagnostic and control functions interactively, as well as executing automated sequences of commands fed into it as test recipes. An independent tool for generating test recipes, named Recipe Editor, has been developed to simplify the process of designing command sequences for automatic sperm isolation and extraction. This Recipe Editor program, allows the user to select items from a comprehensive list of instructions, set relevant parameters, and then place them into the main window in the required order. These commands range from simple, one-step instructions such as "Move Stage," "Turn LED On," and "Turn UV On," to macro-instructions containing long sequences of commands for executing more complicated functions such as autofocusing, alignment, and running pump sequences for the microfluidic subsystem. Additionally, some macro-instructions have been designed as separate modules that can execute just the sperm identification and/or extraction process.

Sperm isolation is carried out in a disposable, multilayer microfluidic chip. For developing and testing the sperm isolation procedure, we use a passive, valveless version of the chip described above, into which sample and buffer are manually injected. Sample fluid containing sperm is injected from the sample inlet into a central separation junction. Subsequently, sperm are located by means of image analysis, and optical trapping and stage motion are applied to move them to an output channel. In the final, fully-automated version of the system, isolated sperm will be collected by flowing fluid in the output channel to outlets where material can be removed and subjected to STR analysis.

Selection of operation parameters is critical to achieving good performance. Currently, the system uses the values shown in Table 1.

Table 1: Typical values of test parameters for Sperm Isolation System operation

	Parameter Name	Parameter Value	Remarks
1	Input Channel Width	300 μm	This is a nominal value. In practice the width is larger by 50-100 μm.
2	Output Channel Width	250 μm	This is a nominal value. Usually the width is larger by 50-100 μm.
3	Stage Movement Speed	35 μm/s	Higher speeds may be possible.
4	Number of Fields of View (FOVs) Tested	20-40	
5	Trap Lift Height	10 μm	This is a typical value. More testing is needed to determine the best value.
6	Field of View Area	367μm x 275μm	FOV for imaging
7	Effective FOV Area	140μm x 222μm	Effective FOV for trapping
8	Movement Trajectory Length	>1400 μm	Depends on the active FOV

Under these conditions, the automated system is able to isolate on the order of 80 sperm in one hour, although efficiency is expected to decrease after the first hour. There is much room for improvement in this value, including improvement of trapping efficiency, sample quality control, and parameter optimization. Additionally, the reliability and stability of the system must be improved.

Conclusions

During the research phase covered by this report, we have made significant progress in several key areas towards the development of a holographic optical trapping-based device for forensic cell isolation. A foundation for development of a commercially viable automated system has been provided through development of (1) a suitable microfluidic chip with active fluid control, (2) a sperm-identification strategy combining fluorescent staining with customized image analysis software, and (3) hardware automation software that allows test routines to be quickly created and modified.

The next stage of development, which will take place under NIJ award 2011-DN-BX-K562, addresses the remaining issues involved in developing a robust system suitable for integration with varied forensic lab workflows. This will require improvements to most aspects of the system. In particular, we plan to revisit the challenge of identifying and isolating unlabeled sperm, in order to allow for flexibility in different forensic workflows. Upcoming development will explore more robust software algorithms for identifying both labeled and unlabeled sperm, as well as modifications to system hardware and possibly dye selection so that the same device can reliably use fluorescent or brightfield imaging methods, individually or simultaneously. Two other key areas of the research will be minimizing loss of sperm during processing, and accurate quantitation of the sperm in the output. Finally, integrating the active microfluidic chip with the sperm identification and HOT-based isolation system is a high priority, in order to allow for fully automated processing of samples.

Implications for Criminal Justice

If eventually realized as a commercial device, the cell isolation technology presented in this report can be integrated into existing sample processing workflows to improve genetic identity testing by automating the isolation of sperm eluted from sexual assault evidence swabs and by reducing carryover of victim DNA, thus improving the likelihood of obtaining a reliable STR profile. Additionally, such a system can provide video-based and image-based screening, quantitation, and record-keeping of a sample and how it was processed. Automation will reduce human intervention which has three potential implications: (1) reduced overall cost by lowering personnel/labor cost, (2) shorter processing time and increased overall throughput and productivity, and (3) decreased chance of human error in sample handling.

1 Introduction and Background

1.1 Statement of Problem

The DNA evidence backlog continues to be an ongoing problem in American crime labs. The forensic casework backlog at the end of 2009 was estimated to be on the order of 110,000 cases, and trends suggest that it has only grown since then.[1] A 2007 survey of U.S. law enforcement agencies indicated that 18% of unsolved rape cases between 2003 and 2007 contained unanalyzed forensic evidence.[2] Despite efforts to reduce and eliminate the DNA backlog, the problem persists. The DNA backlog problem, especially for sexual assault evidence, demands the adoption of new approaches and technology to improve speed, throughput, and results of forensic processing and profiling.

At the level of cell separation and DNA extraction, differential extraction remains the most common method currently used in separating assailant and victim DNA fractions in a sexual assault evidence sample.[3] Once eluted from the sample swab, the mixed cell suspension containing cells from both the assailant and the victim 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 separates the released nuclear material from the epithelial cells from the intact sperm nuclei. After the sperm nuclei have been separated, they then lysed to release their DNA. Under optimal conditions, differential extraction aims to achieve two isolated and pure fractions of DNA. However, this technique has a significant frequency of carryover of female DNA into male fractions and generally involves a significant amount of labor and elapsed time for sample processing.

An informal survey, conducted by Applied Biosystems in 2007, estimated that such carryover occurs in 19% of sexual assault forensic samples.[4] This generally occurs because commonly used differential extraction does not fully isolate the DNA of the assailant (typically from sperm) from that of the victim (typically from vaginal or other epithelial cells). As a result, during the PCR amplification process, DNA from both victim and assailant get amplified and STR analysis yields a mixed profile rather than a unique profile to match the assailant. The Differex system from Promega[5] offers several improvements over traditional differential extraction including parallel processing of several samples. However, the mixture of victim DNA into the assailant profile remains an important problem.

Forensic DNA analysis for sexual assault evidence would greatly benefit from improvements in the following three areas: (1) cell separation techniques, (2) incorporation of microfluidics for higher throughput and reduced reagent cost, and (3) automation to reduce manual handling, minimize costs, and increase throughput.

1.2 Review of Literature and Related Work

This review of relevant literature primarily discusses two key areas of forensic DNA analysis:

1. Existing alternatives to differential extraction for the separation of sperm DNA from epithelial cell DNA
2. The use of microfluidics for sample handling as applicable to forensic DNA analysis and separation of cells on such devices.

1.2.1 Isolating sperm from epithelial cells

Various groups have investigated alternatives to differential cell extraction. For example, Elliott, *et al*. has suggested using laser capture micro-dissection (LCM) to selectively capture sperm cells from slides prepared from swabs.[6] This method has met with some success under controlled laboratory conditions,[7, 8] and may represent a viable approach. However, the technique has yet to be widely adopted by forensics labs, perhaps due to cost or sample preparation requirements.[9]

Employing an alternate separation technique, Chen, *et al,* used a nylon mesh membrane (8 micron pore size) to demonstrate separation of sperm cells from epithelial cells. This method suffered from clogging of the membranes with epithelial cells and therefore incomplete separation of free DNA from sperm cells is commonly encountered.[10] Schoell, *et al,* used flow cytometry to separate sperm from epithelial cells using either fluorescently labeled antibodies to bind to cell surface antigens or by using a DNA stain to distinguish cell types.[11] However, antibody-based recognition faces a problem when the cell membrane is degraded, as is often the case in forensic samples.[10] Eisenberg, *et al*, reported the use of magnetic beads to separate sperm using an antibody to capture spermatozoa.[12] A cocktail of antibodies against sperm cell surface antigens were attached to a magnetic bead surface which adheres to sperm cells and separates them when exposed to a magnetic field. However, the success of this approach lies on the specificity and sensitivity of the antibody-antigen binding for sperm recognition and binding. In addition, this method relies on intact sperm membranes.

1.2.2 Microfluidics for forensic applications

In the area of microfluidics, a variety of devices have been developed and tested for the isolation of male and female cell fractions.[13] Sperm capture on a microdevice was demonstrated using dielectrophoresis, in which sperm and epithelial cells migrate differentially when exposed to a concentrated electric field. However, a potential drawback of this technique is the adherence of sperm to the chip at the site of trapping.[14] Additionally, such electrode-based devices are known to often cause significant currents, heating, and reaction within the fluid which may degrade samples. Norris, *et al*, used acoustic standing waves to trap and levitate sperm in a microchannel away from the

surface of the channel while DNA from lysed epithelial cells was washed away.[15] Finally, separation of sperm from epithelial cells has been demonstrated on a microfluidic device containing a single channel where difference in sedimentation rates of sperm and epithelial cells was exploited. After five minutes, epithelial cells settled at the bottom and sperm were moved and separated using a negative pressure syringe pump.[16]

1.3 Rationale for Research and Project Objectives

There are presently few options for automated screening of sexual assault evidence kit elutes for precise sperm isolation. Sexual assault evidence samples still require significant manual processing, and are subject to variability and false negative results for weakly positive samples. We have proposed to develop a powerful platform for automated microscopy which leverages machine vision for object recognition and holographic optical trapping (HOT) for cell separation within aqueous cell samples on slides and active fluidic disposables. This technology, coupled to a suitable microfluidic cartridge compatible with HOT holds great promise to advance forensic science.

Optical trapping, also known as laser tweezers, is a technique which can isolate sperm from epithelial or other cell types and is compatible with downstream STR analysis.[17] It offers scope for automation, can work with cells in suspension without requiring additional or alternative media support, and has the potential to be faster than typical differential extraction techniques. It also promises greatly increased purity of the extracted sperm fraction DNA, providing optimal results even for cases in which the number of sperm cells is relatively very low. Optical trapping also reduces concerns about cell damage since the cells are not physically touched, and it has been shown that—so long as a suitable laser wavelength is used—optical trapping does not significantly damage human sperm.[18, 19] Holographic optical trapping (HOT) is an improved and advanced variant of optical trapping which offers scope for complete automation. Such automation of HOT for processing forensic evidence promises to provide faster turnover and less labor intervention, thereby mitigating, or even eliminating, bottlenecks often experienced in the current approaches used in STR analysis.

The ultimate goal of the research discussed in this report is to develop a prototype system for the automated isolation of sperm from eluted mixed cell solutions from mock evidence swabs, and to develop a disposable cartridge for sample handling and withdrawal of the sorted fraction. Our proposal directly addressed the priority area of improving the "physical separation of cells in mixtures from two or more individuals" as defined in the NIJ solicitation "Forensics DNA research and development." In particular, we aim to provide a viable and cost-effective technology which can be automated for the physical separation of sperm from other cells in a forensic sexual assault sample.

To achieve these goals and objectives, the project has been divided into several areas:

1. Developing a microfluidic cartridge for small volume sample handling which is compatible with HOT

2. Developing various modules for automation such as auto-focusing, computer recognition of sperm from a mock forensic mixed elute, and movement of sperm from one site to another on a microfluidic device using a combination of HOT and microscope stage motion

3. Documenting requirements for integration of an anticipated commercial device into an existing crime lab workflow for processing and analysis of sexual assault evidence samples

4. Testing each individual automation module, and modifying and improving them to attain suitability for integration into the anticipated commercial device while both prioritizing compatibility with typical workflows and routinely testing compatibility with downstream PCR/STR profiling.

The work performed during this phase of the project focused on the first two areas. Compilation of a formal requirements document and systematic testing against those requirements must necessarily wait until device development has progressed to a more advanced stage.

1.4 Goals for the Current Phase

This phase of our research program focused on:

1. Developing a disposable cartridge for introduction of the mixed cell solution, processing, and withdrawal of the solution containing the sorted sperm fraction,

2. Investigating the ability of our system to optically trap sperm through polymers rather than glass, with a view towards reducing the cost and complexity of the microfluidic cartridge,

3. Implementing fluorescent staining strategies to simplify machine vision and automation,

4. Developing of machine vision based automatic sperm recognition, and assessing the efficiency, speed and reliability of automated sperm recognition using brightfield and fluorescent imaging,

5. Developing, testing and integrating key software modules important for building an automated prototype device for HOT based sperm separation.

2. Methods and techniques

This section of the report covers key methods that we have used and developed to pursue the goals of this research phase, as laid out in Section 1.4. We discuss the key holographic optical trapping (HOT) and microfluidic-device technologies that lie at the core of approach to sperm isolation. We also summarize the results of the previous phase,[18] which have been built upon and further developed in the current phase. Please note that many additional methods are detailed in later sections of the report, for the sake of clarity and to avoid repetition.

2.1 Holographic Optical Trapping

Optical trapping, also known as laser tweezers, was developed by Arthur Ashkin at Bell Labs in 1970.[20] This technique 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, and so optical trapping has proven to have great utility in cellular biology.[21] A variety of cell types, including bacteria, yeast, and mammalian cells have been successfully manipulated 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. The near-infrared laser light commonly used in optical trapping of biological material is readily transmitted through most glass, making manipulation possible within optically clear, open or sealed chambers. Sealed chambers allow samples to be manipulated without contamination, damage, or drying. This powerful capability has led to applications for optical trapping in studies of sperm motility.[22] Other biological applications of optical trapping include studies on a variety of motor proteins such as various classes of myosin, kinesin, and polymerases,[23] as well as in studies of DNA structure and conformation.[24]

Holographic optical trapping (HOT), invented in 1997,[25] was developed to overcome certain limitations of traditional optical trapping techniques. These included: inability to create large numbers of optical traps for sophisticated manipulations involving many particles, a lack of methods to increase throughput by parallel processing, limited ability to manipulate objects in three dimensions, and a lack of flexibility in shaping the light to form optimized optical traps which perform better than simple point traps.

HOT employs a phase mask (hologram) to shape a laser beam's wave-front, splitting the single beam into many optical traps. For computer-controlled dynamic holographic optical trapping, Arryx uses a liquid-crystal spatial light modulator (SLM) to generate the necessary phase masks. With recent advances, SLMs are now available with refresh rates of up to 100 frames per second and can withstand several Watts of laser light power.[26] 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.

Figure 1 illustrates how an SLM is used to generate multiple, arbitrarily-located optical traps from a single laser beam, with no moving mechanical parts. The laser beam which is reflected from the SLM is imaged onto the back aperture plane of the objective lens using a telescope and forms the pattern of traps in and around the objective's imaging 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 micro-manipulation system that provides the user with the ability to simultaneously move many cells or other particles along arbitrary paths in three dimensions. Addition of a CCD camera and computer-based image processing produces a machine-vision guided system that can automatically execute sophisticated manipulations and isolations.[27]

Holographic optical trapping technology therefore can simultaneously trap many objects in arbitrary positions for sorting and isolation. It also has the capability to steer each object in three dimensions, allowing each trap to easily be lifted above the bottom of the sample chamber and moved over other cells and debris which would otherwise obstruct the cell's path. Arryx's BioRyx® 200 research instrument,[28] commercially sold since 2002, enables multiple cells to be manipulated in three dimensions and in real time by clicking and dragging a computer mouse. Trapping of human sperm with this system has been demonstrated on numerous occasions. This provided a strong foundation for developing HOT for automated forensic cell separation.

The core of our study and development efforts is a modified version of Arryx's BioRyx 200 instrument that has been internally developed for automated applications. This device employs a 512x512 pixel liquid-crystal SLM (Boulder Nonlinear Systems 512-SLM-HSPDM-1064), a 1064 nm CW laser with at least 3 W of output power (IPG Photonics YLM-3-1064-LP), and a Nikon 40x, 0.95 NA air-immersion objective lens (Nikon MRD00400). Each component is controlled using software that was custom-developed for this project, using National Instruments' LabVIEW™. Laser and SLM control also use proprietary programming interfaces developed by Arryx for our commercial instruments.

For detailed information about traditional optical trapping, we recommend Reference 22. For discussion of holographic optical trapping, please consult Reference 28. Device automation for forensic evidence processing is discussed in detail in Section 3.4.

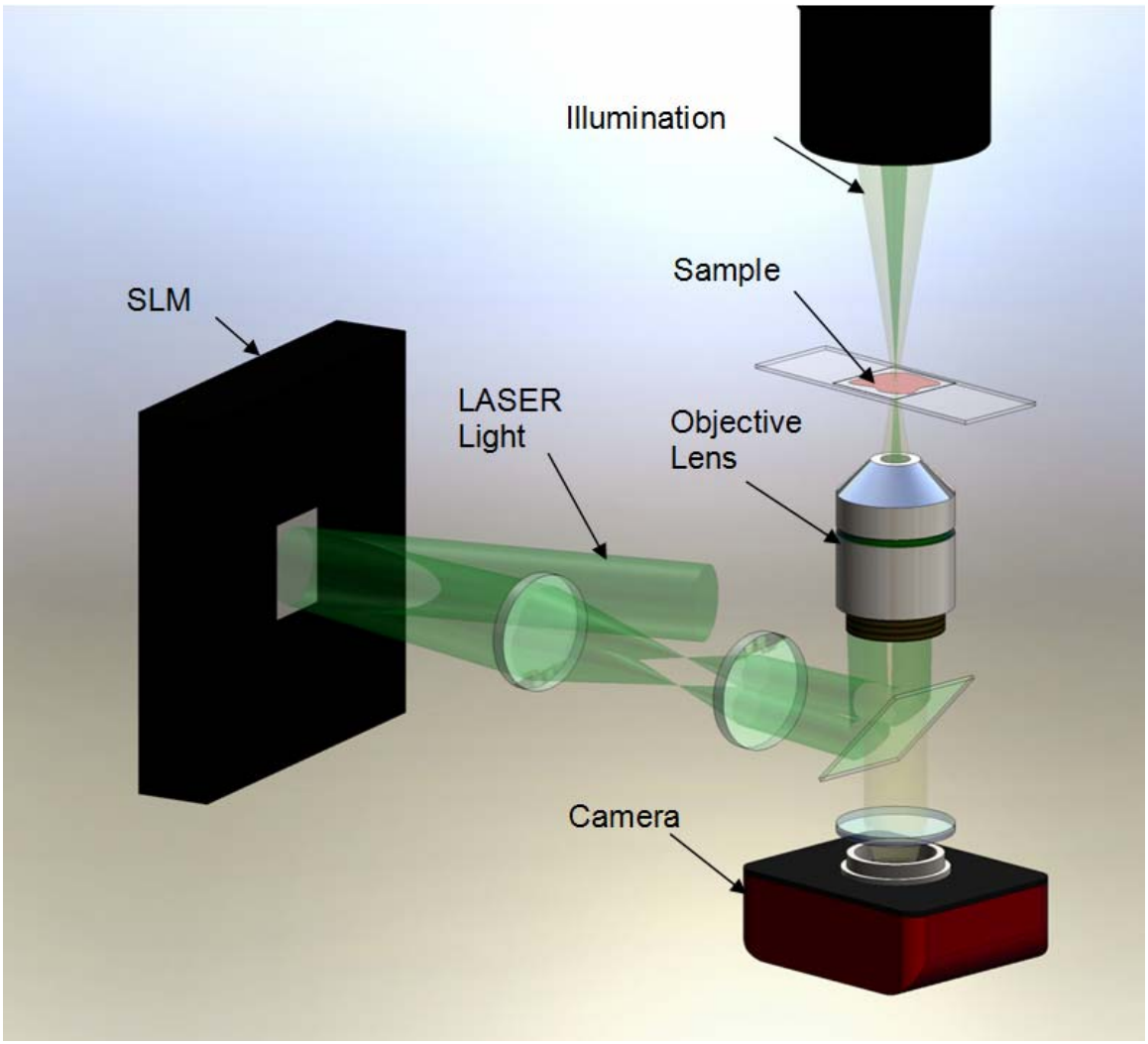


Figure 1: Holographic Optical Trapping (HOT) using a spatial light modulator (SLM): collimated laser light is incident on the face of a liquid-crystal SLM, which imposes a phase profile on it. The light is then transferred to the back aperture of a microscope objective lens, using a series of lenses and mirrors. The objective lens focuses the light into a number of optical traps, which can be manipulated by changing the SLM's phase profile. Images of the trapped particles in the sample are collected by a CCD camera.

2.2 Microfluidics

2.2.1 Microfluidics background

The field of microfluidics is concerned with the manipulation of fluids on very small (nanoliter and picoliter) scales. The typical microfluidic platform is the so-called "lab on a chip"—a system of micro-scale channels that is custom-designed to move fluids in a controlled manner for a particular application. Such a microfluidic device will contain a combination of the following generic components:[29]

- A means of introducing fluids and microscopic particles to the system
- A means of moving fluids through the channels of the chip—this could be simple capillary flow,[30] a system of programmable valves,[31, 32] or electro-osmotic flow[33]
- Application-specific devices, such as detectors, filters, and fluid-extraction points

Additionally, one generally includes a means of monitoring the fluid and particles in the chip. This is usually done by incorporating optically-clear regions into the chip design, and observing these regions with an optical microscope.

Over the past decade, lab-on-a-chip techniques have become increasingly popular in cell biology, and the trend appears to be accelerating.[34] The field is extremely diverse, both in terms of applications, and chip-fabrication techniques. Reviewing the range of microfluidic-enabled cell biology research is beyond the scope of this report; we refer interested readers to References 30 and 34.

2.2.2 Previous microfluidic device development at Arryx for HOT-based sperm isolation

For an application such as this, which involves micro-scale manipulation, microfluidic chips provide the link between the separation abilities of optical trapping and the necessary inputs and outputs accessible to pipettes and other typical sample transport mechanisms. Some of the key challenges with microfluidics are the following:

1) The materials used in fabrication must have low infrared (IR) light absorption, or else the optical traps may either become blocked or may heat the microfluidic chip to an unacceptable level.

2) The bottom surface of the chip must be sufficiently thin to allow a short working distance objective lens to focus on the sample contained within the fluidic channels.

3) Sample loss in the chip – in this case, sperm becoming trapped in corners or adhering to surfaces – must be kept to a low level.

4) Bubble formation and leaks must be prevented since they can create undesired flows and inhibit desired ones.

5) Materials in the chip should not fluoresce to the point of obscuring fluorescence measurements.

6) Microfluidic pump speeds must be reasonably consistent.

During the previous phase of this project, Arryx developed a simple two-layer microfluidic chip. This chip, shown in Figure 2, consists of a laser-cut acrylic layer containing input and output inlets, bonded to a molded cyclic olefin copolymer (COC) layer containing the channels. This chip contains a single input channel, into which sperm-containing fluid is manually loaded, and two output channels, which are filled with buffer solution. Typically, only one of the two output channels is used in a separation. Sample solution, containing mostly sperm, is introduced into the chamber through the Sample port. Laser traps can be projected inside the chamber, so that target cells may be transferred from chamber to the side channels over a short distance. Once the cells are moved to the side channel, more buffer solution is manually injected into one port (either Epi-buffer or Sp-buffer), and the cells were carried to the other port (Epi-out or Sp-out).

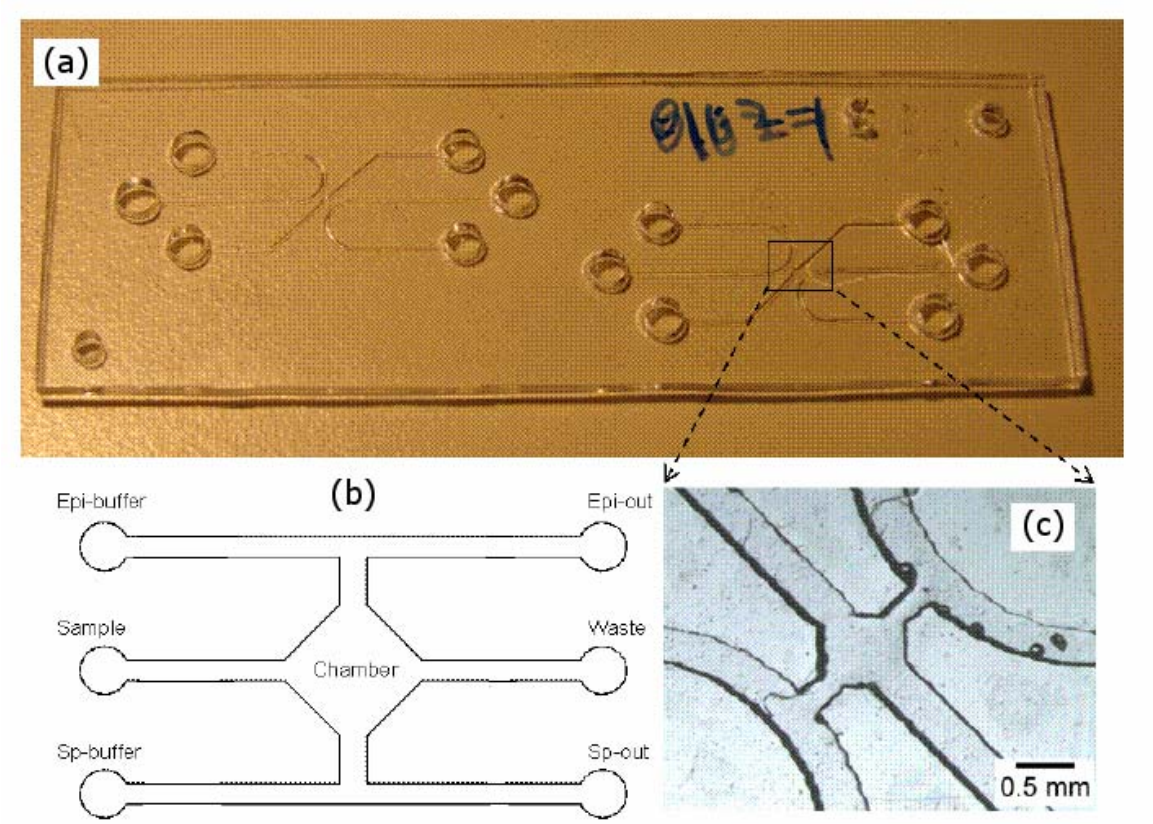


Figure 2: Two-layer passive chip for HOT-based sperm isolation. (a) Image of chip fabricated from laser-cut acrylic and molded COC (b) Schematic diagram of channel layout, showing the connections between channels and inputs/outputs. Not to scale (c) Optical micrograph showing the input channel (straight channel), output channels (curved channels), and separation chamber (center)[18]

The development of this "passive" chip—so called because it does not incorporate active pumping of fluid—is detailed in our final technical report for NIJ grant 2008-DN-BX-K123.[18] When fabricated and filled according to specifications, it fulfills requirements 1-5 above. (Requirement 6 is not applicable for a passive chip.)

This chip design was created to serve as an experiment and testing platform to be used in developing various components of and procedures for HOT-based sperm isolation. All of the automation and image analysis work described in Sections 3.3 and 3.4 employed chips with a similar design.

Development of a more sophisticated microfluidic chip that is compatible with active, computer-automated fluid control was one of the major objectives of the current research phase. The largely successful results of this effort are described in Section 3.1 of this report.

2.3 Fluorescent Labeling of Sperm

Early in our development process, we realized that computer identification of sperm could be greatly simplified by labeling the sperm with a fluorescent dye, and imaging the sample with fluorescence microscopy, rather than using unlabeled sperm, and brightfield imaging. Therefore, as part of the previous research phase, we investigated the use of several dyes for this purpose. Dyes were tested for compatibility with HOT and STR profiling, and compared on the basis of toxicity, photostability (minimal photobleaching), and quantum yield (brightness). The details of this investigation, including the STR compatibility tests, can be found in the final technical report for NIJ award 2008-DN-BX-K123.[18]

The results of these tests indicated that propidium iodide (PI), SYBR 14, DAPI, syto-red, and Hoechst would be suitable. The best results came from PI, SYBR 14, and DAPI, so these dyes were selected for use in the current research phase.

2.4 Sperm Sample Acquisition

Testing of both HOT and microfluidic manipulation of sperm was performed using samples acquired from Orchid Cellmark. Most tests used sperm eluted from swabs containing sperm only, because the first priority is to make sure our methods are appropriate and effective for sperm manipulation. All microfluidic work was performed using these sperm-only samples. The automation work used both sperm-only samples and material eluted from mock forensic samples containing both sperm and epithelial cells. Elution was performed using a proprietary protocol supplied by Orchid Cellmark.

3 Results

The research phase covered by this report was devoted to building upon the preliminary work done in the previous phase[18] to develop the core elements of an automated system for processing sexual assault evidence swabs. The central elements were developing a microfluidic cartridge for HOT-based sperm isolation, and creating automation software for imaging, identifying, trapping, and moving sperm within a microfluidic cartridge. Both areas of work saw significant progress during this phase, the results of which are documented in the following sections.

In the area of microfluidics, we built upon the simple passive chip described in Section 2.2 to develop an actively-driven chip in which sample flow can be automated and controlled by the computer. Development of this chip involved multiple iterations of channel design, material selection, and sample treatments, before a device that performed acceptably in all areas was achieved. Section 3.1 discusses the requirements, design, operation, and testing of the current incarnation of the active chip.

The area of HOT automation can be further subdivided into two functions: machine-vision identification of sperm and automated trapping and hardware control for isolating the particles that are identified as sperm. General considerations regarding sperm identification, and discussion of the techniques used to perform the identification are covered in Sections 3.3. Automation of the sperm trapping and segregation process, including the integration of the sperm identification computer vision procedures, is discussed in Sections 3.4.

3.1 Active Microfluidic Device for Sperm Isolation

This microfluidic research phase for the project has focused on addressing three fundamental challenges:

1. Is there a method of fabricating devices that does not interfere with holographic optical trapping (HOT)?
2. Can we fabricate devices that have adequate and robust fluid manipulation capabilities that will enable correct sample injection and separation?
3. Do these devices allow for adequate sample throughput over the course of an experiment?

3.1.1 Principles of operation

The microfluidic devices that we have been developing use the technique of on-chip, active valving and pumping, which was originally developed by Grover *et al*.[31] These active devices are based on valves incorporated into the microfluidic channels. Such a device, illustrated schematically in Figure 3a, incorporates two layers of channels: a fluidic layer which contains the experimental sample, and a pneumatic layer that is used

to control the valves in the fluid layer. The two channels are separated by an elastomer membrane. The valves are controlled by applying either positive or negative pressure to the pneumatic channel. When a positive pressure is applied to the pneumatic channel, as shown in Figure 3b, a seal is formed between the elastomer membrane and a "plug" in the fluidic channel. This prevents fluid from moving past the valve. When negative pressure is applied to the pneumatic channel, as shown in Figure 3c, the elastomer membrane is pulled away from the "plug," which creates an open fluid path.

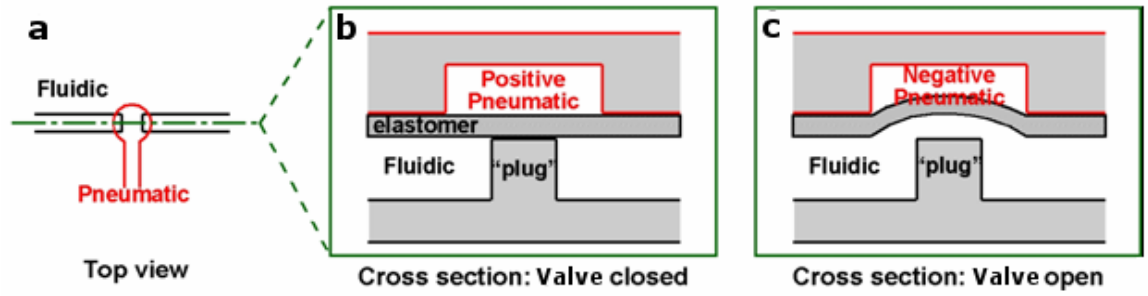
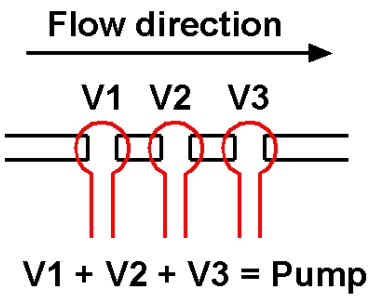


Figure 3: Schematic drawing of a valve in a microfluidic channel. (a) Top view drawing of the two channels of a microfluidic valve. The fluidic channel (black) and the pneumatic channel (red) are separated by an elastomer membrane. (b) Cross-sectional drawing of a valve in a closed state (positive pneumatic pressure) (c) Cross-sectional drawing of a valve in an open state (negative pneumatic pressure)

To generate a pump, three such valves are operated in combination, according to the scheme shown in Figure 4. Initially, all valves are in a closed state (positive pressure applied to V1, V2, and V3). Then valve 1 is opened, followed by valve 2. Next, valve 1 is closed and valve 3 is opened. Finally, valve 2 is closed followed by valve 3. This sequence results in a directional fluid flow and can be repeated multiple times to generate bulk fluid movement.



Step	V1	V2	V3
1	closed	closed	closed
2	open	closed	closed
3	open	open	closed
4	closed	open	closed
5	closed	open	open
6	closed	closed	open
7	closed	closed	closed

Figure 4: Schematic representation of three valves in combination to form a pump. Valve 1 = V1, valve 2 =V2, and valve 3 = V3. Left-to-right bulk fluid flow would be generated by repeating the sequence of steps outlined in the table.

3.1.2 HOT-compatible device fabrication

The active microfluidic devices used with our HOT platform must meet the following two minimal requirements: the materials used in fabrication must have low infrared (IR) light absorption, and the bottom surface of the device must be sufficiently thin to allow a short working distance objective to focus on the sample contained within the fluidic channels.

To meet these two requirements we implemented a fabrication technique that allowed us to make devices entirely of poly (methyl methacrylate) (PMMA), silicone, and glass. These materials have low IR absorption and can be assembled such that all optical trapping is carried out through a #1 glass coverslip of thickness ~ 150 μm. All features in these devices are generated using laser cutting and laser etching, which allowed us to easily experiment with different channel layouts and make rapid revisions to the chip design, compared to press-molding. The design and fabrication of this microfluidic chip is shown in Figure 5.

The pneumatic layer consists of two sections, which are bonded using a silicone-based pressure sensitive adhesive (PSA). The top section, shown in Figure 5(a), consists of PMMA laminated with PSA (PMMA/PSA). It contains seven through holes which serve as inlets and outlets for fluid or as air vents. This section is bonded to the top of the bottom PMMA/PSA section. This bottom section, shown in Figure 5(b) contains through holes and laser-etched pneumatic channels that supply pressure to the fluid valves, as described in Section 3.1.1. Seven of the through holes align with the holes in the top section, and the 24 through holes on the left-hand side interface with the pneumatic sub-stage (see discussion on device control in Section 3.1.4). Twelve of these 24 holes interface with the etched pneumatic channels. The top and bottom sections are then bonded to an elastomer membrane, shown in Figure 5(c) with corresponding through holes. This assembly comprises the "pneumatic layer" of our microfluidic device.

The fluidic layer consists of two sections that are bonded using plasma oxidation (plasma bonding). Plasma bonding is a well-established technique for bonding silicone to glass, as well as to itself.[35] The top section, shown in Figure 5(d), consists of silicone with laser-cut fluid channels. This section is plasma bonded to the bottom section, a #1 glass coverslip, shown in Figure 5(e). These two bonded sections are collectively referred to as the fluidic layer. The final device was achieved by plasma bonding the pneumatic layer to the fluidic layer. A top-down view of the fully-assembled chip, with labeled inputs and outputs, is shown in Figure 6.

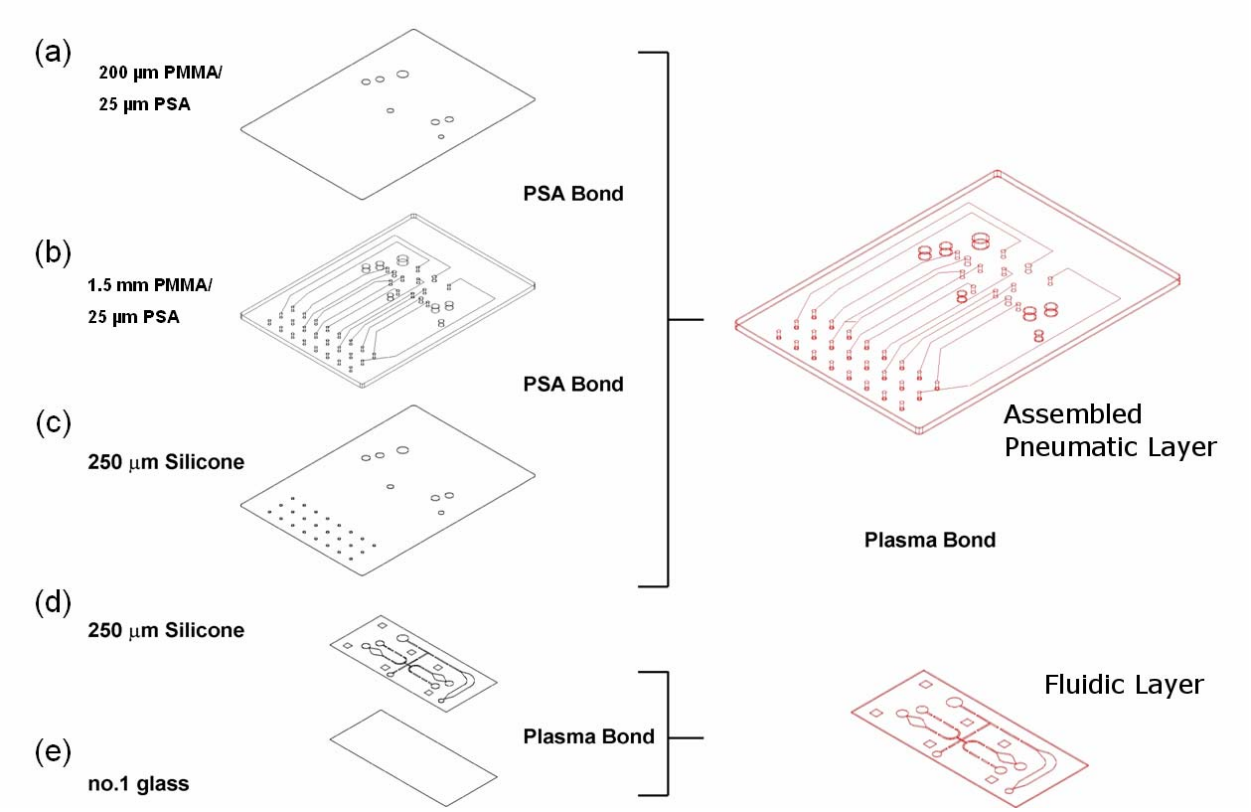


Figure 5 Fabrication of active microfluidic devices. The pneumatic layer (a-c) consists of three sections that are bonded using pressure sensitive adhesive (PSA). The fluidic (d-e) layer consists of two sections that are bonded using plasma oxidation (plasma bond).

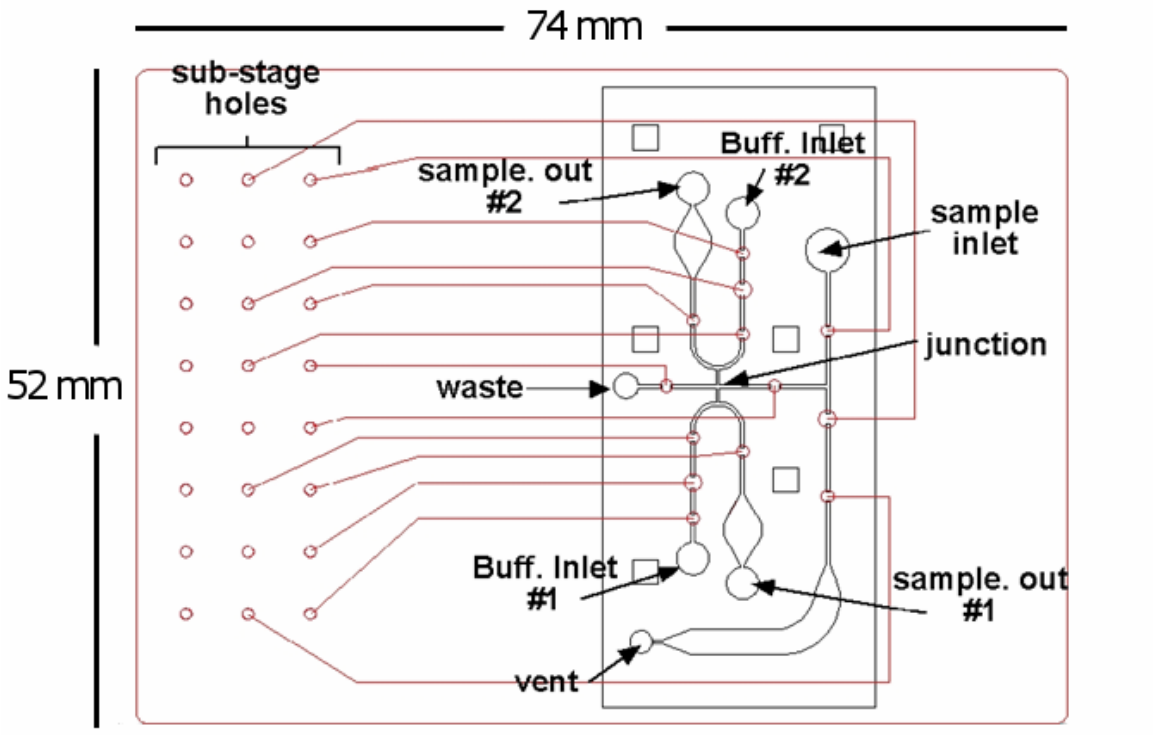


Figure 6: Assembled active microfluidic chip, showing fluid inlets and outlets. Fluid channel widths are typically 300 µm

3.1.3 Device control

On-chip valves and pumps are controlled using the test system shown in Figure 7. This system consists of four main parts:

1. Two diaphragm pumps that are used as positive and negative pressure sources,
2. A computer controlled circuit board with three Lee valve manifolds, for a total of 24 valves,
3. Tubing that connects the Lee valves to the sub-stage,
4. A sub-stage that interfaces with the pneumatic sub-stage holes on the device.

Device control begins with the two diaphragm pumps (KNF Neuberger NMP850.1.2KNDCB). One diaphragm pump supplies positive pressure, and the second pump negative pressure, to the valve manifolds. The Lee valves (The Lee Company LHD0511111H) are two-state, three-way valves, configured with two inputs and a common output. In this test system, one input connects to the positive pressure source and the second input connects to the negative pressure source. In state 1 the positive pressure input was connected to the output, and in the state 2 the negative pressure input was connected to the output. The output of each Lee valve is connected to the substage via tubing, and therefore to a single pneumatic line in the microfluidic chip. In other words, one Lee valve corresponds to one pneumatic channel in the chip.

When a Lee valve is in state 1, a positive pressure is supplied to the corresponding pneumatic channel on the chip. When a Lee valve is in state 2, a negative pressure is

supplied to the corresponding pneumatic channel on the chip. Using this set-up we can control 24 Lee valves independently and therefore, 24 pneumatic channels in a microfluidic device. Note that a given chip design need not use all 24 channels. The state of each Lee valve is controlled using custom software developed by Arryx.

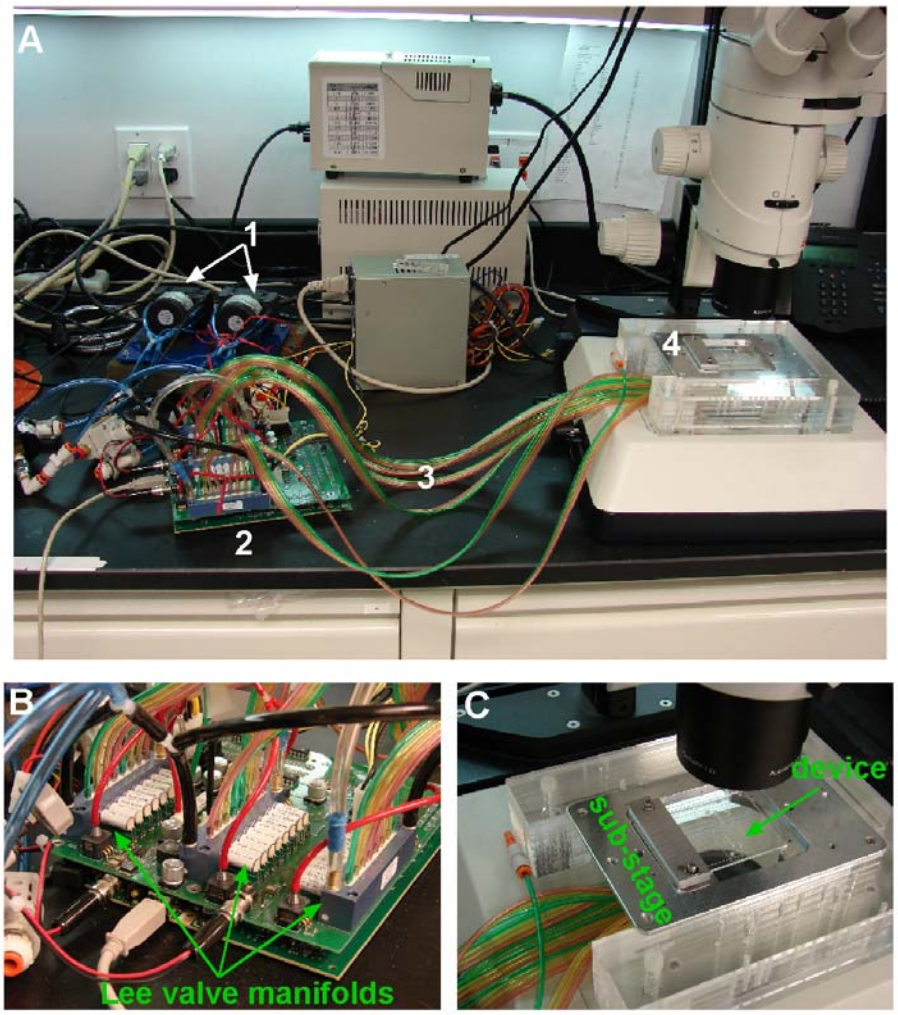


Figure 7: Test system for controlling active microfluidic devices. A) Photograph showing the major components of the test system: (1) Pumps used as positive and negative pressure source, (2) Computer controlled circuit board and Lee valve manifolds , (3) Tubing that connects the valve manifold to the sub-stage, (4) Substage used for interfacing the microfluidic chip to positive and negative pressure sources. B) Close-up view of the circuit board and Lee valve manifolds. C) Close-up view of the sub-stage with a mounted device.

3.1.4 Testing of controlled fluid manipulation in active microfluidic devices

Testing of on-chip fluid manipulation was broken down into two categories. The first was variability in pumping, that is, testing multiple devices to determine the variation in volume of fluid pumped. The second concern was spatial control of fluid control, that is,

testing whether sample location and movement within a device could be controlled in a manner consistent with the application.

3.1.4.1 Variability in pumping

The variation in pumping was determined for seven different devices using deionized water as the test fluid. First, DI water was loaded into the device via the sample inlet and pumped toward the air vent (see Figure 6 above for location identifications). Next, the water was pumped using a fixed number of pump cycles from the air vent to the junction. Pumping was stopped and the final position of the air/water interface along the input channel was measured. A difference in fluid volume pumped corresponds to a different end position of the air/water interface, indicated by the dashed red line in Figure 8. Using the known dimensions of the channel (300 µm diameter) to convert distance to volume, the mean volume of fluid that was pumped from the air vent to the junction was found to be 1.79 µL, with a standard deviation of 0.09 µL. Therefore, assuming the tested devices were representative, we can expect that in 95% of cases, the volume of pumped fluid will not vary more than ±13% from the mean value. (This estimate is derived from the 95% confidence interval for the volume of pumped fluid in the seven-device test.) This range is expected to be sufficient to meet the needs of the next phases of the project.

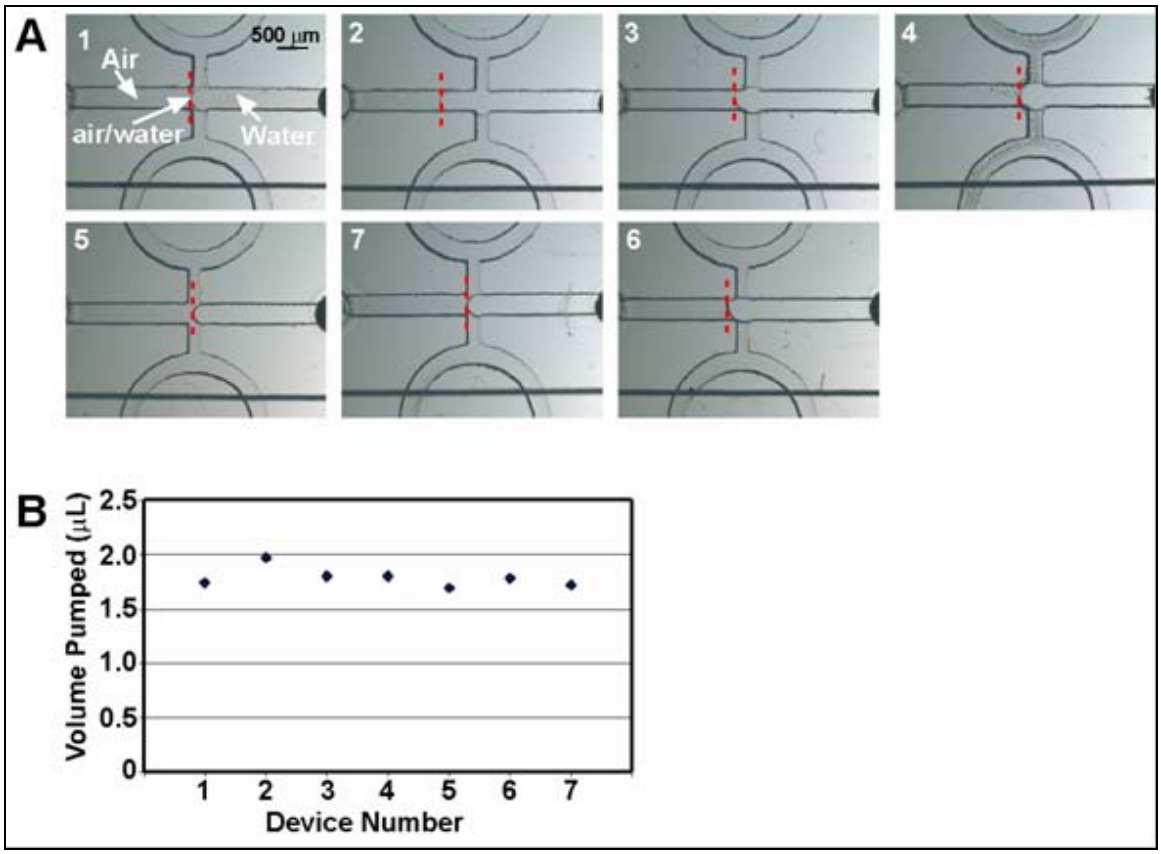


Figure 8: Testing variability in pumping between devices. (A) Microphotographs of the junction region of seven different devices. These microphotographs highlight the small variability in

volume of water pumped under the same pumping conditions. (B) Graph quantifying the volume of water pumped in the seven devices. The average volume was 1.79 μL and the standard deviation was 0.09 μL.

3.1.4.2 Spatial control of fluid

To test the ability to selectively pump a sample from the input to the junction region of the microfluidic chip, without contaminating the output regions, we used a suspension containing 4 μm polystyrene colloid with conjugated Alexa Flour-488 dye. The following description refers to the chip region identifications in Figure 6. First, a buffered solution was pumped from Buffer inlet #1 and Buffer inlet #2 through to the waste region. These buffered solutions were then pumped to sample outlet # 1 and sample outlet #2, so that all the output channels were filled with fluid. Next, the colloid suspension was pumped from the sample inlet to the air vent region. Finally, the colloid was pumped from the air vent through the junction and pumping was stopped. Figure 9 shows the state of a device after this pumping procedure. Although there is a small amount of colloid in the side-channels near the junction (Region B in Figure 9), the colloid has not mixed into the output channels (Regions A and C in Figure 9). This behavior was observed across several tests, leading us to conclude that the channel design and pumping behavior was sufficiently well-controlled to allow for further development and integration with HOT.

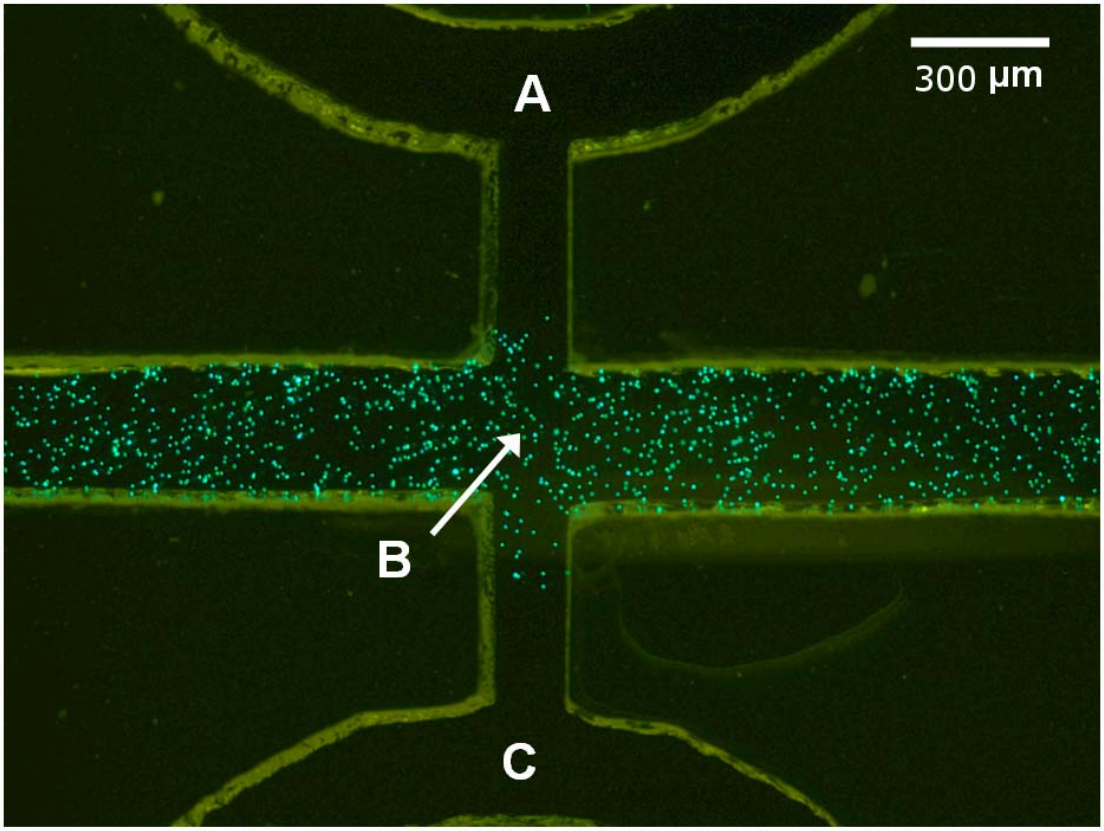


Figure 9: Selective pumping of a fluorescent colloid to a specific location within the microfluidic device, imaged under combined brightfield and fluorescent illumination. This illumination scheme allows the channel structure (yellow) and the 4 µm colloids with conjugated Alexa Flour-488 (green) to be visualized simultaneously. The colloidal suspension was selectively pumped through region B of the device without contaminating output Regions A and C.

3.1.5 Minimizing sample loss during flow through the device

Initial testing of the chip design described in Section 3.1.2, revealed a tendency for sperm to adhere to the channel walls and substrate. Consequently, when a sample was injected into the chip, pumped to the reservoir, and then from the reservoir to the separation junction, a much lower density of sperm was seen in the junction, in comparison to the density near the sample inlet. The situation worsened if the sample was allowed to sediment over time, thus allowing more sperm to contact the channel surfaces and stick. This problem was addressed on two fronts: (i) coating the channel surfaces to mitigate sticking, and (ii) modifying the chip design and operation to mitigate sedimentation. The goal was to achieve a similar density of trappable sperm in the separation junction area to the density observed near the sample inlet.

Note that the work discussed in this section does not address total sample throughput for the device, from sample injection to retrieval of separated sperm. Rather, it covers the

more fundamental work that was done in order to engineer a device that can move sample around as desired, without excessive sperm loss inside the chip.

3.1.5.1 Surface coatings to reduce sperm adhesion to channel surfaces

To reduce the adhesion of sperm to the channel surfaces, we introduced surface coatings to the channels. Our first coating attempt used bovine serum albumin (BSA) mixed in with the sperm suspension (~2.5 mg/ml concentration). While the BSA prevented significant sticking, its presence seemed to promote the formation of air bubbles within the microfluidic channels, which interfered with fluid flow, optical trapping, and imaging.

We did not investigate the causal mechanism for this phenomenon. Rather, we changed our coating material to Pluronic® F-127 from BASF. The Pluronic was injected into the fluid channels of fabricated chips, washed out with water, and the chips were dried completely. This procedure successfully reduced the sperm adhesion to a negligible level. Figure 10 shows the results of measurements demonstrating the improvement for a Pluronic-coated chip, compared to an uncoated ship. In this experiment, sperm were pumped into the chips, and then left to sediment with no further fluid. If a sperm cell is stuck to the channel surface, it cannot be moved with an optical trap, so the ability to trap the sperm was used as a measure of adhesion. The fraction of trappable sperm was measured every 30 minutes for three hours. On the uncoated chip, the fraction of trappable sperm decreased rapidly over two hours, down to near zero over three hours. In contrast, the fraction of trappable sperm in the Pluronic-coated chip remained approximately constant over two hours, and only decreased moderately in three hours. Although the data presented in Figure 10 is for three chips of each type only, the practical functionality of the Pluronic-coated chips was repeatedly observed to be dramatically superior to either the uncoated or BSA-coated chips, in terms of mitigating both bubble formation and sperm loss through surface adhesion, and so we believe the presented data is qualitatively representative of the improvement, if not quantitatively so.

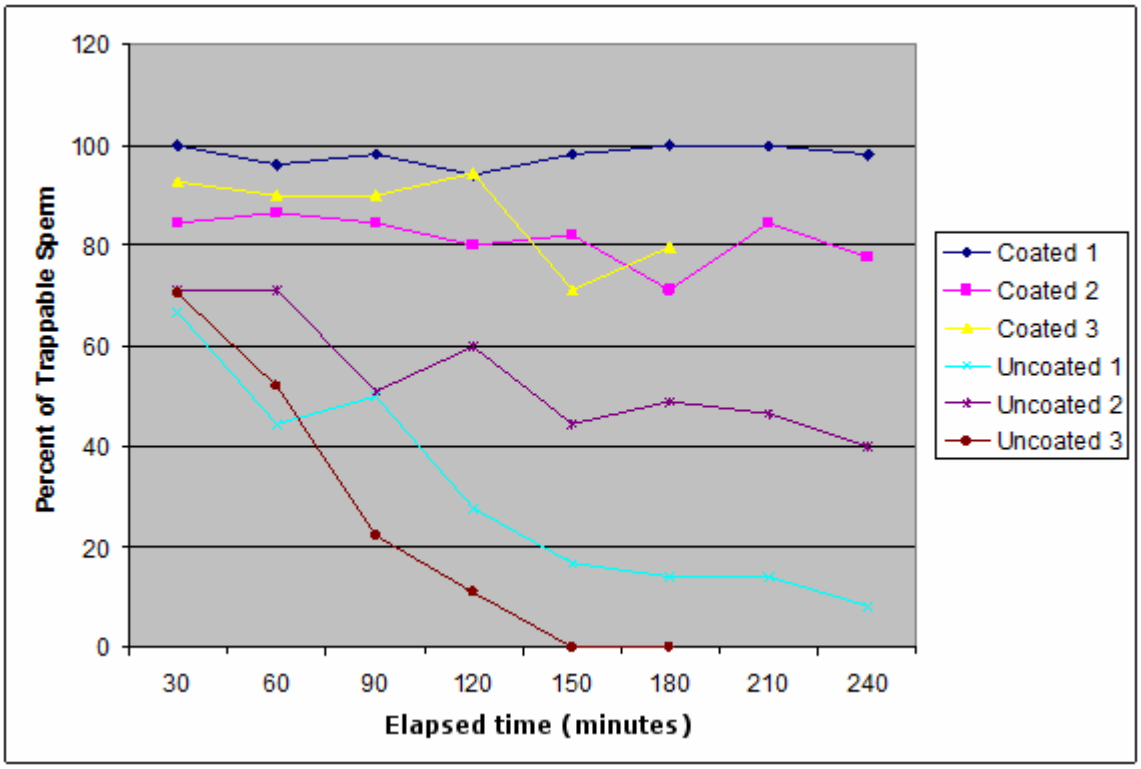


Figure 10 Comparison of sperm adhesion to channel surfaces for Pluronic-coated chips and uncoated chips. The Pluronic coating was found to strongly mitigate the sperm-sticking problem.

3.1.5.2 Modifying chip design and function to prevent sedimentation

In order to separate a sufficient quantity of sperm to obtain an STR profile, we anticipate that it may be necessary to sample fluid into the separation junction of the chip in several iterations. Our initial channel design, shown in Figure 6, contained a reservoir area for storing sample fluid after its initial introduction to the chip. However, even with anti-adhesion coating, we observed a decreased concentration of sperm between the sample inlet and the separation junction, which we found to be due to sperm sedimenting to the bottom of the reservoir area. These sedimented sperm were not necessarily stuck to the chamber walls—the problem was that after they had sedimented inside the reservoir, they were not easily re-suspended by pumping to the junction alone. This situation is shown in Figure 11a, where the sperm density at the sample inlet is clearly higher than the density in the junction.

Therefore, we modified the chip design and operation in order to reduce sperm sedimentation by keeping the fluid in motion. An initial approach was to constantly pump the fluid back and forth between the inlet and the reservoir, as shown in Figure 11b. This indeed prevented sedimentation, and produced comparable sperm density in the junction to that in the inlet. Finally, the reservoir was redesigned to allow for constant circulation of sperm in a closed channel. This design produces equivalent junction sperm density to the “back and forth” model, shown in Figure 11c. The final fluid channel layout is shown

in Figure 12. Since adopting this design, we have not experienced a high degree of sperm loss due to sedimentation.

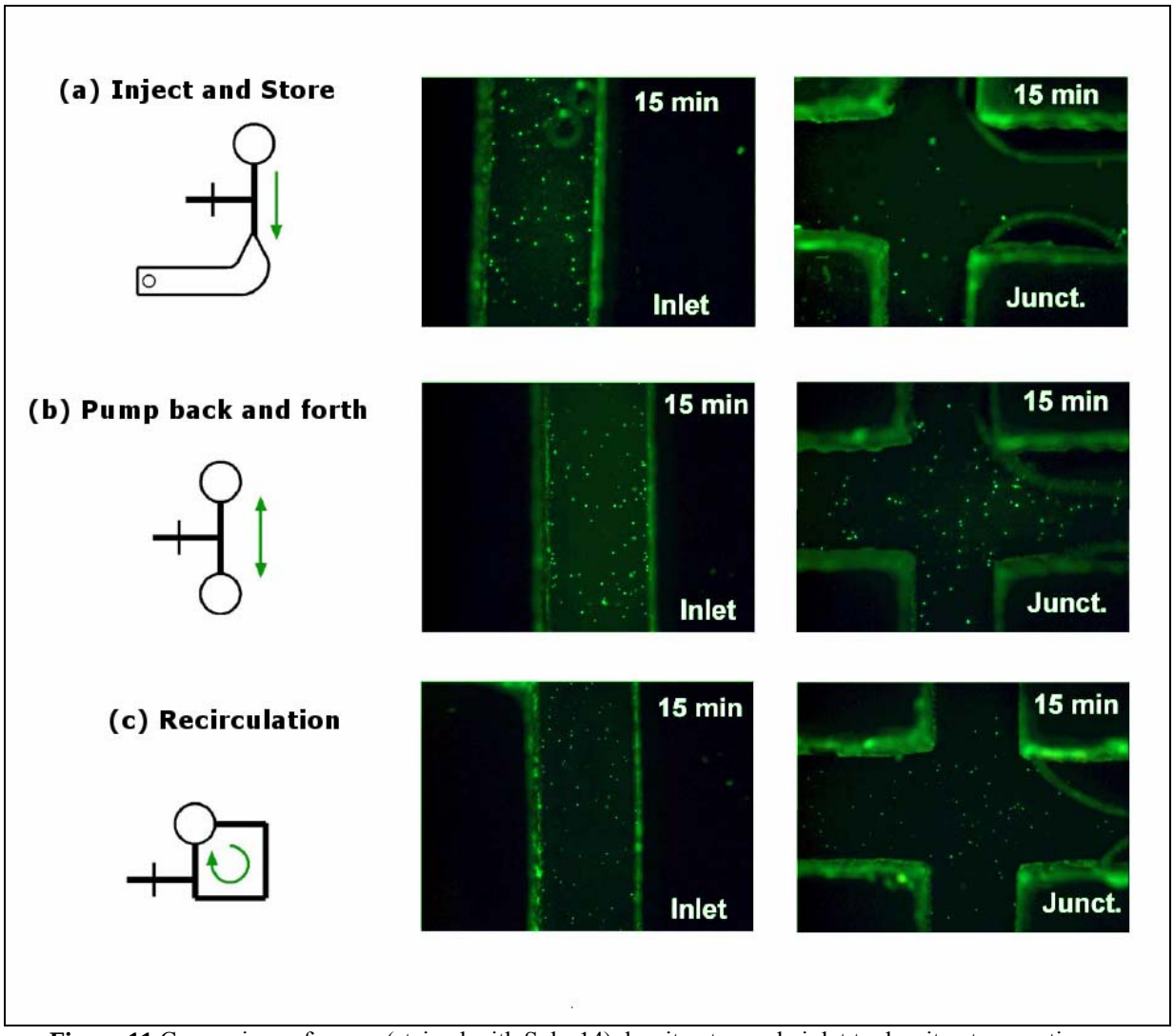


Figure 11 Comparison of sperm (stained with Sybr 14) density at sample inlet to density at separation junction. Constant circulation of the sample fluid greatly reduces sperm loss due to sedimentation. Note that the particles in (c) are dimmer due to a higher degree of photobleaching in this experiment, compared to those depicted in (a) and (b).

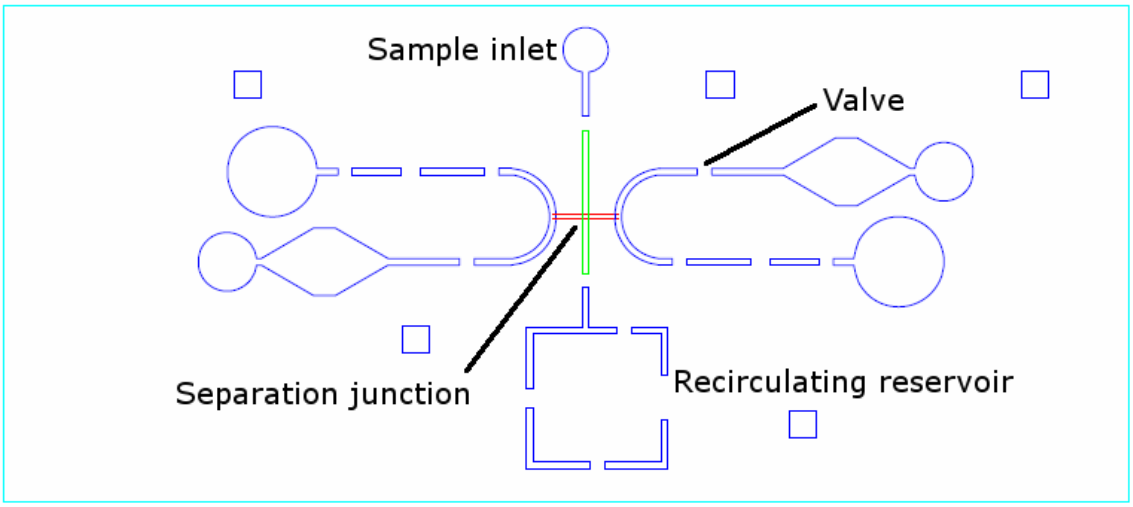


Figure 12 AutoCAD drawing of final fluid channel design, including a recirculating reservoir. The gaps in the channels represent valves. Channel widths are 300 µm.

3.2 Optical Trapping of Sperm through a Plastic Substrate

The majority of the substantial progress made during the research phase covered in this report was accomplished using glass-bottom sample chambers, fabricated in the same manner as the active chips described in Section 3.1.2. Although this fabrication method offered some key benefits for the development work done in this phase, we would ultimately like to switch to a scheme using channels directly molded in cyclic olefin copolymer (COC) and thereby eliminate the need for a separate glass coverslip. Such a development would improve the ease of device fabrication, improve device durability, and may eliminate the need for Pluronic coating of the fluid channels.

However, a potential challenge that we anticipated was that the difference in optical qualities between COC and glass could result in less-efficient optical trapping of sperm inside the device, and therefore, less-efficient processing of swab evidence. Therefore, we performed some feasibility tests to determine the extent to which optical trapping and moving of sperm might be impeded in a COC-bottomed channel.

Sperm eluted from mock forensic swabs were injected into a simple passive microfluidic chip, similar to the one described in Section 2.2.2. Operating the automated HOT system in manual mode, we trapped sperm in the separation area, and moved them to the output area. Because these tests were done at an early point in this development phase, the chip design and optical trapping configurations were far from optimal, and therefore results were variable. However, when a good sample chip—low debris, smooth channels, no leaks--was used, and when the HOT device was configured well, we were able to trap and move between 3 and 7 sperm at once over an average distance of 1.4 mm, using an average laser power of 0.2-0.4 W per trapped sperm, as measured at the laser source. In the best conditions, we were manually able to move approximately 100 sperm per hour into the output area. This rate is comparable to the 80 sperm in one hour that we have more recently achieved using automated HOT in glass-bottom samples, work which is discussed in Section 3.4.5.

Based on these tests, we anticipate that the use of COC-molded chips instead of glass-bottomed ones will not have a great negative effect on the automated system's ability to isolate sperm, although some adjustment of the automated routines will undoubtedly be necessary to obtain best performance.

3.3 Computer Image Analysis for Identification of Sperm

3.3.1 Introduction

Sperm detection software is widely used in andrology and the animal breeding industry, with the goal of distinguishing healthy from unhealthy sperm. Such detection software relies on the characteristic morphology of full-length sperm with attached tails. However, after elution--as is the case when handling sperm in the context of forensic samples--sperm often lose their tails. For efficient detection of an object using machine vision, one of the key elements is to ensure that the object that needs identification spans over a significant number of pixels on the detection device, which is typically a CCD camera. Therefore a large object that spans over several pixels on a camera is much easier to tell apart from a smaller object that is limited to few pixels. Therefore, generally speaking, an intact sperm is much easier to detect than a tail-less one as obtained post-elution. Further, the variations in the preservation of the samples and the elution methods used to prepare the cell mixture elute (which require use of different chemicals) result in significant variation in sperm morphology. This poses a challenge to developing automated sperm detection.

Nevertheless, image-based computer identification of sperm is a necessary prerequisite for an automated sperm isolation system, and so developing a strategy for machine-vision recognition of tailless sperm from an eluted forensic swab was a critical component of our work during this research phase. We initially pursued two strategies, one based on standard brightfield microscopy of eluted sperm, and one based on fluorescence microscopy of sperm that had been stained with a fluorescent dye. Although we saw some preliminary success with the bright field approach, fluorescence microscopy was found to be simpler and more straightforward, and so that is the approach we chose to use in developing the automated trapping and sperm separation methods. We intend to revisit the question of bright field sperm identification in future work, which is discussed briefly in Section 3.3.2.

3.3.2 Brightfield sperm detection

Sperm detection software modules can be developed based on intensity, contrast, and geometry, among other things. Analysis can be based on pattern matching to a library of template images using cross-correlation image methods, or can be computed based on objective algorithms that look for model characteristics and features. For dealing with unlabeled sperm, we developed a software module using normalized image correlation for sperm detection where w(x,y) is the template image and f(x,y) is the acquired image. Then, the normalized correlation coefficient is:

$$R(i,j)=\frac{\sum_{x=0}^{L-1}\sum_{y=0}^{K-1}(w(x,y)-\overline{w})(f(x+i,y+j)-\bar{f}(i,j))}{\left[\sum_{x=0}^{L-1}\sum_{y=0}^{K-1}(w(x,y)-\overline{w})^2\right]^{\frac{1}{2}}\left[\sum_{x=0}^{L-1}\sum_{y=0}^{K-1}(f(x+i,y+i)-\bar{f}(i,j))^2\right]^{\frac{1}{2}}}$$

This preliminary software for brightfield automatic sperm detection is built in National Instruments' LabVIEW and uses the NI Vision development module. The pattern-matching library is based on cross-correlation of two images, namely the template and the sample images that are to be scanned for sperm. The software provides the correlation result that parameterizes the correlation as a number between 0 and 1000 (0 is no match at all and 1000 is for a perfect match). Using an arbitrary threshold value that would accept the result as a suitable fit (possible sperm) of 700, we were able to detect most sperm in some test images, as shown in Figure 14. However, it is possible to add extra image processing to further filter unwanted objects (reject false positives). These template images can be changed easily. In the future, we will repeat the test of detection efficacy by adding more templates. Adding more templates will increase the accuracy of detection but it will also increase the time taken for detection. We will compare detection efficiency versus speed and use of different sets of templates. Another parameter that will be investigated is the threshold factor, which affects how similar an object must be to the templates in order to be identified as sperm.

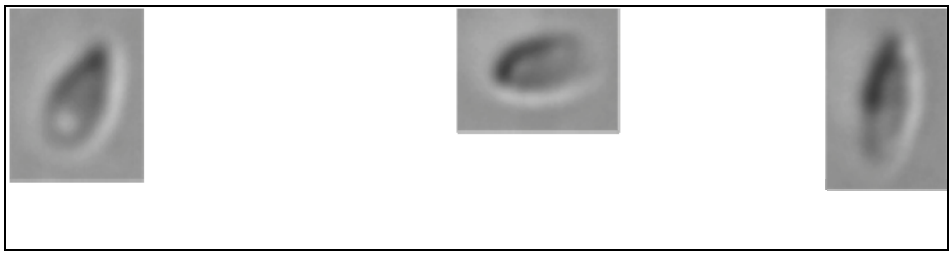


Figure 13: Three example templates that were used for brightfield automated sperm detection based on normalized correlation

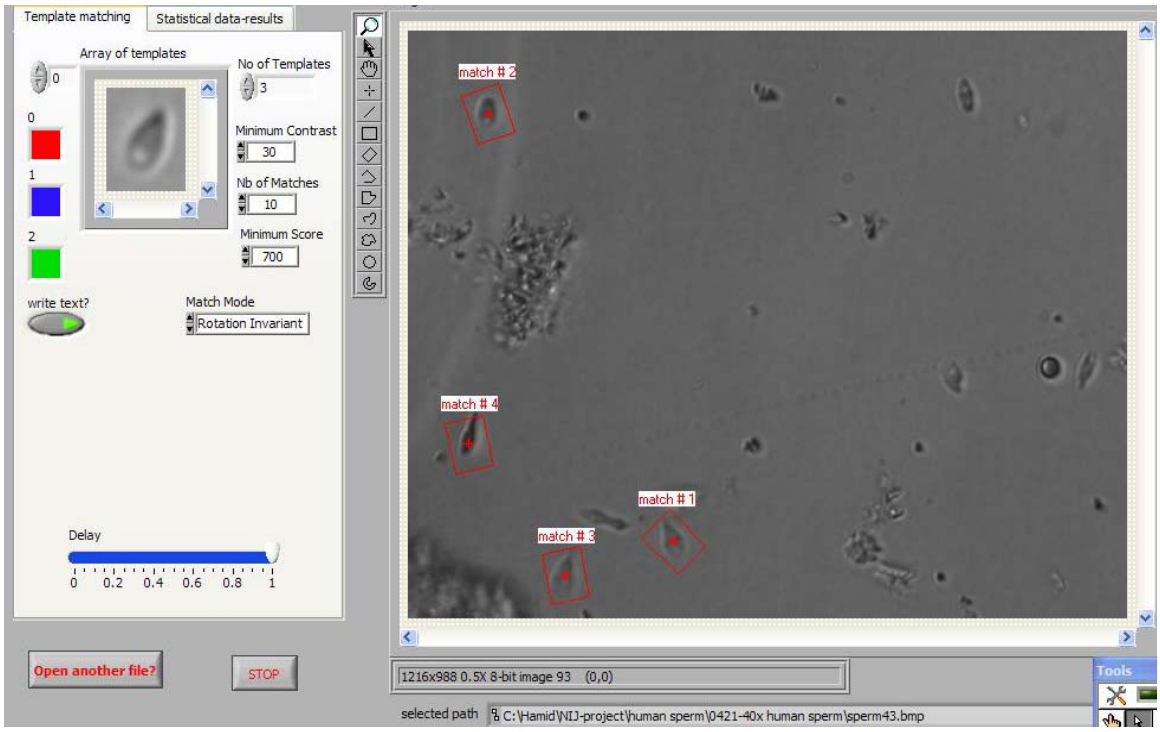


Figure 14: Sperm detection using template matching on a brightfield microscope image

3.3.3 Detection of fluorescently stained sperm

While automated sperm recognition and trapping was found to be possible with unstained samples, fluorescent staining offers greatly improved contrast and simplifies the features in an image of an eluted sample. Consequently, automated detection of sperm was found to be faster and more reliable with our fluorescence-based recognition routines than with the preliminary brightfield method discussed in Section 3.3.2. Even though such fluorescence labeling incorporates an extra step in sample handling, the overall impact is minimal given that the speed of sperm separation can be improved many-fold. Previous work, discussed in Section 2.3, indicated that PI was a good choice of dye for this application, and so the majority of our work used that. Our communications with a number of forensic laboratories have led us to believe that most laboratories are fairly open to the use of fluorescent dyes (PI being one of them) in their forensic workflows.

What follows in this section is a description of the technical issues we encountered in developing image analysis routines for identifying sperm from eluted swabs.

Image processing of swabs involves a series of steps geared toward accurately identifying fluorescently-dyed sperm amongst other DNA-containing dyed objects on a black background that may have camera noise. Some of the key challenges are as follows:

1. The algorithm must be robust against moderate variations in the intensity of sperm and other objects. Dyeing effectiveness, photobleaching, illumination intensity, camera-to-camera variation, thickness variation of the bottom chamber surface, and occluding objects are some factors that can cause sample-to-sample intensity variation. Because variations in intensity can have an effect on apparent object size as well, both intensity thresholding and object classification by size must account for a level of variation.

2. The algorithm must be robust against samples in which no objects exist. This challenge arises as a result of unpredictable object intensities. An algorithm that permits variations in intensity can easily be so sensitive that in an empty sample, camera noise fluctuations in the intensity background may be misinterpreted as objects. This was a frequent problem earlier in the project. So in addition to a requirement that objects have an intensity that is relatively greater than the surrounding background, objects must also have a minimum intensity or greater, when judged on an absolute level.

3. Epithelial cells should be distinguished from sperm. Although the design of the system is not tailored to move epithelial cells, and consequently they would not be separated into the output chamber accidentally, system performance is reduced if epithelial cell nuclei are identified as sperm. While epithelial cell nuclei are substantially larger than sperm and can be discriminated based on size, the nuclei can also illuminate non-fluorescing structures in the cell nearby, so the algorithm must be tailored as closely as possible to the characteristic appearance of sperm.

4. Yeast can appear similar to sperm under fluorescence (and often brightfield imaging as well). While they should not impact the STR analysis if separated to the output chamber with the sperm, this would inevitably result in wasted time during the sorting process. Consequently, size criteria for the sperm need be carefully selected such that yeast are unlikely to be misidentified as sperm. Nevertheless, the variation in yeast sizes makes it very difficult or impossible to distinguish correctly between the two every time, at least using fluorescence imaging and the dyes presently in use for this work.

5. Clusters of more than two or three sperm should be ignored by the algorithm, as these are likely to be stuck together and difficult or impossible to move.

6. The algorithm must be designed to handle lower-than-ideal fluorescence intensities because exposure times of half a second or more would substantially add to the process duration.

A variety of approaches have been examined during algorithm development and refined over time in response to varying hardware conditions. Magnification, illumination intensity, angles of illumination, camera exposure, camera gain, camera offset, objective correction collar adjustment, chamber bottom thickness, laser-blocking filters, and filter cube spectral characteristics all have an impact on the resulting images and affect the algorithm requirements. The software used for image processing is National Instruments' LabVIEW 8.6 Full Development System with NI Vision 2010. This package was selected for its ease of integration with other system components and multitude of image processing functionality.

The approach currently in use has five steps:

1. **Color Threshold**: This module applies a threshold to the three planes of the initial RGB image and places the result into an 8-bit image (grayscale). All pixels with low values (0-50) are then replaced with a value "1", and all remaining with "0". This essentially generates a binary image with black spots representing particles.

2. **Absolute Difference**: This module subtracts a constant value of 1 from from the image (pixel by pixel) and returns the absolute value of the difference, i.e. it reverses the input image.

3. **Low-Pass Filter**: This module applies a low-pass filter to the image, effectively reducing the size of particles and making their shapes more regular.

4. **Particle Analysis**: This module returns the number of particles detected in a binary image and a 2D array of measurements about the particle. The measurements selected are the center of mass (X), the center of mass (Y), and the particle area (size measured in pixels).

5. **Reduce Data Set**: This module rejects very small or very large objects.

Figure 15 shows an example of the process. It shows an original image, followed by the same image after key steps of the processing and analysis. The remaining objects are those identified by the software as sperm.

When applied to correctly prepared samples, this image analysis software, running on our automated HOT system, has proven capable of correctly identifying over 95% of PI-dyed sperm in a field of view. For example, of the 56 sperm cells present in the image of Figure 16, 54 were found correctly, and only one object, a cluster of two closely spaced particles, was mistakenly identified as a single particle. The efficacy of the method has been further demonstrated by a significant increase in sperm isolation speed (number of sperm moved per hour), compared to the previously used, simple particle counting algorithm which did not possess the ability to analyze background.

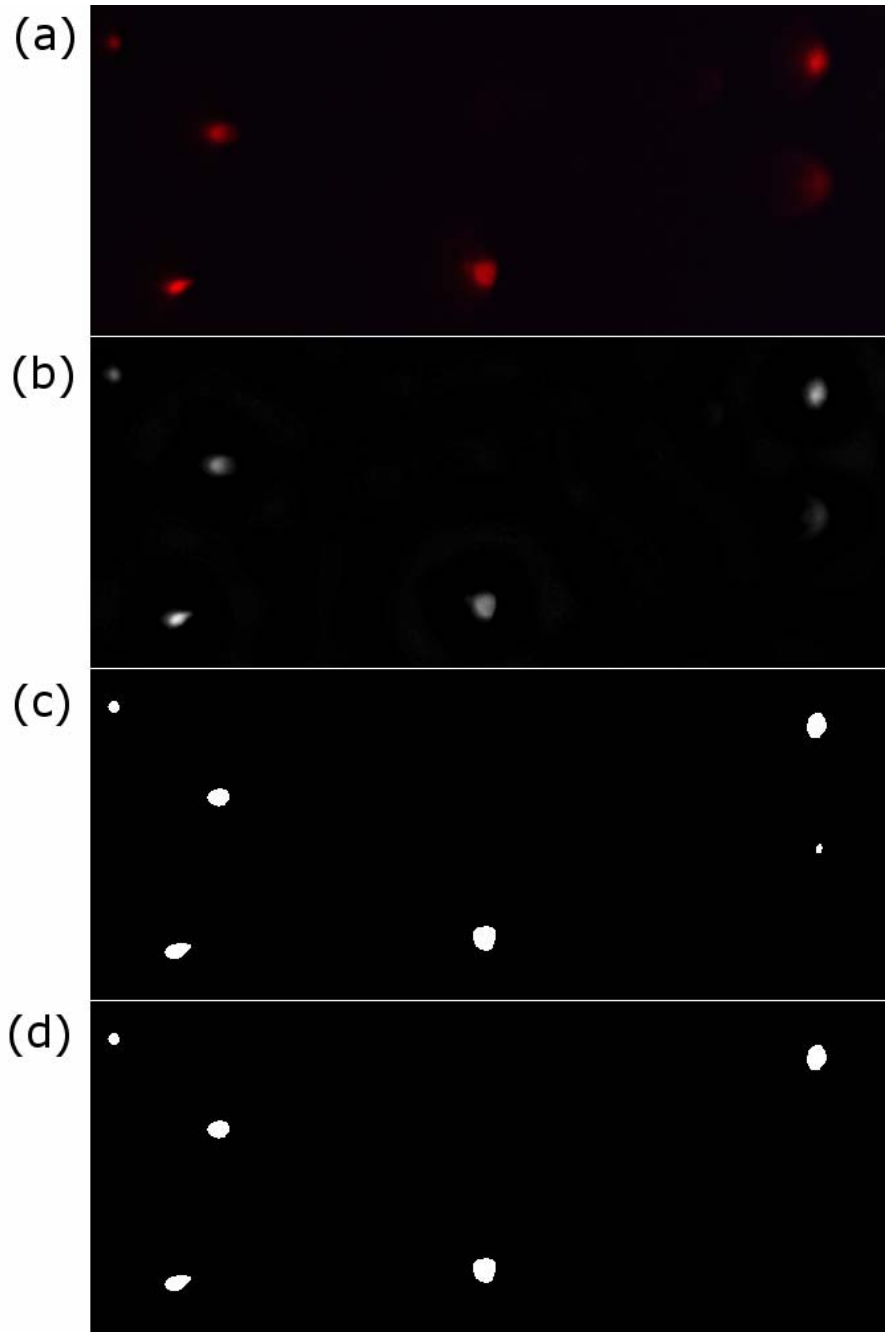


Figure 15: Example of sperm identification in a fluorescent image. The sperm are dyed with PI. (a) Original microscope image (b) Conversion to grayscale (c) Thresholding with color inversion (d) Rejection of features that do not fit the size criteria

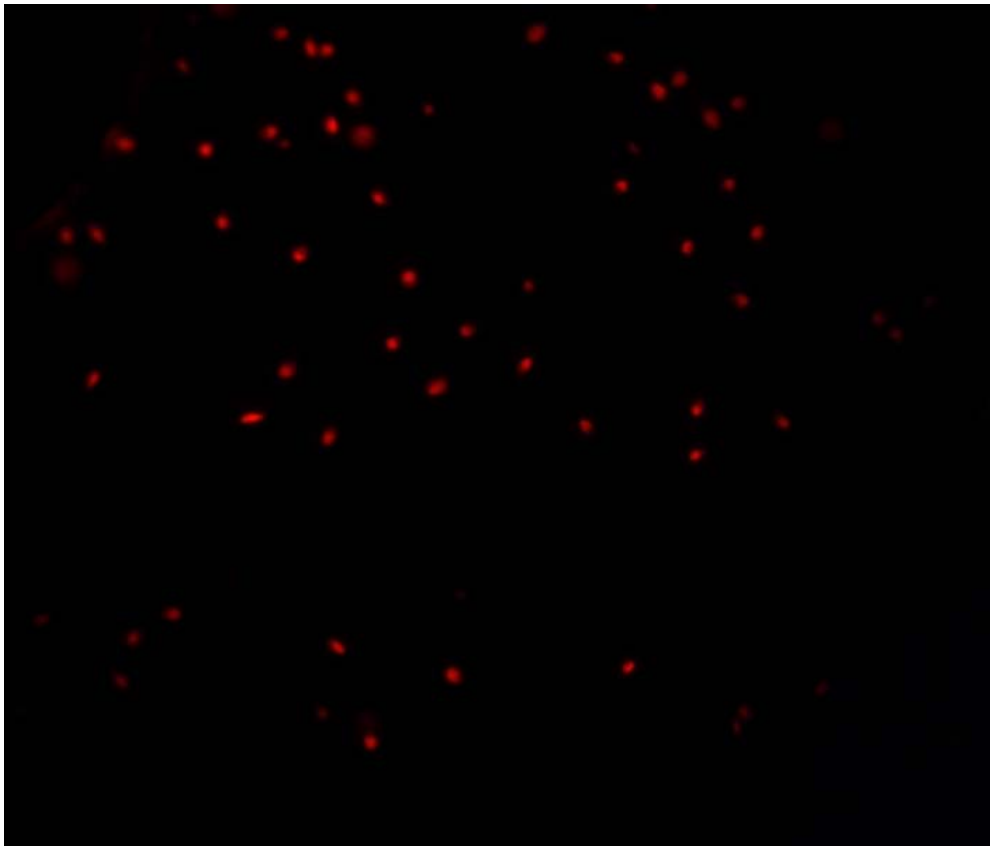


Figure 16: Typical fluorescence image obtained during the sperm isolation procedure. The variation in particle sizes, intensities, and shapes is characteristic, as is a variation in background intensity which is not clearly noticeable at this resolution.

3.4 Automated Forensic Sperm Isolation System

In conjunction with the work on sperm imaging and identification, we have developed an automated system for trapping and moving sperm within a microfluidic chip. This work leverages a pre-existing hardware platform and software architecture that Arryx developed for general automated HOT applications. Our development efforts for this project focused on creating software routines that would enable this system to identify, trap, and isolate sperm. This work involved adapting existing hardware control routines and overall software architecture to the present application, as well as the development of new routines for processes specific to processing of sperm samples.

3.4.1 Hardware platform

Figure 17 shows the automated holographic optical trapping (HOT) platform used in this development program. Its key components are:

- HOT optics module, including spatial light modulator and microscope objective lens
- Infrared laser (IPG Photonics YLM-3-1064-LP, 1064 nm, 3 W)
- CCD camera (QImaging Retiga EXi)
- Motorized XY translation stage
- Motorized focusing drive
- Broadband UV epi-illumination source for exciting fluorescent dyes (EXFO X-Cite PC 120)
- Bright field light source (Thorlabs LIU004)
- Auto-focus guidance module, including red diode laser
- National Instruments Data Acquisistion Card + electronics for micro-fluidic subsystem control
- Computer workstation for system control, data acquisition, and user interface

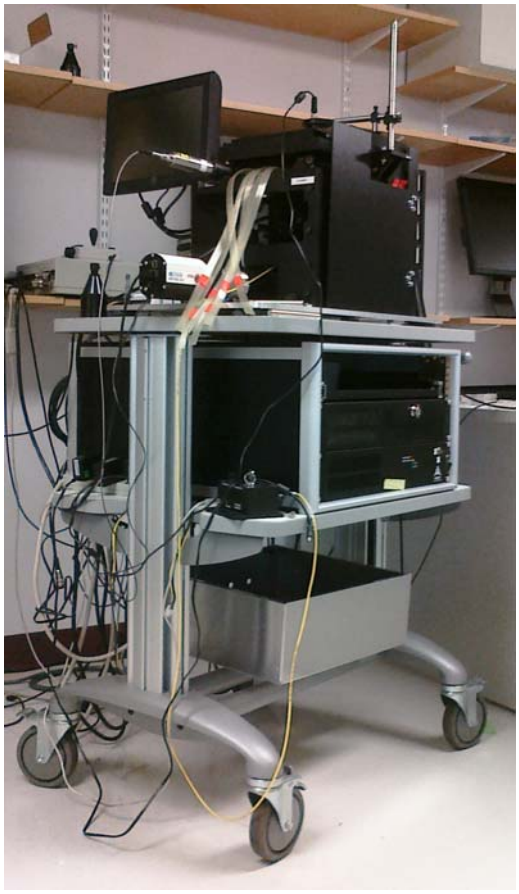


Figure 17: Arryx's automated holographic optical trapping system

3.4.2 Software architecture

The key software modules we have developed for sperm isolation on our automated HOT platform are:

- Main program (recipe-driven) for the automated execution of test sequences
- User-accessible editor for generating test recipes
- User login module with 2 access levels
- Automatic test log generation

The software architecture, which was originally developed by Arryx as a general platform for automated HOT, is shown in Figure 18. The whole system is controlled by a main progam named Forensic Automation System (FAS). FAS is a software tool capable of running several diagnostic and control functions interactively (via user accessible buttons), as well as executing sequences of commands fed into it in the form of test recipes. An independent tool for generating test recipes, named Recipe Editor, has been developed to simplify the process of designing sequences of commands needed to do automatic sperm isolation and extraction. Recipe Editor, shown in Figure 19, allows the user to select items from a comprehensive list of available instructions and place them

into the main window in the required order. Every instruction contains its name and a list of all parameters necessary to execute it. Currently, there are over 60 commands available in the Recipe Editor. These commands range from simple, one-step instructions such as “Move Stage”, “Turn LED On” , and “Turn UV On”, to macro-instructions containing long sequences of commands for executing more complicated functions. Some examples of such multi-step routines are:

- Perform Image Stitching (generates a composite image from several fields of view)
- Run Autofocus (for auto-focusing in Z)
- Find Registration Mark (for x,y alignment)
- Run Pump Sequence (for controlling micro-fluidic sub-system)

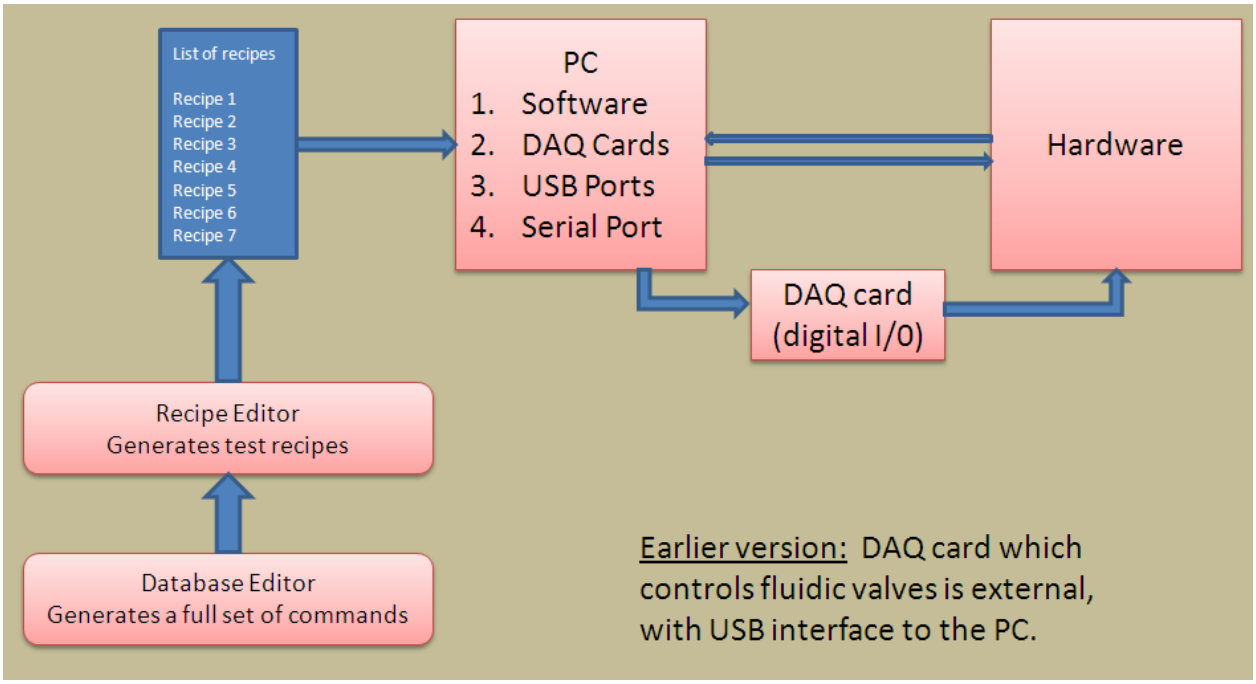


Figure 18: Forensic Automation Software (FAS) architecture overview

There is also a group of macro-instructions which initialize and shutdown hardware components such as the trapping laser, UV light source, camera, and motion stage. Additionally, macro-instructions have been designed (as separate modules) to execute just the sperm identification and extraction process. In general, a test recipe contains an initialization section, the main routine which executes sperm isolation routine, and a shutdown section at the end of the process. Once the recipe is selected and started, the process runs automatically without any need of intervention by the operator.

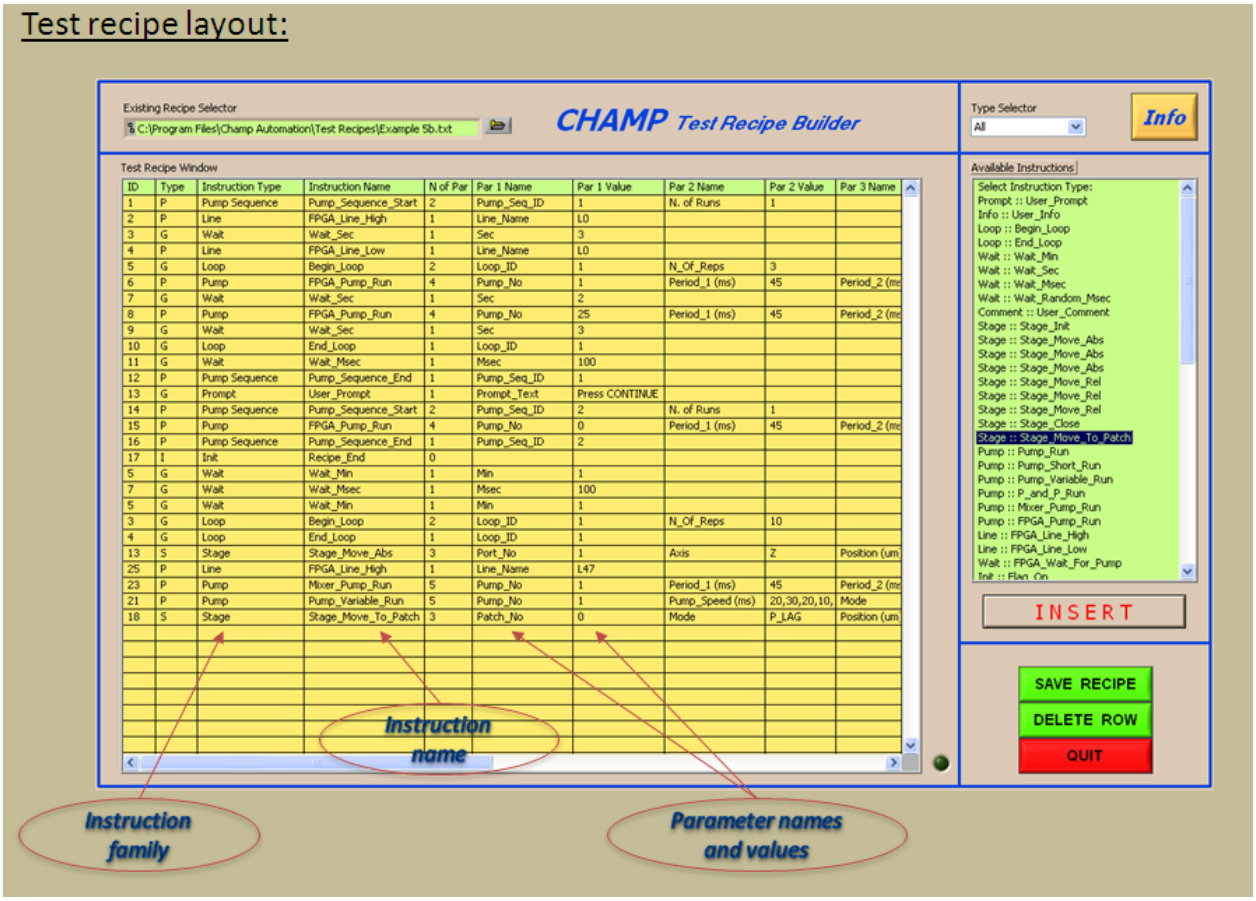


Figure 19: Test Recipe Editor interface. The list of available instructions is displayed in the green window on the right. Instructions are copied to the recipe window by a single mouse click.

3.4.3 Description of sperm isolation process

Sperm isolation is carried out in a disposable, multilayer chip with a fluid channel layout similar to that shown in Figure 20, and fabricated with materials and methods similar to those described in Section 3.1.2. Initially, the forensic evidence, i.e. fluid containing sperm, is injected from the sample inlet into the separation junction at the region in the center of the chip, shown as the vertical green channel in the figure. Subsequently, sperm are identified by means of image analysis, and optical trapping is applied to move them to either or both of the ends of the output area, which is shown as the horizontal red channel in Figure 20. When a sufficient number of sperm is collected, all are flowed to the sample outlets to be removed and submitted for STR analysis.

In software, the sperm isolation routine is carried out by a macro-instruction which, after initial system setup, automatically identifies sperm present in the separation junction and uses optical traps to transfer them to the output area. This is done in a repetitive fashion until either the required number of sperm is collected, or until there are no more sperm present in the junction. In the future, once the actively pumped microfluidics described in Section 3.1 have been integrated with the automated separation discussed in this section,

situations when the junction is completely depleted of sperm that can be moved and the number of isolated sperm in the output area is still insufficient will be dealt with by purging the input channel, pumping in more sample, and continuing with sperm isolation. The process may be repeated as many times as necessary to acquire a sufficient number of sperm for STR analysis. Sperm identification is carried out by an image processing algorithm that is capable of recognizing sperm particles previously stained with a fluorescent dye. This algorithm is described in Section 3.3.3.

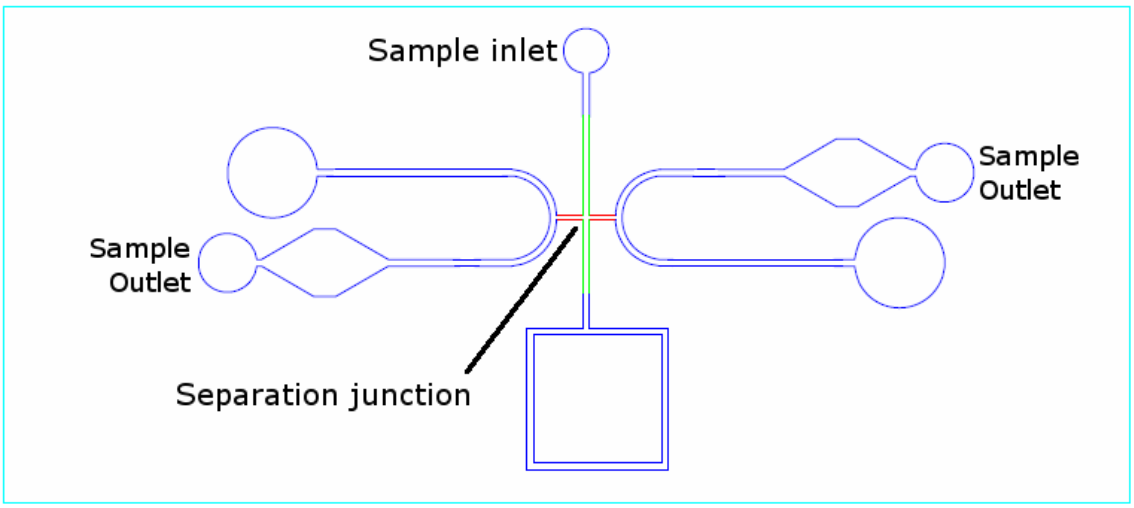


Figure 20: Disposable chip channel layout for sperm isolation. Input channel is shown in green. Output channel is shown in red. The channel layout is identical to that of the active chip shown in Figure 12, except for the absence of valves.

The software searches the input channel for sperm particles in an orderly manner, moving from one field of view to another, according to a pre-selected search pattern. Multiple search patterns have been implemented as part of the current device testing stage, since the total isolation speed--defined as the number of particles moved per hour--depends strongly on the movement trajectory and the order in which subsequent fields of view are processed.

An additional factor affecting the sperm isolation speed is the need for the software routine to return several times to one particular field of view (FOV). This is due to the fact that often the initial number of sperm available in one FOV is greater than the maximum number of traps that can be created. In the current system this number is set to four, i.e. 1, 2, 3 or 4 traps can be used to move sperm. When examining a particular field of view, the software initially determines how many individual sperm are present. Then, it keeps removing the sperm, up to 4 in each cycle, until the remaining number of movable sperm is zero.

3.4.4 Functionality of the sperm isolation routine

The sperm isolation routine consists of many steps, which are executed automatically as part of the user-selectable test recipe. When the routine is executed, it controls the

functionality of all hardware componenets. It also performs image analysis, and based on the results, makes decisions such as when to move to a new field of view, pump in a new sample, and so forth. From the functional point of view, the routine can be described as a series of logical steps, listed below:

1. Pump in a new sample
2. Determine the total number of sperm in the region of interest, consisting of a large section of the input channel and comprising multiple microscope fields of view
3. Move stage (x,y) to a new field of view (FOV)
4. Re-focus (adjust the "Z")
5. Identify sperm in the current FOV
6. Trap up to 4 sperm
7. Lift the trapped sperm above the surface by a distance "d"
8. Move the trapped sperm to the output area
9. Release the sperm by disabling traps
10. Identify and count sperm in the output area
11. Continue, or EXIT (go to "16") if a sufficient number of sperm has been collected
12. Return to the current FOV
13. Re-focus
14. Identify the remaining sperm
15. Go either to "4" (more sperm available) or to "3" (no sperm available)
16. EXIT

The routine completes execution when the number of isolated sperm is equal to the required number, or when the maximum total quantity of sample that is to be pumped in is reached. The sperm counting step (10) uses the same image analysis algorithm as step (5) in the input area. However, in this case, its accuracy is lower due to the fact that sperm are released at a height of a few μm above the surface, and it takes some time before they sediment to the bottom. Thus, the sperm count in the output area, obtained while the isolation routine still runs, must be treated as a rough estimate only. After the routine is completed, the entire output area is examined again, and the final, exact number of isolated sperm can be obtained. More details on the image analysis algorithm currently in use are given in Section 3.3.3.

3.4.5 Test results

The basic functionality of the Sperm Isolation System has been successfully tested on several passive chips, which have similar construction to that described in Section 3.1.2. Figure 21 shows the central region of the chip, where sperm identification, trapping and movement take place. As indicated in the figure, several contiguous areas of the input channel are scanned for sperm presence. These areas are defined as effective fields of view. Effective field of view is the portion of the full field of view where the trapping strength is sufficient to lift the sperm above the surface and move them to the output area. The most important test parameters are listed in Table 2.

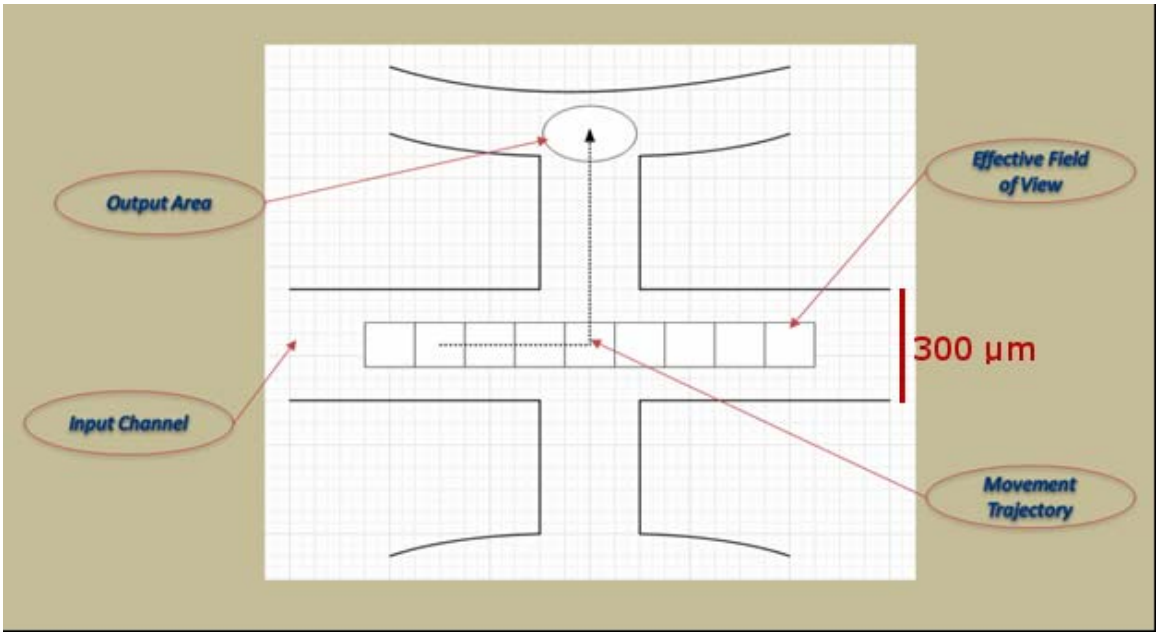


Figure 21: Central sperm isolation region of microfluidic channels. The dotted line indicates the sperm movement trajectory from a given field of view to the output area.

Table 2: Typical values of test parameters for Sperm Isolation System operation

	Parameter Name	Parameter Value	Remarks
1	Input Channel Width	300 µm	This is a nominal value. In practice the width is larger by 50-100 µm.
2	Output Channel Width	250 µm	This is a nominal value. Usually the width is larger by 50-100 µm.
3	Stage Movement Speed	35 µm/s	Higher speeds may be possible.
4	Number of Fields of View (FOVs) Tested	20-40	
5	Trap Lift Height	10 µm	This is a typical value. More testing is needed to determine the best value.
6	FOV Area	367µm x 275µm	FOV for imaging
7	Effective FOV Area	140µm x 222µm	Effective FOB for trapping
8	Movement Trajectory Length	>1400 µm	Depends on the FOV selected.

3.4.5.1 Isolation speed

The final, most important result is the overall sperm isolation speed, defined as the number of sperm that can be moved per hour from the input channel input to the output area. When this capability initially became operational, the system's best performance was moving 60 sperm in 1.5 hours, or IS = 40 sperm/hour. After improving the search and trapping routines, and adjusting system parameters, we have been able to isolate up to 80 sperm in one hour, from a fairly dense sample, under the conditions given in Table 2. Although the isolation speed is expected to be lower after the first hour, we also expect that, as the hardware and software are developed further, this rate can be improved.

3.4.5.2 Isolation efficiency

For the purpose of system characterization, another property named Isolation Efficiency has been defined. Isolation efficiency (IE) is the ratio of the total number of sperm moved (NM) to the total number of sperm trapped and attempted to move (NT). The isolation efficiency can be expressed by the following simple equation:

$$IE = \frac{NM}{NT}$$

Typical IE values obtained so far are approximately IE = 0.5. Thus, about 50% of the sperm initially identified and trapped are transferred to the output area. At this point it is not possible to determine whether the efficiency obtained is the best for the current system. As in the case of isolation speed, isolation efficiency depends on many factors and more research is needed to understand and evaluate all physical/chemical phenomena occurring during sperm trapping and movement. Some of these issues are discussed in Section 4.1.2.

4 Conclusions

4.1 Summary and Discussion of Results

During the research phase covered by this report, we have made significant progress in several key areas towards the development of a holographic optical trapping-based device for forensic cell isolation. A credible foundation for development of a commercially viable automated system has been created through development of a suitable microfluidic chip with active fluid control, a sperm-identification strategy combining fluorescent staining with customized image analysis software, and hardware automation software that allows test routines to be quickly created and modified.

4.1.1 Microfluidic chip

In the area of microfluidics, we created a multilayer microfluidic device that incorporates on-chip active valving and pumping to control fluid flow throughout the chip. It can move a consistent quantity of sample from the fluid input to the separation area, flow the separated sperm from the collection area to an output area, and continually circulate untested sample to prevent sedimentation, as well as preventing undesired cross-flow between the input and output channels. The addition of Pluronic coating to the channels decreases sperm adhesion to the channel walls to a manageable level.

While this chip design is effective, there is still room for improvement, both in terms of performance and manufacturing. The five-layer design discussed in Section 3.1.2 allows for easy modification of the flow channels, but it also introduces more opportunity for contamination, and thus requires more cleaning of all the pieces prior to assembly. More layers makes assembly itself more difficult and prone to variation, and the need to coat the channels with Pluronic after assembly adds an additional degree of difficulty. Therefore, we intend to move to a three-layer design, in which the fluidic layer is press-molded as a single layer of optically clear COC plastic, as well as consolidating the top two PMMA layers. Our tests on trapping sperm inside simple COC chips indicate that changing the fluidic layer substrate in this way will still allow for sufficiently strong optical trapping to trap and move sperm, and there is reason to believe that it may eliminate the need for the Pluronic channel coating.

4.1.2 Sperm identification and system automation

The software package named Forensic Automation System (FAS) for the automated detection and isolation of sperm stained with a fluorescent dye has been developed and tested on several passive chips. The image processing algorithm devised for sperm identification and counting has proven to be very efficient. Over 95% of the sperm

present in each image taken are routinely recognized, regardless of variations in shape and fluorescence intensity. Initial tests show that isolation speeds of 80 sperm in one hour can be achieved. The whole device still requires a significant amount of development in the areas of process optimization and micro-fluidic subsystem control. In particular, the active-control microfluidic system must be integrated with the HOT system, so that fully automated processing of samples can be achieved.

It has been found that both isolation speed and isolation efficiency depend on many factors which need to be thoroughly studied and understood. Some of these factors are:

- **Movement Trajectory (Z component)**: The distance "d" between trapped particle and the underlying surface determines the trap strength and also probability of collisions with other sperm and/or any debris stuck to the surface. Larger values of "d" make it easier to carry the trapped sperm over any surface-bound objects but at the same time the traps become weaker, thus it is easier to drop the sperm. The optimum value of "d" has to be determined.

- **Movement Trajectory (XY component)**: The order in which fields of view are depleted of sperm (i.e. the scan pattern) is a very important factor affecting the final isolation speed and efficiency. Best results are achieved if the areas closest to the chip center are scanned first.

- **Movement Speed**: Human sperm cells, due to their asymmetric shape, are not easily trapped objects. They can be often dropped due to fluid drag from the suspending fluid. Optimum movement speed for every height "d" that may be used must be determined.

- **Chip Manufacturing Process and Quality**: It is very important to make sure that input and output channels are free of any debris that might be produced during chip manufacturing. Such unwanted objects become obstacles in the trap trajectory and significantly reduce isolation efficiency. The ratio of "unstuck" to stuck sperm, determined by the surface chemistry, is another factor affecting isolation speed and isolation efficiency. Stuck sperm become obstacles and effectively make the movement trajectories longer for the "unstuck" sperm, since fields of view which are more distant from the separation junction have to be examined. Although the addition of Pluronic coating has mitigated the sperm-sticking problem to a level where we can trap and isolate a nontrivial quantity of sperm, there is still room for improvement. We have reason to believe that the planned modifications to chip manufacturing discussed in Section 4.1.1 will lead to improvements in both these areas.

4.2 Direction for Future Research and Development

The research and development to date have gone far toward developing a device suitable for daily use in forensic labs. The next stage of the process, which will take place under grant 2011-DN-BX-K562, addresses the remaining issues involved in developing a robust system suitable for integration with varied forensic lab workflows. This will require improvements to most aspects of the system.

To maximize usefulness to a forensic lab, ideally the device user will be given a flexible platform that permits selection of an imaging and labeling strategy. The user would select from a list of established protocols or use a protocol that the lab develops and validates itself. Through our research, we have identified promising approaches for separating both labeled and unlabeled sperm. While we believe that labeled sperm are easier to discriminate, it would be valuable for the device to handle unlabeled samples as well for labs that cannot or do not wish to use fluorescent dyes. Since stained samples have a huge difference in their contrast, brightness, and distinguishing characteristics compared to unstained samples, the detection approach for the two methods will vary in many ways. Additionally, for labeled cells, the choice of dyes is critical, as the efficiency of labeling, the ease and speed of labeling, the dye stability, and the dye quantum yield all play important roles. Upcoming development, then, will explore more robust software algorithms for identifying both labeled and unlabeled sperm, as well as modify system hardware and possibly dye selection so that the same device can reliably use fluorescent or brightfield imaging methods, individually or simultaneously.

One focus of the research will be on minimizing loss of sperm during processing and accurately counting the number of sperm in the output. Inaccurate DNA quantitation often leads to PCR failures because the reagents in the PCR mix either greatly exceed the amount needed for the quantity of DNA actually present, or are in much lower amounts than are required to carry out the amplification. This wastes reagents as well. However, an accurate sperm count based on machine vision can lead to much better estimates of DNA expected post-extraction. Ensuring that the system is tailored to provide accurate estimates of DNA in the output will be an important development goal.

A great deal of effort will be required to bring the current pre-prototype device to one that could work largely maintenance-free in a lab for months or ideally years at a time. On both the hardware side and the microfluidic disposable sides, many imperfections presently preclude long-term reliability. All aspects of the hardware need to be examined for durability and process repeatability and improved upon where necessary. This includes the opto-mechanical components, camera, and various control electronics. On the software side, in addition to the sperm detection approaches mentioned above, autofocusing algorithms will be improved to allow more accurate focusing both initially and during the separation process. Further integration of the software steps involved in the separation process, from pumping to imaging to separation, will be necessary.

Once good hardware and software are in place, SOPs for integration with differing lab workflows will need to be tested extensively and optimized. Robustness against the wide variation in sample characteristics will be an important component of this testing. Through consultation with forensic labs, software and hardware user interfaces will be developed to minimize the chance of user error and the skill level required to use or maintain the machine.

4.3 Implications for Criminal Justice Policy and Practice

If eventually realized as a commercial device, the cell isolation technology presented in this report can be integrated into existing sample processing workflows to improve genetic identity testing of sexual assault evidence by:

1. Automating the isolation of sperm from all other cells and free DNA fragments, and thereby automating the fractionation process, which is often done manually
2. Preventing carryover of victim DNA into the assailant DNA fraction, via improved separation
3. Obtaining reliable STR results even in cases where DNA from non-sperm sources (e.g. epithelial cells) would normally overwhelm the sperm DNA,
4. Obtaining visual measurements of the cellular composition of a forensic sample including sperm and epithelial cell counts,
5. Obtaining STR profiles using fewer sperm thereby enabling the processing of samples with very low sperm count and also preserving a greater portion of evidence for repeat testing if necessary
6. Reducing chances for evidence contamination, since processing is done in a closed chamber
7. Obtaining video-based and image-based screening, quantitation, and record-keeping of the sample and how it is processed (e.g. quantitation of cell counts of epithelial cells and sperm).

Automation will reduce human intervention which has three possible implications: reduced overall cost by lowering labor cost, shorter processing time and increased overall throughput and productivity, and decreased chance of human error in sample handling.

Our past work[18] demonstrated the compatibility of HOT with downstream PCR-based STR profiling. Optical trapping of sperm has been shown to cause no discernable damage to their DNA.[19, 20] By using automated trapping in microfluidic chambers, one can simultaneously characterize and sort a forensic sample, providing feedback such as cell counts and allowing unused sample to be preserved (e.g. sperm cells beyond the several hundred required for downstream analysis). Further, the output given to the downstream process may be controlled by delivering a precise cell count. Alternatively, if a successful STR profile is not possible, one can identify the source of the problem—likely in advance--such as having fewer cells than required. Since nearly one in five unsolved sexual assault cases involve unanalyzed DNA evidence,[3] and approximately 19% of analyzed samples result in victim DNA crossover,[5] the practical impact of this

technology can be dramatic. Automation and enhanced overall speed and throughput of the separation technology will contribute to solving the current DNA evidence backlog problem, which, as of the end of 2009, stood at over 110,000 unprocessed cases, and trends suggest that it has only grown since then.[2]

The ability to perform the sorting in an enclosed microfluidics cartridge opens new vistas for integration of the sperm sorting process with downstream DNA extraction and PCR, such as performing on-chip PCR.[14] Since HOT offers the ability to handle and analyze single cells, a variety of single cell analyses including DNA analysis[36] may be achievable using automated HOT and on-chip assays. Such applications would involve a significant extension of the proposed plan and are currently beyond the scope of the proposal in the defined time frame. However, the ability of HOT to handle and analyze single cells provides exciting long-term opportunities. Thus, the HOT technology not only promises near-term advances, but forms a platform for even greater advances moving forward. Various applications outside the area of sexual assault evidence sample handling in the forensics field, in which precise and automated sorting is needed, could also benefit greatly from a microfluidics platform on an automated HOT instrument.

5 References

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6 Dissemination of Findings

We plan to present the results of our work at national and regional meetings relevant to forensic science, such as those organized by AAFS (American Academy of Forensic Sciences) or the Symposium on Human Identification sponsored by Promega. We recently presented a poster on our work at the annual NIJ conference in June 2011 in Arlington, VA. Additionally, our work was presented by the principal investigator, Dr. Chakrabarty, at several separate meetings with a number of forensic lab directors and/or their teams, namely Dr. Eric Buel of Vermont, Joanne Sgegulia of MA crime lab, Dr. Roger Kahn of Harris County, Texas, Dr. Rick Staub and his colleagues at Orchid Cellmark. Dr. Chakrabarty has also discussed her work with other forensic scientists such as Dr. John Butler from NIST. We have also presented work-in-progress at internal meetings and discussed our work with various forensic scientists throughout the course of the project. We maintain close communication with our collaborators at Orchid Cellmark 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 presentations at both the 2010 and 2011 NIJ annual meetings were received with great enthusiasm and garnered a lot of interest in our HOT technology and its unique application in forensic research and practice. In order to eventually build a commercially viable automated machine, we have initiated discussions with an optical device design and manufacturing company in California. The PI recently attended the 2011 AAFS meeting in Chicago and the DNA mixtures workshop in order to further expand her interaction with forensic lab practitioners and for evaluating the performance and implication of the described work in typical forensic lab workflows.

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 fabrication of microfluidic devices and on the development of various automation modules. Most recently, the principal investigator attended the 2011 LabAutomation Conference in Palm Springs, CA. Such meetings will continue to provide wider exposure for our work and may generate broader interest in HOT and forensic science. This strategy allows 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. Arryx will also support the installation of a HOT-based instrument at Orchid Cellmark for testing. The principal investigator has also initiated the process for validation studies and understanding the requirements for working with NFSTC. Arryx 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. Arryx's past experience with local companies with business focus in forensic analysis will also benefit the dissemination of knowledge about this exciting technology.

Significant new inventions conceived during the course of the proposed work have been filed in patent applications in accordance with existing guidelines. Most recently, two patent applications have been submitted to the U.S. Patent Office for review.