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Optimization of a Microfluidic Device for Cell Capture Using Optical Trapping

Samantha C. Pagel

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Williams and Dawson Green Forensic Molecular Biology Research Laboratories
Virginia Commonwealth University, Richmond VA
Department of Forensic Science

Sarah J Seashols-Williams, Ph.D.
Graduate Program Director and Associate Professor
VCU Department of Forensic Science

Joseph Reiner, Ph.D.
Associate Professor Experimental biophysics, Experimental nanoscience
VCU Department of Physics

Tracey Dawson Green, Ph.D.
Professor & Department Chair
VCU Department of Forensic Science

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Abstract

Biological mixtures which result from evidence produced in sexual assault cases are one of the most common types of mixtures observed in forensic biology casework and have contributed to evidence backlogs across the country. In the forensic community, this has highlighted the need for a more efficient cell separation method at the beginning of the DNA analysis workflow than the currently accepted method. One promising novel front-end cell separation technique is optical trapping. In heterogeneous mixtures, optical tweezers have been demonstrated to manipulate and isolate cells of interest from other cell types.

For this research, a range of 5 to 25 sperm cells were isolated in triplicate from 1:20 diluted neat semen or a male-female mixture of sperm and resuspended vaginal epithelial cells. Spermatozoa were isolated using a previously designed microfluidic device and an optical trap produced from a 700mW, 1064nm continuous wave laser. The average amount of time spent trapping per sperm cell was 2.30±1.17 minutes for the diluted semen samples and 2.90±1.40 and for the mixture samples. None of the average quantified total DNA yields were statistically different from the theoretical yields, and little or no degradation amongst trapped samples was indicated. When a simple linear regression was fit to the sample data, the number of sperm cells optically trapped explained >56% of the variability observed in the percentage of expected alleles for both sample types.

Using an updated microfluidic device design and a 5W ytterbium linearly polarized laser split into two optical traps, sperm cells were again isolated from a 1:20 diluted neat semen solution. The average time per cell spent trapping decreased to 1.38min/cell. Average total DNA yield was not significantly different from the calculated theoretical yields, and the average degradation index indicated that no DNA degradation was present in the optically trapped samples. On average 97.37 ± 2.63% of the expected alleles in the samples were observed. Additionally, resuspended vaginal epithelial cells were able to be captured with the dual optical trap setup using an attenuated laser power output.

This research has demonstrated with the use of a dual optical trap setup, a sufficient number of spermatozoa to generate a full STR profile can be captured after approximately 1 hour of optical trapping. The use of a microfluidic device minimizes the possibility for contamination events and allows for easy transfer of trapped cells to subsequent DNA analysis steps. Further, this research has demonstrated the ability to capture vaginal epithelial cells, which could eventually allow for dual capture and isolation of forensically relevant cell types on a single microfluidic device. Overall, optical trapping is a promising alternative technique for isolating forensically relevant cells of interest, such as spermatozoa, compared to the traditional differential cell lysis method.

Keywords: optical trapping, microfluidic device, laser, spermatozoa, vaginal epithelial cells, differential cell lysis, cell separation

Introduction

The 2009 National Academy of Sciences report 'Strengthening Forensic Science in the United States: A Path Forward' stated that for all individualization techniques used in forensic science, analysis of nuclear DNA was what set the standard for objective unbiased analysis (1). However, the field of forensic biology is not free of complications. Mixture profiles are generated when more than one individual has deposited their DNA on a collected sample. Interpretation of DNA profiles with more than one contributor is one such area of forensic biology that is a continuous challenge for the field. Variations in interpretation of mixture profiles are known to exist both between laboratories and amongst analysts within the same laboratory (2–4). Advances to DNA analysis technologies over the last few years have led to an increase in the observance of mixture profiles due to increased sensitivities of the technology that allow for the detection of low-level contributors. Often, in two-person mixtures, if the major component can be clearly differentiated from the minor component, individual profiles can be easily deconvoluted by a DNA analyst. However, not all mixtures have clear major/minor contributors, and some mixture profiles may be complex (i.e., consisting of three or more contributors), which can make individual profile deconvolution challenging or impossible.

One of the most common types of DNA mixtures observed in crime laboratories are those that come from evidence in sexual assault cases which consist of spermatozoa (sperm) and epithelial cells. In 2019, the National Crime Victimization Survey reported 459,310 rape/sexual assault incidents which made up 33.9% of violent crimes reported for the year (5). Studies have found that the majority of individuals who perpetrate rape or sexual assault crimes, and have not been convicted, are repeat offenders of the same crime and commit other acts of interpersonal violence (6). In addition, those who are convicted and released have been found to be more likely

than other released prisoners to be rearrested for rape or sexual assault (7). The annual high rates of reported sexual assaults, as well as the lengthy turnaround time for such cases has led to a sexual assault kit backlog in several states. While Virginia has since eliminated its sexual assault kit backlog (8), in 2018 the Department of Forensic Science (DFS) reported a 25% increase in submissions to the forensic biology section which resulted in a backlog of all cases submitted to the department (9). Virginia's DFS has handled the increased workload by streamlining the screening of kits and adding more trained DNA analysts to the workforce, but not all states have the resources to do this. State backlogs, as well as the known repeat offense and recidivism rates for rape and sexual assault, highlight the need for a more efficient processing method at the beginning of the DNA analysis workflow to handle biological mixtures that often result from sexual assault cases.

Currently Accepted Methods for Handling DNA Mixtures

Currently, the most common and widely used method for cell separation is a differential cell lysis. Differential cell lyses separate sperm and epithelial cells into two separate fractions by exploiting differences in the stability of the cell membranes. Epithelial cells, presumed to be primarily from the female contributor, are first lysed using proteinase K and sodium dodecyl sulphate (SDS) reagents (10). After an incubation period and centrifugation, sperm cells pellet, and lysed epithelial cell components (including the DNA) are removed to create the non-sperm fraction. Sperm cells are then washed before being lysed with proteinase K, SDS, and dithiothreitol (DTT) – a reagent which breaks down the disulfide bonds that make sperm impervious to proteinase K – creating the sperm fraction (10). Techniques such as organic or silica-based methods are used to isolate and purify the DNA present in each fraction (10). The

two purified cell fractions are subsequently carried through the rest of the DNA analysis workflow.

Unfortunately, differential cell lysis does not always result in two complete single-source profiles. Carryover of epithelial cell DNA from the female contributor has been observed in the sperm fraction DNA profile after a weak initial lysis or insufficient washing of the sperm pellet (11). Sexual assault cases are not always reported immediately after they occur, and therefore the collection of samples may be delayed. Time, as well as a harsh initial lysis step, can compromise sperm cell membranes and cause premature lysis of sperm cells resulting in male contributor DNA possibly being detected in the non-sperm fraction DNA profile (11,12). Further, the differential cell lysis process is typically performed manually, has been difficult to automate, and may give variable results depending on the skill level of the analyst performing the technique. Differential cell lysis is time consuming and can cost even more time and resources when there is inefficient separation resulting in the need for downstream mixture deconvolution.

Probabilistic genotyping is a method used by some crime laboratories that can be used at the end of the DNA analysis workflow to help with the interpretation of DNA mixture profiles, as well as degraded and low copy number samples. Instead of the traditional inclusion/exclusion binary method of interpretation, probabilistic genotyping software calculates the probability of different genotype combinations for competing hypotheses at each locus based on the alleles that are present/absent (13). There are two main approaches to the probabilistic method: semi-continuous (discrete) and fully continuous. The semi-continuous approach does not consider peak heights for probability calculations, whereas the fully continuous approach considers peak height ratios, mixture ratios, and stutter percentages using Markov-chain Monte Carlo algorithms (13,14). Both approaches consider the possibility for drop-in and drop-out alleles (13).

Given that probabilistic genotyping software is a more recently adopted method by the forensic community and involves complex bioinformatic algorithms, the approach has been subject to several court admissibility arguments in the last decade. Some software systems are open source, while others consist of a proprietary source code that can only be made available to attorneys upon request by the court (14). Different probabilistic genotyping software systems have been found to give variable results between and within laboratories when analyzing the same mixture sample due, in part, to subjective decisions made early on by analysts and/or the underlying source code of the software (2,4,14). A crime laboratory wanting to implement probabilistic genotyping into their workflow will have to spend extensive time and resources (usually monetary) to obtain, internally validate, and train analysts on the software. Even once this capital is spent, many analysts struggle to understand and/or coherently explain probabilistic genotyping in court due to its complex 'black-box' nature. While some concerns over probabilistic genotyping can be solved with uniform practices, the need for a front-end separation method prior to DNA extraction that can eliminate time intensive back-end mixture interpretation remains.

Other Cell Separation Methods

Several methods for cell separation of sexual assault type samples have been developed over the past two decades. One method employs the same preferential lysis steps as a traditional differential cell lysis, but instead of washing, the sperm pellet is treated with DNase I to remove residual epithelial DNA from the fraction (15). While this nuclease method is promising for producing single source male profiles, the method yields lower quantities of DNA than the standard differential method and there is risk for degradation to the male DNA if the nuclease is not completely inhibited or removed before sperm lysis (15). Acoustic differential cell lysis

methods trap sperm cells as they enter a standing wave set at a specific frequency. Similar to a differential cell lysis, the acoustic trapping method requires prior lysis of the epithelial cell component before sperm cells can be separated from the mixture; trapping is based on the size and density of the particle (16,17). Norris et al. found that to produce an interpretable short tandem repeat (STR) profile, up to three trapping nodes must be generated to capture a sufficient number of sperm cells (16). Norris et al. also stated that the epithelial cell lysate could be a source of contamination if the lysate became trapped with the sperm cells (16). Clark et al. noted that variance from the optimal frequency of the standing wave resulted in sample loss (17).

Magnetic bead-based separation methods use immunomagnetic beads (IMB) with a conjugated, sperm-specific antibody to isolate sperm cells from a mixture. Zhao et al. was able to obtain single-source male profiles from a 100:1 epithelial to sperm cell mixture using anti-PH-20 coupled IMBs (18). Anti-PH-20 was found to be more sensitive than previously tested antibodies, however, this method was not tested on aged forensic type samples which are known to affect sperm isolation efficiencies when using antibody-based methods (18,19). Additionally, researchers have used a bio-inspired oligosaccharide called Sialyl-Lewis^X (SLeX) attached to magnetic beads to capture spermatozoa in 25 minutes with >81% capture efficiency (20). However, this method has had issues with non-specific binding of epithelial cells (8.33-16.67%) (20).

Fluorescence activated cell sorting (FACS) uses immunofluorescent tagging of cells to target specific cells in a mixture. Single cells are interrogated with a laser, which results in the scattering of light in all directions. Cells are sorted based on how the light is scattered and how population-specific gates are assigned during calibration (21). FACS has been used to separate forensically relevant mixtures of blood and saliva, however, the separated samples did not

always result in single source profiles (21). Similarly, Xu et al. used anti-AKAP3 immunomagnetic beads to separate a mixture of vaginal epithelial cells and two seminal fluid donors (22). Separation of sperm cells via magnetic beads was followed by FACS separation of the two male contributors based on blood type (22). Successful separation of the male contributors' DNA was highly dependent on the ratio of cellular components, as well as secretor status and disparate blood types of the contributors (22).

Dielectrophoretic (DEP) trap arrays manipulate single cells using an array of electrodes which together create a nonuniform electric field (23). With this method, cells of interest are first stained and fixed using fluorescent tags before being moved to an isolation area by the DEP cages (23). This method relies on the dipole properties of cells to trap, sort, and isolate cells of interest in the field (24). When sperm cells from a mock sexual assault mixture were targeted using DEP, Williamson et al. was able to obtain single source profiles 96.2% of the time and identified a 1:10,000 dilution of neat semen to buccal epithelial cells as the limit of detection for the DEPArray™ system (23). While the DEPArray™ system performed better than a standard differential approach, the method requires multiple preparation steps and has a lengthy processing time.

Finally, laser capture microdissection (LCM) is another separation technique that allows for targeting of single cells of interest in a mixture. Cell isolation can be performed using an infrared (IR) or ultraviolet (UV) laser under direct microscopic visualization (25). The IR laser method captures targeted cells via adherence to a removable thermolabile polymer film, however there is risk for contamination from non-targeted cellular material (25). Elliot et al. was the first to use IR-LCM for separation of sperm from sexual assault type samples (26). While Elliot et al. found LCM to outperform the differential cell lysis method, the method still produced mixture

profiles of the male and female contributors (26). The UV laser LCM technique is a contact-free method which catapults targeted cells into a collection area using a laser pulse (25,27). UV methods are less likely to result in DNA mixtures, however the LCM method is both laborious and time consuming (25).

While several methods have been developed for the separation of forensically relevant mixtures such as sperm and epithelial cells, no single method has yet to completely replace the traditional differential cell lysis method which continues to be used by most modern crime laboratories. Thus, there is a continued search within the forensic research community for a more robust cell separation method that can be used to more quickly and efficiently separate cells from different contributors.

Optical Trapping

Optical trapping, also known as optical tweezing, is a promising cell separation method that could address several of the issues discussed above. Physicist Arthur Ashkin was awarded the Nobel Prize in 2018 for inventing the optical tweezer method, as his discovery provided many avenues for studying and investigating biological systems (28). Optical trapping uses an IR laser focused through the lens of a high numerical aperture objective on an inverted microscope to capture dielectric particles, such as cells, via optical forces (29). The optical force is generated from the scattering force of the laser beam photons when they hit a particle and the gradient force which results from the nonuniform optical field applying a net force on the sample's dipoles (either induced or permanent) (29). This gradient force yields a localized trap near the focal point of the laser and moving this focal point with respect to the remaining solution is what allows for the manipulation of particles in the X, Y, and Z directions (29). Ashkin et al. reported the first use of a single-beam gradient force optical trap in 1986 which could be used to capture

dielectric particles ranging in size from 10μm – 25nm (30). In 1987, Ashkin et al. further demonstrated that optical tweezers could be used to manipulate biological organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* without causing damage to the reproductive abilities of the living cells (31). Since then, optical tweezers have been routinely used in the fields of biology, chemistry, physics, nanoscience, and medical science to study microscale particles and systems (32). Nascimento et al. and Tadir et al. used optical trapping to quantitatively study the motility of human sperm cells after being trapped under different laser power outputs and time conditions (33,34). In 2010, Reiner et al. was able to detect mitochondrial heteroplasmies from HL-60 cell lines using optical tweezers to isolate individual mitochondria (35).

Previous research at Virginia Commonwealth University (VCU) has also investigated the use of optical trapping for separating forensically relevant cells from mock evidence mixtures. Auka et al. used optical tweezers to trap sperm cells from both diluted semen and mock sexual assault type mixtures using an open droplet method (36). Liquid samples were deposited onto a custom microscope slide and individual cells were moved to the edge of the droplet where they could be collected using a glass capillary then deposited onto a clean coverslip for subsequent DNA analysis. Auka et al. determined that 50 or more sperm cells were needed to obtain a complete STR profile, and they reported the ability to obtain single source male profiles for two-thirds of samples collected (36). Valle further demonstrated that optical tweezers could be used to capture other forensically relevant cell types, such as leukocytes from enriched whole blood samples (37). Using a modified direct injection into lysis buffer method in place of the coverslip transfer method, Valle reported that only 10 leukocytes from a liquid enriched leukocyte sample were required to obtain a complete single-source profile. When sexual assault type samples were

examined using the same optimized collection method, it was found that 85% or more of expected STR alleles could be observed in a DNA profile generated from 20 or more sperm cells (37). The 50 sperm cell threshold for a complete STR profile was reaffirmed by O'Brien after comparing the Auka et al. coverslip transfer method and the Valle direct injection method (38). However, O'Brien observed that the direct injection method had a better predictive power for determining the profile completeness from the number of trapped cells when a non-linear regression model was fit to the data (38).

In the previous VCU studies detailed above, it was consistently noted that the capillary collection method sometimes resulted in the collection of cells which were not intentionally placed at the edge of the sample droplet, and the open droplet setup left the sample vulnerable to contamination (i.e., 'drop-in' alleles) (36–38). Sample contamination is a prominent issue for the forensic DNA community because exogenous DNA can cause otherwise single source samples to become mixtures and/or result in more challenging STR profile interpretation downstream. A microfluidic device could offer an appropriate alternative to the open droplet approach, as microdevices allow for the control and manipulation of fluids and particles on a microscale within a closed system. The closed system nature of microdevices has been shown to reduce the potential for contamination (16). Microfluidic devices are also advantageous in that they can reduce sample handling time, require smaller sample amounts, and have the ability to be integrated into multi-step automated systems (16). Several of the previously discussed cell separation methods have implemented microdevices into their workflow (16,17,24,39,40).

At VCU in 2020, Lally utilized a microfluidic device that could be incorporated into the existing optical trapping setup, replacing the open droplet microscope slide setup (41). The microfluidic device design consisted of five polyethylene terephthalate (PET) layers. The first

and fifth layers consisted of plain PET, while the second and fourth layers were coated with heat-sensitive adhesive on both sides, and the middle third layer was coated in black printer toner. Negative space was incorporated into the bottom three layers to adhere a plastic microscope cover slip onto the device for optical trapping. Ports and channels were arranged in a T-shape orientation for control of fluid and particle flow (Figure 1A). Autoclaved ddH_2O was added to Port A to prepare channels, and Kwik-Cast™ sealant was then added to Port C to prevent cells from flowing down the channel from the trapping zone to the extraction zone. Sample solution was pipetted into Port A, and water was added to, then removed from Port B so the overall net flow of cellular material was from Port A to Port B. Cells were optically trapped and moved from trapping zone area to the extraction zone. Optical trapping with the microfluidic device relied entirely on the passive flow of fluid and cellular material from Port A to Port B.

Lally examined both 1:20 diluted semen and mock sexual assault type samples, trapping 25-50 sperm cells per sample. The minimum number of sperm cells needed to produce a complete STR profile with 1:20 diluted semen was reported to be 41 cells, while the minimum number of cells for mock sexual assault samples was 31 (41). These cell numbers are less than those reported by Auka et al. and O'Brien. Additionally, Lally reported a positive correlation between the number of sperm cells trapped and the percentage of expected STR alleles in the profile, and a linear regression fitted to the data explained 70-80% of the variability observed (41). Lally's research provided minimal or no data for trapped sperm cell counts less than 25.

A few drawbacks were observed using the microfluidic device design for optical trapping. One to three drop-in alleles were observed in 36% of profiles (41). Some of the drop-in alleles were attributed to analyst contamination from excessive handling and manipulation of the extraction zone (41). Although no impact on total DNA yield or STR profile completeness was

observed, Lally stated that future projects should explore more efficient methods to excise the extraction zone. Additionally, although it had been previously reported that sperm cells could be trapped at a rate of 30 seconds per cell (38), optical trapping with the microfluidic device resulted in an increased sample processing time which ranged from 0.93-2.3 minutes per cell (41). This is likely due to the more complicated manipulation of sperm cells through channels within the device. When flow of sperm cells at the trapping zone ceased, Lally had to add more sample to the microfluidic device to trap additional cells (41). This could be problematic for forensic cases with limited sample volumes.

Furthermore, the standard differential cell lysis method analyzes both the non-sperm and sperm fractions. None of the previously discussed optical trapping work examined the epithelial cell fraction from the female contributor. This in part was due to the inability to trap epithelial cells using the existing laser setup with a 25mW power output (41). Based on the calculated degradation index (DI) after quantification, Lally reported that the manipulation of cells using an optical trapping laser resulted in no or negligible DNA degradation (41). These results were concordant with the findings of previous research using optical trapping (37,42). It is unknown whether a different laser setup or increased power output used to trap epithelial cells would impact DNA integrity and subsequent STR profile generation. The possibility for epithelial cell capture in addition to sperm cell capture, as well as further optimization of the microfluidic device design needs to be explored.

The main objective of this research project was to expand upon previous optical trapping research by further optimizing and exploring the limitations of a microfluidic device designed for separation of sexual assault type samples. The first goal was to collect low copy number (LCN) cell replicates (i.e., 5-25 sperm cells) using both diluted semen and male-female mixture types.

This would expand upon the previous research conducted by Lally (41), to better understand the relationship between the number of sperm cells trapped and the percentage of expected alleles observed in the STR profile. The second goal was to explore and determine an optimal mechanism for excising the extraction zone from the microfluidic device to address concerns expressed in Lally's research (41). After implementation of a new laser, the final goal was to test and determine the optimal power output for optical trapping of epithelial cells and assess the impact of the laser on DNA integrity and STR profile generation.

Materials and Methods

Biological Fluid Collection

Virginia Commonwealth University's Human Subjects Institutional Review Board (IRB) approved protocols (HM20014405 + HM2002931) were followed for the collection and use of all samples in this study. Semen and vaginal fluid samples were collected from anonymous volunteer donors using either sterile collection cups (semen) or sterile cotton swabs (vaginal fluid). Semen was aliquoted into multiple 1.5ml microcentrifuge tubes and stored at -80°C prior to use. Vaginal fluid swabs were allowed to air-dry overnight inside a laminar flow hood and were stored at room temperature in swab boxes within biohazard bags prior to use.

Biological Fluid Preparation

Previously collected and stored semen samples were thawed and gently vortexed to ensure proper mixing before use. Neat semen was diluted twenty-fold using 4mg/ml bovine serum albumin (BSA) in phosphate buffered saline, 1X, pH 7.4 (PBS). The entire cotton swab used to collect vaginal epithelial cells was added to 300µl deionized water (diH_2O), gently vortexed, and incubated for five minutes at room temperature to resuspend the epithelial cells. The cotton swab was placed and a spin basket and centrifuged to remove any residual liquid. A

mixture of the 1:20 diluted semen and resuspended vaginal epithelial cells was created by combining equal volumes of each biological fluid to generate a male-female mixture sample.

The resuspended vaginal epithelial cell sample was used to test the newly integrated laser's ability to trap epithelial cells. A buccal swab from each sample contributor was processed via DNA analysis, as described below, and used to develop a known reference DNA profile. All biological samples were stored at -20°C between uses.

Sample Types Collected via Optical Trapping

Three replicates each of 5, 10, 15, 20, and 25 sperm cells were collected from both the prepared 1:20 diluted semen and male-female mixture samples to complete the low copy number (LCN) cell replicates. A substrate control chip was analyzed along with the LCN samples following the existing optical trapping procedure described below, using only autoclaved water added to the microfluidic device. LCN replicates and reference swabs were carried through DNA analysis as described below.

After a new laser was installed into the optical trapping set up, resuspended vaginal epithelial cells were used to test the laser's ability to trap this cell type. Three replicates each of 40, 45, and 50 sperm cells were trapped using 1:20 diluted semen. Replicate samples were carried through DNA analysis as described below. Control samples using neat semen from the same donor were serially diluted to approximately the same cell count as the number of cells trapped to assess the impact of the new laser on DNA integrity and STR profile generation. The approximate cell count was determined using a Neubauer-improved hemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany) and Trypan Blue staining (Corning Inc., Corning, New York) of the controls following previously established protocols (43,44).

Microfluidic Device Design

Microfluidic devices used for optical trapping of LCN samples were prepared following the procedure and design previously described by Lally (41). Devices were designed using AutoCAD LT® 2018 (Autodesk, San Rafael, CA) software as shown in figure 1 and exported to a VLS 3.50 software system. The design was cut using a VersaLaser® CO_2 laser ablation instrument (Universal Laser Systems, Scottsdale, AZ), and layers were bonded together using a AL13Ps laminator (Apache Laminators, Humacao, PR).

Existing Optical Trapping & Cell Separation Procedure

The LCN cell replicate samples were manipulated and collected using an AxioObserver D1 inverted microscope (Zeiss, Thornwood, NY). The microscope was affixed onto an air-floated 3'x4' vibration isolation table with a motorized staged controlled via a joystick. A 700mW, 1064nm continuous wave (CW) laser (CrystaLaser, Reno, NV) was focused through the back aperture of a 100x oil immersion objective. The focused laser trapped sperm cells in the trapping zone using a measured attenuated power output of 25mW reduced by an OD1 neutral density filter. Enough autoclaved water dispensed from a Direct-Q 3 UV Water Purification System (MilliporeSigma, Burlington, MA) was added to Ports A (10µl ddH_2O) and B (5µl ddH_2O) to flow through the channels of the entire microfluidic device (Figure 1A). Port C was sealed with Kwik-Cast™ Sealant (World Precision Instruments, Sarasota, FL), and the corners of the microfluidic device were attached to the stage using the same sealant (Figure 1A). After the sealant solidified, 2µl of sample was added to Port A and the flow of cells was verified microscopically under a 10X objective. Under a 100X oil immersion objective, after the laser was turned on, cells were moved from the trapping zone and released, one at a time in the extraction zone until the desired number of cells were captured.

DNA Analysis – LCN Samples

Trapped sperm cells for LCN cell replicates were removed from the microfluidic device using the previously described scissor excision method to cut out the extraction zone (41, Figure 2). After excision of the extraction zone from the microdevice, DNA from the collected samples was extracted using the QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for 'Isolation of Total DNA from Surface and Buccal Swabs'. For all optically trapped sperm cell samples, 20µl DTT was added to the proteinase K and Buffer ATL mixture. Carrier RNA was not added to the lysates. The excised extraction zone was placed into a spin basket (Promega, Madison, WI), centrifuged at 13,000rpm for one minute, and discarded to ensure maximum sample removal from the microfluidic device piece before DNA isolation and purification with the silica column. DNA was eluted to a final volume of 30µl in Buffer ATE. LCN samples were quantified on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the Quantifiler™ Trio Kit (Applied Biosystems) according to the manufacturer's protocol modified for half volume reactions.

Prior to amplification, lysed samples were vacuum centrifuged to a volume of 7.5µl using a Savant DNA120 SpeedVac concentrator (ThermoFisher Scientific, Waltham, MA). The PowerPlex® Fusion 5C kit (Promega) and manufacturer's protocols (modified for half volume reactions) were used to amplify the entire extracted DNA sample volume on a ProFlex PCR System (Applied Biosystems). Amplicons were separated and analyzed using capillary electrophoresis (CE) on an ABI Prism 3130 Genetic Analyzer® (ThermoFisher Scientific). Amplified samples were prepared for CE on a 96-well plate, and each well contained 9.7µl of Hi-Di™ Formamide (ThermoFisher Scientific), 0.3µl of WEN ILS 500 (Promega), and either 1µl of PowerPlex® Fusion 5C allelic ladder or 1µl amplified sample. The plate was heat denatured for three minutes at 95°C and snap cooled for 5-10 minutes at -20°C prior to CE injection. Amplicon separation on the 3130xl Genetic Analyzer was conducted with a 3kV injection for 5sec using 36cm capillaries and POP-4 polymer (ThermoFisher

Scientific) as the separation matrix. STR profiles were analyzed using GeneMapper™ Software v4.1 (ThermoFisher Scientific) with an analytical threshold of 100RFU.

Optimization of Excision Method

Lally expressed concerns regarding the scissor excision method due to collection variability and the possibility for contamination events (41), so excision was evaluated using a variety of alternative tools. Alternative excision tools included a 2mm scissor-style animal ear punch (VWR International, Radnor, PA), paper single-hole punch, 4mm Biopsy Uni-Punch® (Premier Dental, Plymouth Meeting, PA), X-ACTO #2 knife (Newell Brands, Atlanta, GA), and a 1.2mm Harris Uni-Core™ punch (ThermoFisher Scientific). The new methods were assessed for their ability to remove the extraction zone precisely, consistently, and efficiently, as well as for ease of recovering the excised piece and cleaning the excision tool compared the current scissor method. Assessments were performed with unused microfluidic devices that contained no sample or fluids.

Microfluidic Device Redesign

The microfluidic device described above was altered to improve trapping efficiency and allow the operator to better understand their position on the device while trapping under the 100X objective (Figure 1B and Table 1). The middle PET layer coated with black printer toner was replaced with plain PET. The boundaries between the ports and channels were flared/rounded to minimize capillary valving effects observed during the collection of LCN samples. Raster marks were etched into the top first layer of the device to serve as orientation markers for trapping. The radius of the extraction zone was reduced from 0.7mm to 0.4mm and outlined with a 1.2mm in diameter raster circle. Devices were designed, cut, and bonded as described above.

New Optical Tweezer Set Up

After the completion of the LCN cell replicate samples, a newly acquired 5W ytterbium linearly polarized laser (YLR-LP laser; IPG Photonics, Oxford, MA) was installed on the existing microscope setup discussed above. The power output of the YLR-LP laser was attenuated to 1.0W (20% power) to allow for trapping of sperm. The laser was split into two optical traps using a 1" Polarizing Beamsplitter Cube, 1064 nm (Thorlabs, Newton, NJ). Figure 2 depicts the optics setup for the YLR-LP laser. Resuspended vaginal epithelial cells were used to demonstrate the laser's ability to trap epithelial cells. Samples consisting of 1:20 diluted semen in BSA or resuspended vaginal epithelial cells were deposited into the new microfluidic device design following the same procedure previously described (Figure 1). Spermatozoa both with and without tails, as well as epithelial cells, were trapped. Trapped cells were removed from the microfluidic device for cell lysis using the optimized excision method.

DNA Analysis Methods – YLR-LP Trapped Samples

Samples were extracted via an alkaline cell lysis as previously described by Schellhammer (45). Excised extraction zone pieces were incubated in 4.0µl of 1M NaOH (ThermoFisher Scientific) and 16.0µl of PBS (1X, pH 7.4) at 75°C for five minutes. Post incubation, 4.0µL of 1M Tris-HCl pH 7.5 (Invitrogen; Waltham, MA) was added to the samples. Samples were vortexed briefly before the excised piece was transferred to a spin basket and centrifuged for five minutes at 13,000 rpm. All lysates were stored at -20°C until further processing.

All samples and reagent blanks were vacuum centrifuged to a volume of 7.5µl using a Savant DNA120 SpeedVac concentrator (ThermoFisher Scientific). Vacuum centrifugation was followed by PCR amplification of the entire lysed DNA sample volume (7.5 µl) using the GlobalFiler™ PCR Amplification Kit (ThermoFisher Scientific) and manufacturer's protocols

for 29 cycles (modified for half volume reactions) on a ProFlex PCR System (Applied Biosystems).

Samples were prepared for CE on a 96-well plate. Each well contained 9.6µl of Hi-Di™ Formamide (ThermoFisher Scientific), 0.4µl of WEN ILS 500 (GlobalFiler™), and either 1µl of GlobalFiler™ Allelic Ladder or 1µl amplified sample. The plate was heat denatured for three minutes at 95°C and snap cooled for 5-10 minutes at -20°C prior to CE injection. Amplicons were separated and analyzed on an ABI Prism 3500 Genetic Analyzer® (ThermoFisher Scientific). Injection parameters for the 3500 Genetic Analyzer were 1.2kV for 15sec using 36cm capillaries and POP-4 polymer (ThermoFisher Scientific) as the separation matrix. STR profiles were analyzed using GeneMapper™ ID-X Software v1.6 (ThermoFisher Scientific) with an analytical threshold of 100RFU.

Data Analysis

Samples were evaluated using the microscopic and real-time data gathered during optical trapping, as well as the real-time PCR quantification results and STR amplification results. The total time spent trapping each sample, as well as the average amount of time per cell was recorded.

Theoretical DNA yield for each sample was calculated based on the number of cells trapped multiplied by the estimated DNA quantity per haploid cell. Haploid cells were estimated to yield 3.0pg DNA per cell (41,46). Using the real-time PCR quantification results, total DNA yield was calculated by multiplying the small autosomal target concentration (pg/µl) by the final elution volume (30µl). The average total DNA yield and standard deviations for each sample type were calculated and compared to theoretical DNA yield for the number of cells trapped. Substrate controls (number of cells trapped = 0) were not included in average total yield

calculations, and a Student's T-test ($\alpha = 0.05$) was performed to determine statistical significance between calculated total yield and the theoretical DNA yield at each cell count. The average overall DNA yield per sperm cell trapped (pg/cell) was calculated by taking the mean of each sample's calculated total DNA yield over the total number of cells trapped. Degradation index was calculated by dividing the small autosomal target quantity by the large autosomal target quantity. Values >1.5 suggested some degree of DNA degradation, while values ≤1.5 indicated that the sample exhibited no DNA degradation (47).

The STR profile generated from a buccal swab from each contributor was used to determine the expected STR profile for all optically trapped samples. For each experimental sample, the observed STR profile was compared to the expected reference profile. The percentage of STR alleles present was calculated by dividing the number of alleles observed in the sample profile by the total number of alleles expected to be observed. Homozygous alleles were counted once, and the amelogenin locus was not included in the calculation. The number of cells trapped for each experimental sample were then compared to the percentage of expected STR alleles observed in the respective STR profile, and a simple linear regression was fit to the data.

Called alleles in the male-female mixture samples, that were consistent with the female contributor's profile, were assumed to come from the female contributor. Observed alleles that could not be attributed to either of the mixture contributors or the donor of the single source body fluid were counted as drop-in alleles. Average peak height for each sample was calculated by averaging the RFU values from all the called STR allele peaks in the profile. The peak heights from a known homozygous locus were counted as two towards the total number of alleles called

for the denominator in average peak height calculations. The amelogenin locus was not included in the peak height calculation.

The impact of the new laser on DNA integrity was evaluated by comparing the average total DNA yield and percentage of expected alleles that appear in the STR profiles generated from the optically trapped samples to the serially diluted control samples. Averages were compared for statistical significance using a Student's T-test ($\alpha = 0.05$).

Results and Discussion

Low Copy Number Samples

Samples collected included a range of 0, 5, 10, 15, 20, or 25 optically trapped sperm cells. Spermatozoa were isolated from either 1:20 diluted neat semen solution or an equal volume male-female mixture. Over the course of this research project, male-female mixture samples were re-prepared using the separate 1:20 diluted semen and resuspended vaginal fluid swab solutions one month after the initial preparation date. Monthly re-preparation was performed because a large decrease in the number of sperm cells from the mixture samples was observed after the one-month period. The large decrease in observed sperm cells made it difficult or near impossible to trap the targeted number of cells from the mixture.

The average amount of time spent trapping per sperm cell was 2.30±1.17 minutes for the diluted semen samples and 2.90±1.40 minutes for the mixture samples (Table 2). The observed increase in trapping time for sperm cells collected from mixture samples was due the increased presence of non-sperm cell material (e.g., resuspended vaginal epithelial cells) in the sample resulting in more material to manipulate the sperm cells around. These averages are also greater than those observed by Lally using the same microfluidic device design (1:20 diluted semen average = 1.37min/cell and mixture average = 1.67min/cell) (41). The differences in trapping

time could be attributed to differences in individual operator experience and ability to trap. Periodic loss of a sperm cell before being dropped off at the extraction zone was observed while trapping with the 700mW CW laser and could have also contributed to this observed increase in trapping time per cell.

DNA from optically trapped samples was extracted and quantified using Applied Biosystems Quantifiler™ Trio DNA Quantification Kit. The total yield are shown in Tables 3 and 4. Figure 3 depicts the average quantified total DNA yield for each sample type at each targeted cell count versus the expected theoretical DNA yield, assuming one haploid sperm cell is equivalent to 3pg (46). Averaged total DNA yields were not statistically different from the theoretical yields (p-values > 0.05). Therefore, it can be inferred that the optically trapped samples did not statistically differ in the actual amount of human DNA extracted versus the amount of DNA expected to be extracted based on the number of trapped sperm cells.

For the 1:20 diluted semen samples, the calculated average DNA quantity per trapped sperm cell was 2.81 ± 2.85pg/cell, while for male-female mixture samples, it was 4.24 ± 2.94pg/cell (Table 2). Given the standard deviations in the diluted semen and mixture samples, the DNA quantity per trapped sperm cell falls within what would be expected for a single haploid cell (3pg). Results from samples that yielded lower total DNA yields than expected, or 1.5-4x greater total DNA yields than expected caused the wide standard deviation values observed.

One of the 20 cell count replicates collected from 1:20 semen had a total DNA yield (243.7997pg) more than 4x greater than the theoretical yield (60pg). During the optical trapping process, spermatozoa would sometimes get stuck in the channel before reaching the inner area of the extraction zone and would not be counted towards the total number of cells trapped. The scissor cutting method used to excise these samples was not precise in removing the extraction

zone. The scissors could potentially have incorporated sperm which were not included in the total count used for calculating theoretical yield. This would result in a higher total DNA yield than what was expected.

Additionally, the average DI value for the 1:20 diluted semen samples was 1.00 ± 0.52 (Table 3), while the average DI for male-female mixture samples was 1.16 ± 0.52 (Table 4). According to Vernarecci et al., a DI between 0 and 1.5 corresponds to no degradation, while a DI between 1.5 and 4 corresponds to mild degradation (48). Because none of the optically trapped samples had a degradation index greater than 2.5, and most sample DIs were less than 1.5, little or no DNA degradation amongst samples was indicated.

The percentage of expected STR alleles observed in each STR profile was calculated. The results of those comparisons are presented in Tables 5 and 6 for the 1:20 diluted semen and male-female mixture samples, respectively. Ski-sloping, a gradual decrease in the observed peak height values from smaller to larger amplicons in the same dye channel, was not observed in these samples.

The 1:20 diluted sperm sample that had a total DNA yield more than 4x greater than the theoretical also yielded a full DNA profile and demonstrated no signs of contamination (Table 5). The incorporation of sperm cells from outside the extraction zone in the excised and extracted piece of the sample discussed above is possible, especially given that full profiles were not observed with other samples that had 20 or more trapped sperm cells (Table 5). Six of the seventeen male-female mixture samples generated total DNA yields 1.5x greater than the theoretical yield (Table 4). For three of these samples, at least part of the increased total yield can be attributed to contribution of DNA from the female contributor in the mixture; one or two called alleles observed in each of the sample profiles were attributed to the female contributor

(Table 6). However, the entire ≥1.5x increase in total yield cannot entirely be accounted for by contribution from the female contributor in the mixture. The incorporation of uncounted sperm from outside of the extraction zone into the final excised piece more likely explains the increase in total DNA yield.

A full STR profile (100% expected alleles) was observed with as little as 25 trapped sperm cells in the male-female mixture samples (Table 6). However, this sample also had a total DNA yield more than 3 times greater than the theoretical yield. Two alleles consistent only with the female donor's profile were present in this sample's STR profile. However, the increase in total DNA cannot be entirely attributed to contribution from the female donor. The observed increase again could potentially be due to the accidental incorporation of sperm cells stuck outside the extraction zone as discussed previously.

For both the 1:20 diluted semen and male-female mixture sample types, a positive correlation between the number of sperm cells trapped and the percentage of expected STR alleles in the profiles was observed (Figures 4 and 5). One of the main objectives of this research was to expand on previous optical trapping research performed by Lally (41), by trapping sperm cells in the less than 25 cell count range and following the same subsequent DNA analysis methods. After STR profile analysis, a simple linear regression was fit to the LCN sample data, as well as the previously reported Lally data (41). This simple linear regression model was used to test if the number of cells optically trapped significantly predicted the percentage of expected alleles present in the profiles generated from each sample type.

When a simple linear regression was fit to the 1:20 diluted semen data the model was: percentage expected alleles = 0.813 + 2.397*(number of cells trapped), and the overall regression was statistically significant ($R^2 = 0.7245$, $F(1, 24) = 66.74$, p-value < 0.0001). It was

found that the number of cells trapped significantly predicted the percentage of expected alleles observed in a sample's STR profile ($\beta = 2.397$, p-value < 0.0001), and the number of sperm cells optically trapped explained 72.45% of the variability observed in the percentage of expected alleles. This value is less than what was observed by Lally (80.32%), however the regression still explains >50% of the variability in the percentage of expected alleles observed.

When a simple linear regression was fit to the male-female mixture data the model was: percentage expected alleles = 10.236 + 2.118*(number of cells trapped), and the overall regression was statistically significant ($R^2 = 0.5635$, $F(1, 27) = 37.15$, p-value < 0.0001). It was found that the number of cells trapped significantly predicted the percentage of expected alleles observed in a sample's STR profile ($\beta = 2.118$, p-value < 0.0001), and the number of sperm cells optically trapped explained 56.35% of the variability observed in the percentage of expected alleles. This value is less than what was observed by Lally (70.47%), however the regression still explains >50% of the variability in the percentage of expected alleles observed.

Optimization of Excision Method and Microdevice Redesign

Ultimately, the 1.2mm Harris Uni-Core™ punch (Harris punch), over the old scissor cutting method described by Lally, was selected as the new excision method for collecting optically trapped samples (41). When unused microfluidic devices containing no sample or fluids were assessed, the Harris punch was the most time efficient, precise, and had the highest average ratings for ease of excision (i.e., removal of the extraction zone) and ease of recovery (i.e., transfer of the excised piece to the microcentrifuge tube for cell lysis) (data not shown). To help improve the accuracy and precision of extraction zone removal for each collected sample, the radius of the extraction zone was reduced from 0.7mm to 0.4mm and outlined with a 1.2mm in diameter raster circle (Figure 1B). This design change allowed for alignment of the 1.2mm

Harris Uni-Core™ punch with the extraction zone during the excision process. Additionally, the boundaries between the channels and ports were flared on the optimized microfluidic device to minimize capillary valving of water used to prime the channels and fill the device prior to adding any biological sample. Previously during optical trapping of the LCN samples, when water was added to Port A, flow of the water throughout the port channel design was not observed unless the microfluidic device was agitated by the operator. Even then, water would still occasionally slowly progress through the port channel design. Flaring the boundaries alleviated this issue.

YLR-LP Laser Optically Trapped Samples

Samples containing a range of 40, 45, and 50 optically trapped sperm cells isolated from 2μl of a 1:20 diluted neat semen solution were collected in triplicate at each cell count. The optimal power output for trapping spermatozoa using the YLR-LP laser and dual trap setup was determined to be 1.0W (20% power). A power output >1.0W did not demonstrate any improvement in trapping ability or the retention of cells during manipulation. The measured power of each individual optical trap was 20±1mW. A demonstration of the dual optical tweezer setup and optimized microfluidic device while trapping 3.5μm polystyrene beads can be viewed here.

The average time per cell spent trapping with the new YLR-LP laser was 1.38min/cell. This is almost one minute less per cell than the average time calculated using the 700mW CW for the LCN samples, and closer to the time reported by Lally (1.37min/cell) for the 1:20 diluted neat semen sample type. The decrease in average time spent trapping per cell can be attributed to the dual traps generated from the beam splitters and new optics setup, which allows for trapping of at least two sperm per trip between the trapping zone and the extraction zone. Trapping time was likely also reduced due to the addition of raster marks added around the trapping zone and

the extraction zone (Figure 1B and Table 1), which allowed the operator to better understand where on the microfluidic device sperm cells were currently trapped. The operator no longer had to waste time moving up and down the channel to figure out whether they had passed into the extraction zone from the main channel. The raster marks provided two clear shadows that indicate to the operator they have passed into the extraction zone. The raster marks should further be adjusted to the design suggested in Figure 6.

The average trapping time could possibly be additionally reduced by priming the channels with a solution other than ddH_2O, such as 4mg/mL BSA in PBS. Even with the increased optical strength of the new laser, sperm cells would still occasionally get stuck in the channel prior to reaching the extraction zone. After a period of time, following the addition of biological sample to Port A, sperm cells would also adhere to the coverslip surface (third layer of the device) and were not able to be manipulated further (Figure 1B). This led to a decrease of free trappable sperm cells as the net fluid flow to Port B in the channel slowed, and thus an increase in overall trapping time. Occasionally priming/pumping of Port B by adding and removing a few microliters of fluid via pipette to/from the port would shift the fluidics and loosen some sperm cells from the coverslip surface. However, this fluidics adjustment only worked temporarily. Further optimization of the microfluidic device needs to be pursued that would allow for control over the microfluidics during trapping and thus minimize the adhesion of sperm to the coverslip surface.

Additionally, while trapping some of the 40, 45, and 50 replicate samples with the updated microfluidic device design, debris that was reactive with the YLR-LP laser was occasionally observed within the microdevice channels. This resulted in the sporadic generation of air bubbles and/or loss of sperm cells from one or both optical traps (Video Link 2). The

debris was presumed to come from either the sacrificial layer used to keep the layers flat during fabrication of the microfluidic device and/or fragments of the PET layer edges generated from the VersaLaser® CO_2 laser ablation instrument. To remedy this issue device layers were washed with 70% isopropyl alcohol and allowed to dry overnight prior to alignment and lamination. While this did result in significantly less to no reactive debris present in the channels, it also took longer for ddH_2O to prime the channels prior to adding biological sample, and after one week, the adhesive layers of the device started to delaminate. Other methods for 'washing' the layers, such as the use of a compressed air duster, should be explored in future studies.

Optical Trapping of Resuspended Vaginal Epithelial Cells

Another goal of this research project was to explore the possibility for optical trapping of epithelial cells using the new YLR-LP laser. The resuspended vaginal epithelial cell solution used to prepare the male-female mixture samples was deposited on a hollowed out microscope slide with a cemented 0.17nm glass cover slip as described by Auka et al. (36), as well as the optimized microfluidic device described above. Smaller epithelial cell fragments were able to be individually captured in each optical trap and manipulated within the microfluidic device using an attenuated laser power output between 1.0W and 1.85W. Larger fragments, or clumps of cells, could be manipulated using the dual optical traps combined with the same power output, as demonstrated in this video. However, large fragments easily slipped out of the optical traps and smaller fragments were unrecoverable if they were caught in a larger clump of cells. Additionally, unlike with spermatozoa, individual epithelial cells and their nuclei were difficult to identify and subsequently isolate. Further research needs to be conducted to find a method that will allow for the identification of vaginal epithelial cell nuclei while optically trapping to better predict the number of cells captured. Although DAPI is known to successfully stain the nucleus

of cells such as leukocytes and epithelial cells, previous work by O'Brien reported that leukocytes stained with DAPI were repelled by the optical trap (38). Propidium iodide is a red-fluorescent nuclear counterstain for nonviable cells that has been used with laser-based cell manipulation techniques such as flow cytometry and is presumed to be compatible with the YLR-LP laser (49–51).

DNA Analysis of YLR-LP Laser Optically Trapped Samples

The 40, 45, and 50 sperm cell samples collected in triplicate were lysed using the alkaline direct cell lysis method previously described by Schellhammer (45) in attempt to reduce overall DNA analysis workflow time and minimize DNA loss by reducing the number of tube transfers during cell lysis. Samples were then amplified with the GlobalFiler™ PCR Amplification Kit and amplicons were separated on a 3500 Genetic Analyzer. When the samples were analyzed using GeneMapper™ ID-X software, no alleles above the 100RFU analytical threshold were observed (Figure 7). Two changes to the optical trapping workflow were initially considered to be the possible cause of the observed 'blank' STR profiles: 1) the new excision method using the Harris punch pushed cells out of the extraction zone when a downward force was applied; or 2) the alkaline lysis reagents and/or byproducts of the reaction interfered with GlobalFiler™ STR amplification success.

Control Alkaline Lysis Test

To test the considerations discussed above, the 1:20 diluted neat semen solution used for the optically trapped samples was serially diluted with 4mg/mL BSA in PBS to a 1:100, 1:1000, and 1:10,000 dilution from the original neat semen concentration. On microscope slides, 7.5µl of each dilution was stained with Kernechtrot Picroindigocarmine Stain (KPICS) to confirm the presence of spermatozoa in each dilution. Kernechtrot stain was applied to the dried stain for 15

minutes at room temperature and rinsed off with ddH_2O followed by a 5-second application of the Picroindigocarmine stain. Very few sperm were identified in the 1:10,000 dilution, so only two replicates of this dilution were prepared, while four replicates of the other two dilutions were prepared for subsequent testing.

Seven and a half microliters of each replicate were added to a microcentrifuge tube and subjected to the alkaline lysis method previously described in this paper, however the PBS volume was adjusted to obtain the same final lysis volume used for the optically trapped samples (24µl). Half of the replicates were then vacuum centrifuged to a volume of 7.5µl and the entire lysate was added to the amplification reaction – just as the optically trapped samples were – while the other half of the replicates were not concentrated and only 7.5µl out of the total lysate volume was added to the GlobalFiler™ PCR amplification reaction. This step was performed to determine whether lack of STR profile generation in the optically trapped samples was the result of concentrating the DNA via the vacuum centrifuge, and thus the lysis reagents and their reaction byproducts. When an alkaline lysis is performed, 1M NaOH and heat are applied to a biological sample to lyse open the sample's cellular and nuclear membranes (52,53). Tris-HCl (1M) is then added to the reaction to neutralize the NaOH, and water and NaCl salt are formed as byproducts (52,53). Sodium chloride salt is a known PCR amplification inhibitor (54). Further concentration of this inhibitor by vacuum centrifugation is likely to increase the chance of PCR inhibition.

Although it has previously been demonstrated that alkaline lysed semen samples successfully produce STR profiles when amplified with the PowerPlex® Fusion 5C amplification kit (45), when the profiles of the control alkaline lysis were analyzed using GlobalFiler™, no true alleles above analytical threshold were observed (Figure 8). One off

ladder peak above the analytical threshold was called by the analysis software for a 1:100 diluted semen sample replicate that had not been vacuum centrifuged (Figure 8). However, this peak was determined to be a spike artifact. It could then be concluded that the lack of alleles observed in STR profiles generated from optically trapped samples at low cell counts of 40-50 sperm cells was likely due to the alkaline lysis method.

A direct cell lysis method, such as the alkaline lysis, would be useful in a forensic workflow due to the decreased processing time and complexity of the cell lysis procedure (i.e., three lysis reagents and a 5-minute incubation at 75°C, compared to a multiple reagent and multiple hour-long QIAGEN QIAamp extraction). However, the direct cell lysis method needs to further be optimized to achieve successful PCR amplification for optically trapped samples at low cell counts (≤50 cells). This may require an additional lysate purification step, or the use of a completely different direct cell lysis method such as those evaluated by Schellhammer (e.g., Promega® Forensic DNA Testing - Casework Direct System) (45). Alternatively, methods for direct PCR amplification where the excised microfluidic device piece is directly inserted into the PCR reaction could be explored (55).

YLR-LP Laser DNA Integrity Assessment

To assess whether the new YLR-LP laser attenuated to 1.0W power output had a negative impact on DNA integrity and STR profile generation, three replicates at 50 sperm cells each were optically trapped from the 1:20 diluted neat semen solution on separate microfluidic devices. In case the new optimized excision method did cause sperm cell loss due to the downward applied pressure to the device and resultant flushing of fluid around the extraction zone, these samples were also excised using the old scissor cutting method. After extraction zone excision, samples were processed following the LCN sample extraction and qPCR protocols with

the QIAamp DNA Investigator Kit and Quantifiler™ Trio. Alongside these samples three sets of serially diluted control samples of approximately the same cell count were processed using the same DNA analysis methods. Cell count was estimated using a Neubauer-improved hemocytometer and Trypan Blue staining mixed in equal volume to amount of diluted semen added to the mixture.

The average total DNA yield from the 50 sperm cell count replicates was found to be not significantly different from the theoretical yields (150pg) after performing a Student's T-Test (Table 7, p-value > 0.0775). The average degradation index for these samples was 1.10 ± 0.17, and none of the individual sample DIs were greater than 1.4 (Table 7). Both of these assessments are consistent with the quantitation data from the old 700mW, 1064nm CW laser. Thus, based on the qPCR data, it can be inferred that the new YLR-LP laser does not result in DNA degradation that can be detected using real-time PCR human DNA quantification methods.

The serially diluted control samples did not produce the expected total DNA yields or theoretical number of cells present in the sample based on the dilutions performed and hemocytometer cell count estimates (Table 8). Even with diligent mixing of each control solution via vortexing and pipetting up and down, replicates within each control type varied widely in the amount of total human DNA quantified. This may have resulted from clumping of sperm cells even with mixing and agitation. Further optimization using Trypan Blue staining and the hemocytometer should be conducted so that more consistent replicates that are also closer to the target cell count can be acquired.

Optically trapped 50 sperm cell count samples and the 'similar cell count' control samples were amplified using the GlobalFiler™ PCR Amplification Kit. Amplicons were then separated on a 3500 Genetic Analyzer and the generated STR profiles were analyzed to

determine the percentage of expected alleles observed in the profile as well as average peak height. On average, 97.37 ± 2.63% of the expected alleles in the 50 sperm cell count optically trapped samples were observed (Table 9). The average expected allele percentage of the optically trapped sample profiles was not found to be significantly different from the calculated averages of the control samples (p-values > 0.1). Additionally, ski-sloping across loci in the same dye channel was not observed (Figure 9). This further supports the conclusion that the 5W YLR-LP laser did not negatively impact DNA integrity or the ability to generate STR profiles.

Two out of the three 50 optically trapped sperm sample profiles had a single drop-in allele. At least one drop-in allele from a profile was inconsistent with the reference profile of the analyst and thus could not be attributed to accidental analyst contamination. Drop-in alleles were also observed in five of the nine control samples, and for at least three of these STR profiles the drop-in allele was inconsistent with the analyst's reference profile. It is unlikely that these random observed drop-in alleles are the result of analyst contamination.

Notably the optically trapped sample consisting of 19 spermatozoa and excised using the Harris punch, had a total DNA yield (64.49pg) greater than what was expected (57pg) and produced an STR profile with 89.47% of expected alleles and peak heights of 303.80 ± 152.96RFU on average (Table 7). This suggests that the Harris punch excision method may not result in sperm cell loss due to the downward pressure applied and may be a suitable alternative to the scissor cutting method. Further investigation of this excision method is needed.

Conclusions

Significant sexual assault sample backlogs are present in crime laboratories across the US and the current front-end method for separation of cell types from these samples is laborious and inefficient. Existing differential cell lysis methods often result in mixture profiles which require

time consuming back-end interpretation by DNA analysts, adding to the overall evidence backlog observed in many crime laboratories. This has led the research community for more than two decades to search for an efficient novel cell separation method, and while several methods have been developed, none have been universally adopted by the forensic community. The development and optimization of a microfluidic device, such as the device described within this manuscript, using optical trapping has the ability to separate sperm from epithelial cells efficiently and result in single-source STR profiles representative of the male contributor.

Optical trapping of spermatozoa from mixtures such as those commonly found in sexual assault cases, has the potential to decrease the overall time spent processing these types of samples in the forensic workflow. This research has demonstrated with the use of a dual optical trap setup, a sufficient number of spermatozoa can be captured after approximately 1.5 hoursal of optical trapping (~15-minute microfluidic device preparation + 25 trapping evens = 50 cells x 1.38min/cell = 69 minutes + ~5-minute sample collection into microcentrifuge tube). Single source profiles of the male contributor – with no to minimal drop-in alleles (≤1), or alleles attributed to the female contributor in a mixture (≤3) – can be produced using optical trapping and the microfluidic device discussed in this paper. Additionally, optical trapping eliminates the need for a differential cell lysis or analysis of a mixture profile from an inefficiently separated sperm fraction on the back-end of the forensic workflow, thus reducing the overall time spent processing these types of evidentiary samples.

The incorporation of a direct PCR amplification method in place of traditional extraction methods of DNA from sperm cells could further reduce the overall analysis time in the forensic workflow by eliminating the qPCR step and utilizing a more rapid cell lysis method. The amount of human DNA present in an evidence sample would be quantified using the number of trapped

sperm cells multiplied by the expected DNA yield per haploid cell (3pg) to be in compliance with the forensic DNA Quality Assurance Standards. Further optimization of the DNA analysis workflow post optical trapping needs to be conducted.

This research has demonstrated the ability to optically trap resuspended vaginal epithelial cells at an attenuated laser power output between 1.0W and 1.85W. Further exploration into the ability to trap vaginal epithelial cells within a microfluidic device could eventually allow for dual trapping of sperm and epithelial cells from a mixture on the same device. Postcoital samples could then be examined with this dual cell type trapping device to mimic what can be expected from evidence samples such as those that come from sexual assault cases, or other forensically relevant samples that result in cellular mixtures. Finally, it can be concluded that a 5W ytterbium linearly polarized laser attenuated to 1.0 W power output does not negatively impact DNA integrity or STR profile generation.

Closed microfluidic device designs reduce the potential for contamination, which has previously been identified as a major concern using an open-droplet optical trapping method. An optimized microfluidic device using optical trapping for cell capture and separation of sexual assault type samples would allow DNA analysts to dedicate more time towards other cases and would help with the overall biological evidence backlog.

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Tables and Figures

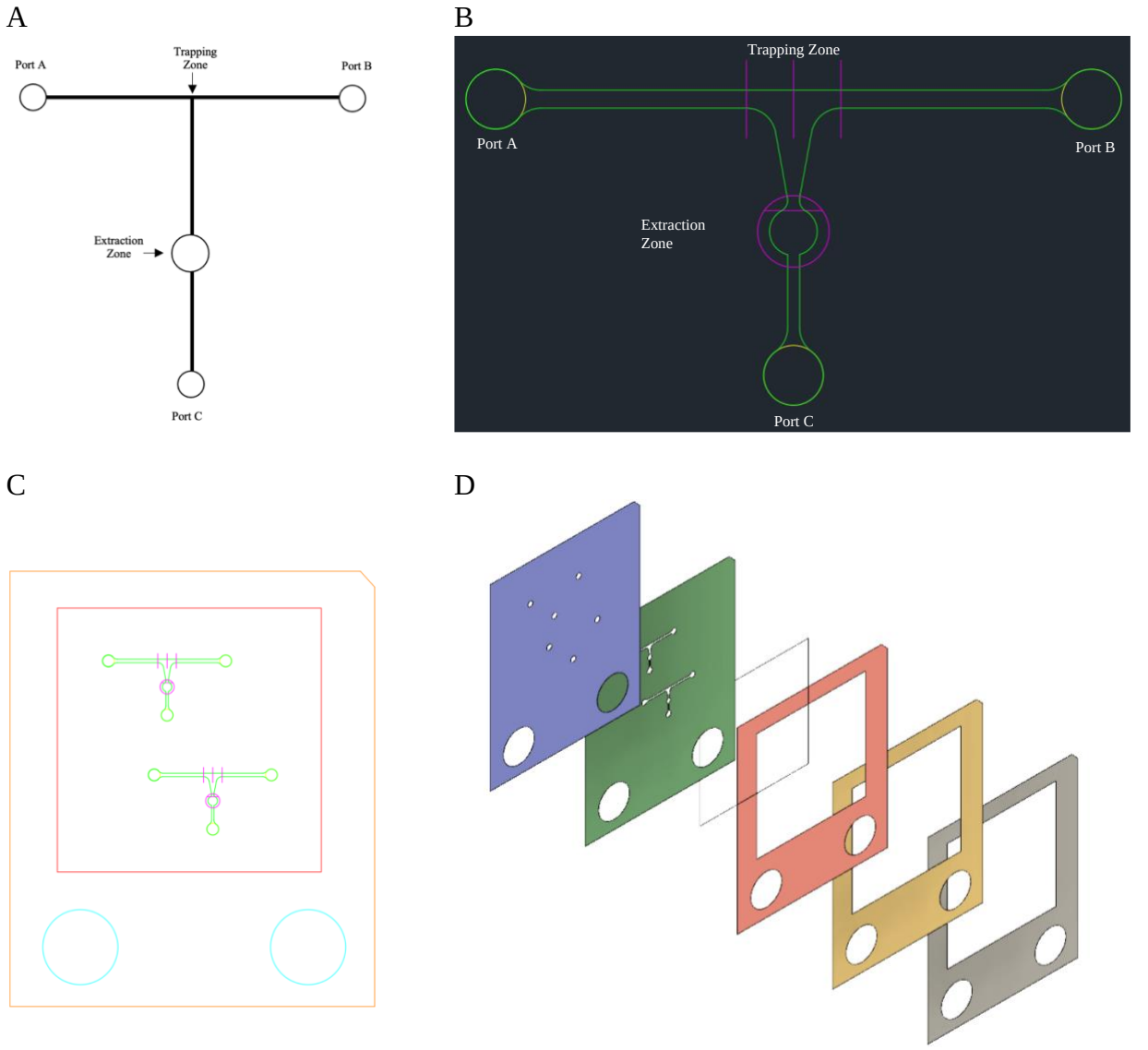

Figure 1: Original port and channel design for microfluidic device and optimized rendering of device design.
A) Original port and channel design used by Lally and for LCN samples. B) Optimized port and channel design. Boundaries between ports and channels flared in Layer 2 to minimize capillary valving. Raster marks in Layer 1 depicted with magenta lines. C) Single microfluidic device with two T-shaped port channel designs. D) Exploded view of microfluidic device layers. Renderings generated by Shane Woolf, Ph.D., using AutoCAD LT® 2018 software.

Table 1: Microfluidic device laser cutter settings.

Layer	Material(s)	Colors*	Power	Speed	Unique features
Layer 1	PET	yellow, cyan, orange	7%	10%	vents and inlets
		magenta (raster cut)	7%	100%	raster marks
Layer 2	PET-HSA	green, cyan, orange	7%	5%	fluidic channel(s)
Layer 3	PET + cover slip	red, cyan, orange	7%	10%	cutout for cover slip
Layer 4	PET-HSA	red, cyan, orange	7%	10%	cutout for spacer
Layer 5	PET	red, cyan, orange	7%	10%	cutout for spacer

PET = polyethylene terephthalate, HSA = heat sensitive adhesive.
*Colors coordinate to the colored lines in Figure 1C.

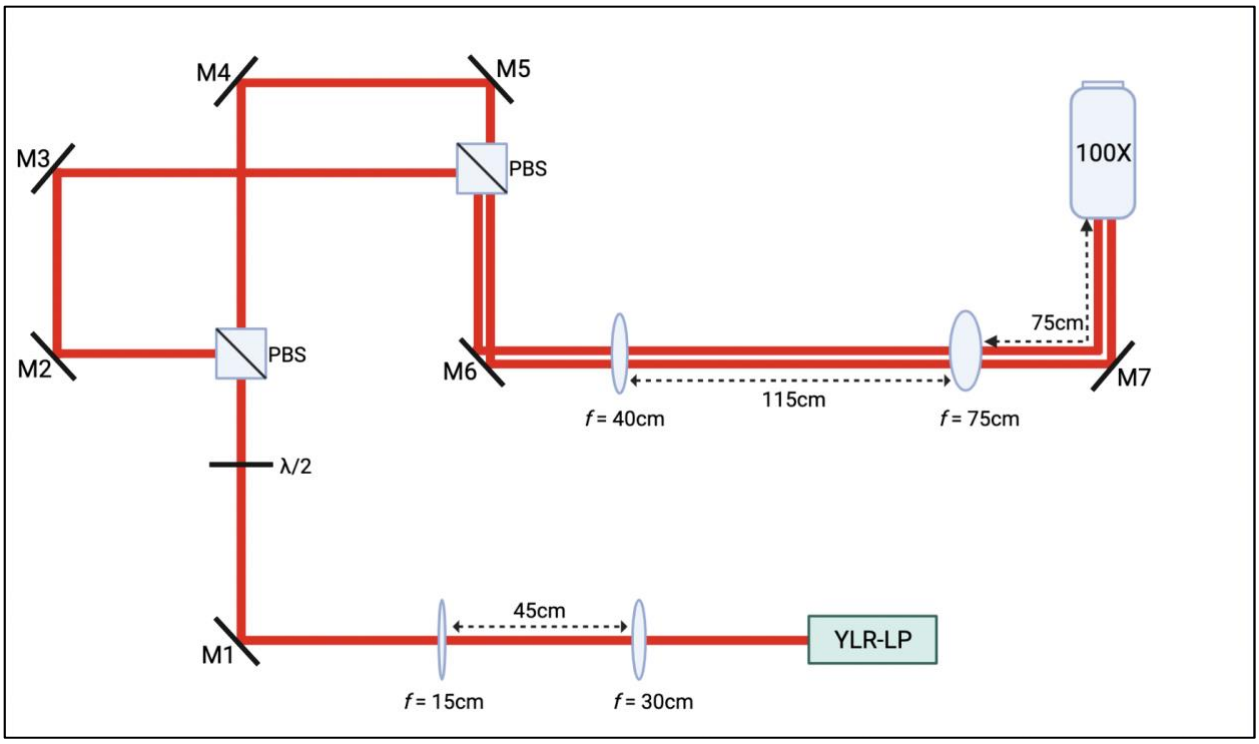

Figure 2: Optics setup for 5W ytterbium linearly polarized laser.
Two optical traps are formed from aligning the laser beam of a 5W ytterbium linearly polarized (YLR-LP) laser through a series of mirrors, lenses, and beam splitters to the mounting thread end of a 100X objective on an inverted AxioObserver D1 microscope. Lenses, f =30cm and f =15cm, are used to decrease the beam size so that they will fit into the optic components. The half-wave plate (λ/2) is present to allow for change to the percentage of power in each beam without changing the total power. Lenses, f =40cm and f =75cm, are used to increase the beam size to overfill objective lens so the trap focus will be as small as possible. Mirrors labeled M1-M7. The two upper most left corner mirrors (M3 and M4) are each about 40 cm away from the 40 cm lens. This is necessary to allow the traps to be positioned independently of each other. When M3 or M4 is adjusted, it changes the location of that corresponding beam/trap on the computer screen without degrading the beam alignment. PBS = polarizing beam splitter. Drawing created in BioRender.com, not to scale.

Table 2: Microdevice optical trapping of low copy number (LCN) cell replicates.

	1:20 Diluted Neat Semen	Mixture*
Average Time Per Cell Spent Optical Trapping (min/cell)	2.30 ± 1.17	2.90 ± 1.40
Average DNA Total Yield Per Cell (pg/cell)	2.81 ± 2.85	4.24 ± 2.94
Average DI Per Sample	1.00 ± 0.52	1.16 ± 0.52

*Mixture is male-female mixture.

Table 3: DNA Quantitation data for optically trapped 1:20 diluted neat semen samples.

Number of Cells	Total Yield (pg)	Theoretical Yield (pg)	Degradation Index*
0	7.63	0	N/A
5	21.50	15	2.36
5	5.18	15	1.70
5	0.00	15	N/A
10	40.70	30	0.79
10	14.15	30	N/A
10	47.49	30	0.97
15	48.95	45	1.07
15	6.81	45	0.84
15	19.89	45	0.42
20	243.80	60	0.68
20	45.72	60	0.54
20	54.27	60	1.15
21	32.31	63	1.25
25	40.46	75	0.94
25	35.00	75	0.49
25	65.64	75	0.76
Average	**2.81pg/cell**	**3.0pg/cell**	**1.00 per sample**

*N/A = degradation index for sample could not be calculated due to large autosomal or small autosomal target quantitation value = 0.00, these values were not included in average degradation index calculation.

Table 4: DNA Quantitation data for optically trapped male-female mixture samples.

Number of Cells	Total Yield (pg)	Theoretical Yield (pg)	Degradation Index*
0	7.63	0	N/A
5	30.67	15	1.71
5	12.98	15	N/A
5	41.58	15	2.44
5	8.10	15	0.40
6	36.49	18	0.66
10	17.20	30	N/A
10	102.13	30	1.16
10	54.00	30	0.87
15	43.22	45	1.37
15	16.17	45	0.52
15	43.89	45	1.60
20	54.04	60	1.25
20	71.10	60	1.52
20	61.44	60	1.08
25	58.82	75	0.77
25	38.03	75	1.08
25	249.06	75	0.93
Average	**4.2431pg/cell**	**3pg/cell**	**1.16 per sample**

*N/A = degradation index for sample could not be calculated do to large autosomal or small autosomal target quantitation value = 0.00, these values were not included in average degradation index calculation.

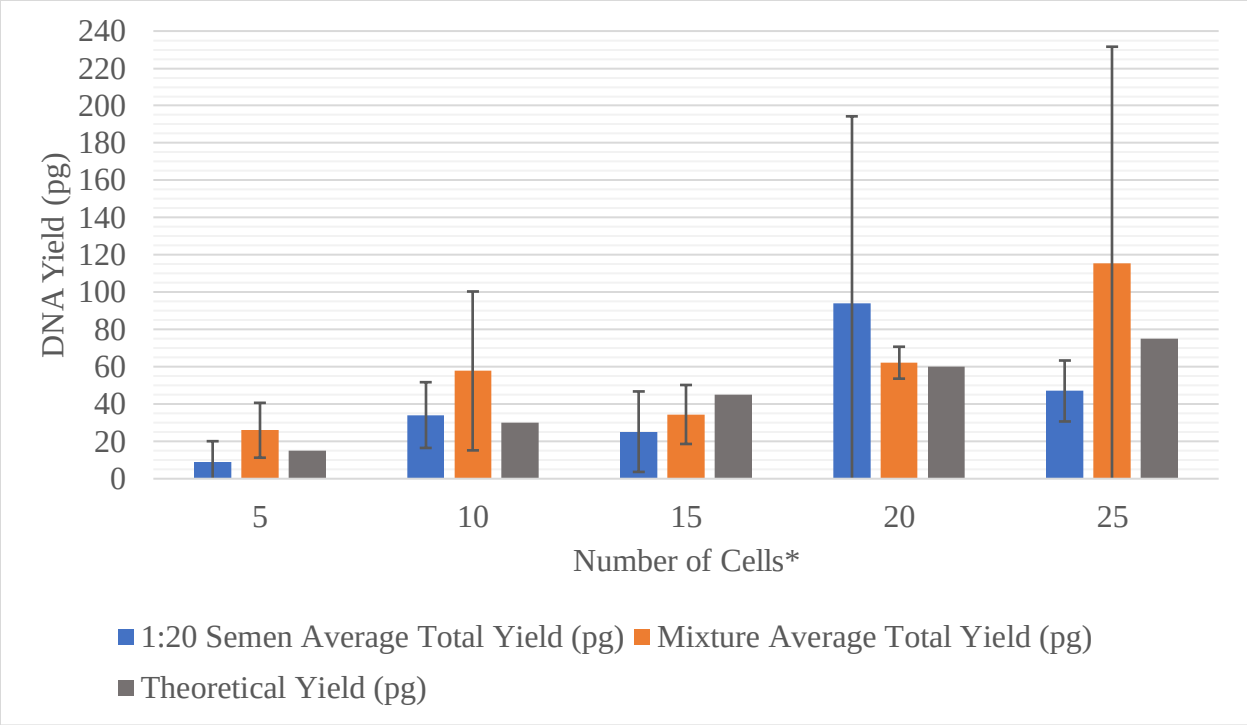

Figure 3: DNA yield for 1:20 diluted neat semen and mixture samples.
Three replicates per cell count except for 5 and 20 cell columns. Error bars represent standard deviation. All p-values were > 0.05.
*For mixture average total yield at 5 cell count, there are 4 replicates of 5 cells and one sample with 6 cells mixed into the average. For 1:20 semen average total yield at 20 cell count, there are 3 replicates of 20 cells and one sample with 21 cells mixed into the average.

Table 5: Results of STR profile analysis for 1:20 diluted semen samples.

Number of Cells	Expected Alleles (%)	Drop-in Alleles	Average Peak Height (RFU)
0	0.00	0	N/A
5	0.00	0	N/A
5	0.00	0	N/A
5	0.00	0	N/A
10	28.21	0	141.00±73.98
10	0.00	0	N/A
10	38.46	0	107.25±37.19
15	51.28	0	134.46±41.90
15	2.56	0	103.00±0.00
15	17.95	0	114.00±22.25
20*	100.00	0	752.09±193.78
20	53.85	0	148.52±44.20
20	64.10	0	121.03±50.72
21	43.59	0	128.83±49.55
25	84.62	0	199.79±83.67
25	74.36	0	161.71±57.84
25	76.92	0	203.08±87.93

N/A = no allele peaks to perform average peak height calculation.
*This sample had a calculated total yield of 243.7997pg.

Table 6: Results of STR profile analysis for male-female mixture samples.

Number of Cells	Expected Alleles (%)	Alleles from Female Contributor	Drop-in Alleles	Average Peak Height (RFU)
0	0.00	0	0	N/A
5	12.82	0	0	118.80±19.91
5	5.13	0	0	113.00±11.00
5	23.08	0	0	104.09±36.89
5	0.00	0	0	N/A
6	43.59	1	0	103.26±45.88
10	0.00	0	0	N/A
10	79.49	1	0	205.89±86.49
10	25.64	0	0	110.62±42.78
15	48.72	0	0	135.88±37.01
15	58.97	0	0	161.62±71.15
15	15.38	0	0	92.33±47.02
20	71.79	0	0	186.12±77.82
20	25.64	3	0	109.13±34.43
20	41.03	1	0	134.68±82.93
25	46.15	1	0	134.32±39.50
25	71.79	0	0	180.82±67.64
25	100.00	2	0	498.73±134.39

N/A = no allele peaks to perform average peak height calculation.

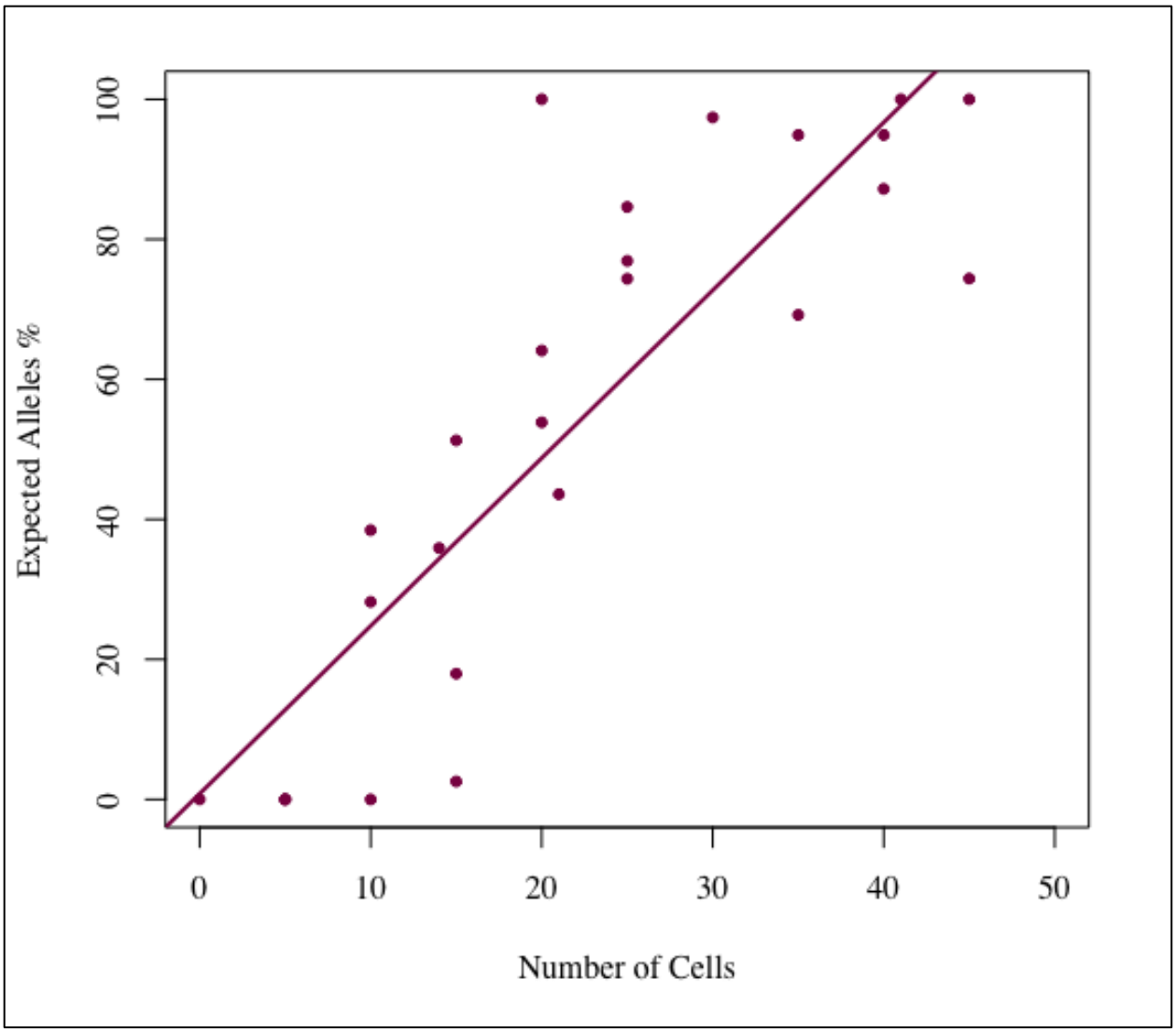

Figure 4: Simple linear regression for 1:20 diluted semen sample STR data.
Linear regression ($R^2 = 0.7245$, $F(1, 24) = 66.74$, p-value < 0.0001) comparing number of cells trapped and percentage of expected alleles observed in the STR profile.

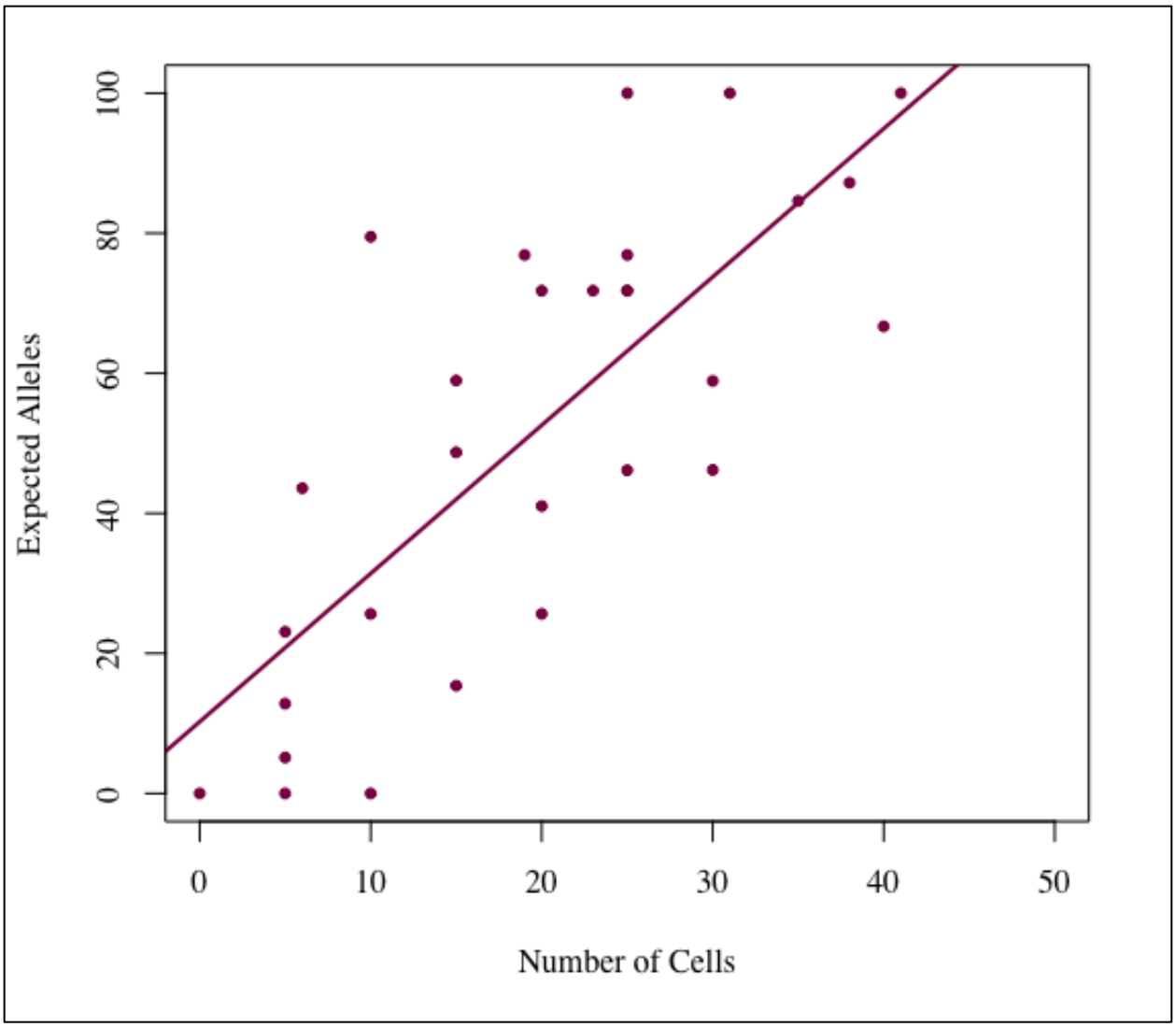

Figure 4: Simple linear regression for male-female sample STR data.
Linear regression ($R^2 = 0.5635$, $F(1, 27) = 37.15$, p-value < 0.0001) comparing number of cells trapped and percentage of expected alleles observed in the STR profile for male-female mixtures.

Video Links

Video Link 1 – https://youtu.be/nIZvJ4vgXCU

Description: Dual trap with optimized microfluidic device and 3.5um polystyrene beads

Video Link 2 – https://www.youtube.com/watch?v=XrkUjeJMvak

Description: Example of debris and air bubbles within microfluidic device channels

Video Link 3 – https://youtu.be/wcvy4MmR0jc

Description: Optical trapping of resuspended vaginal epithelial cells with YLR-LP laser 1.0W power output

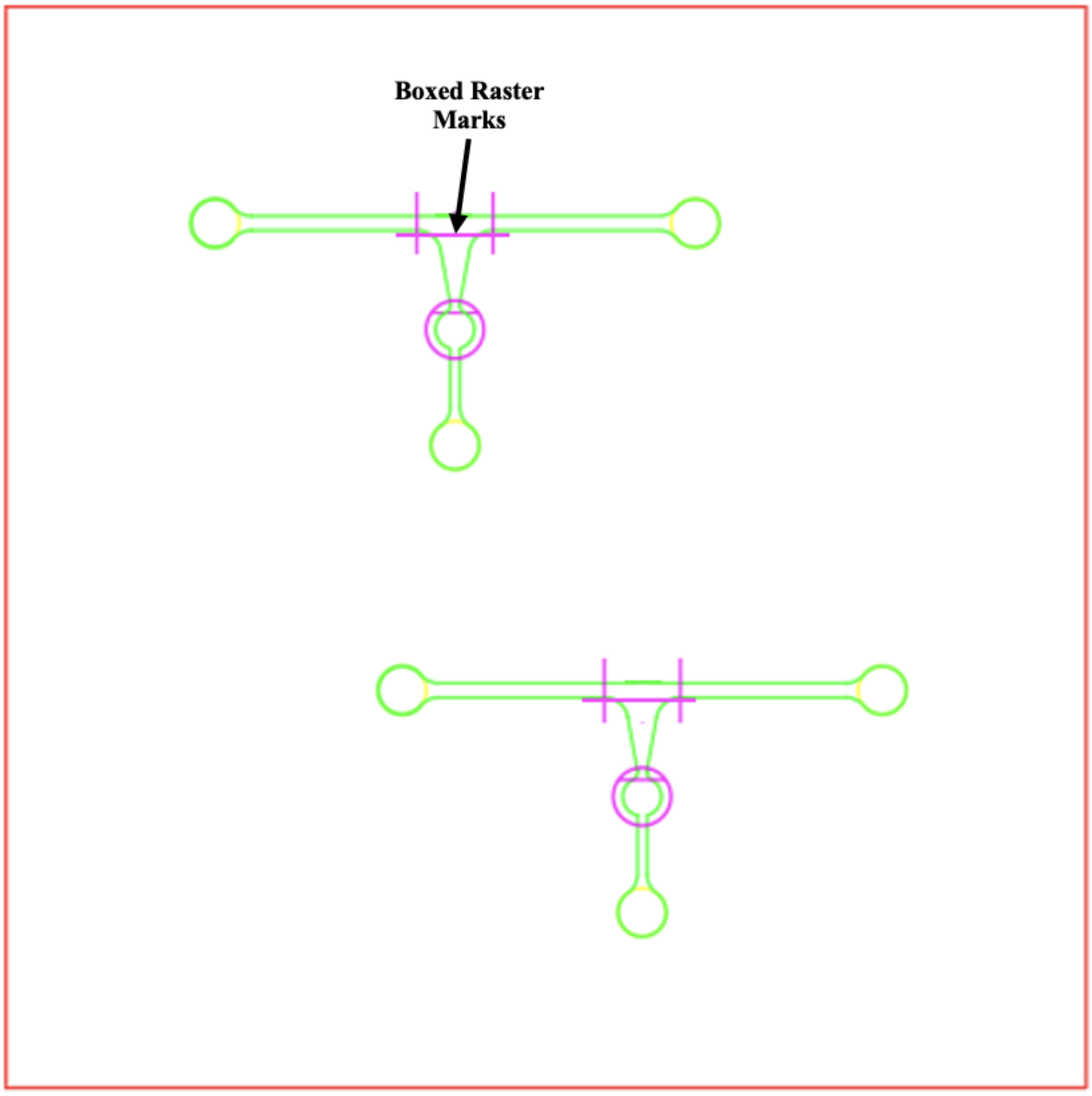

Figure 6: Proposed boxed raster mark design for microfluidic device.
Further optimization of the chip design to allow the operator to better understand where on the microfluidic device the optical traps are under the 100X objective. The majority of the cell flow occurs in the area outlined by the 'boxed' raster marks.

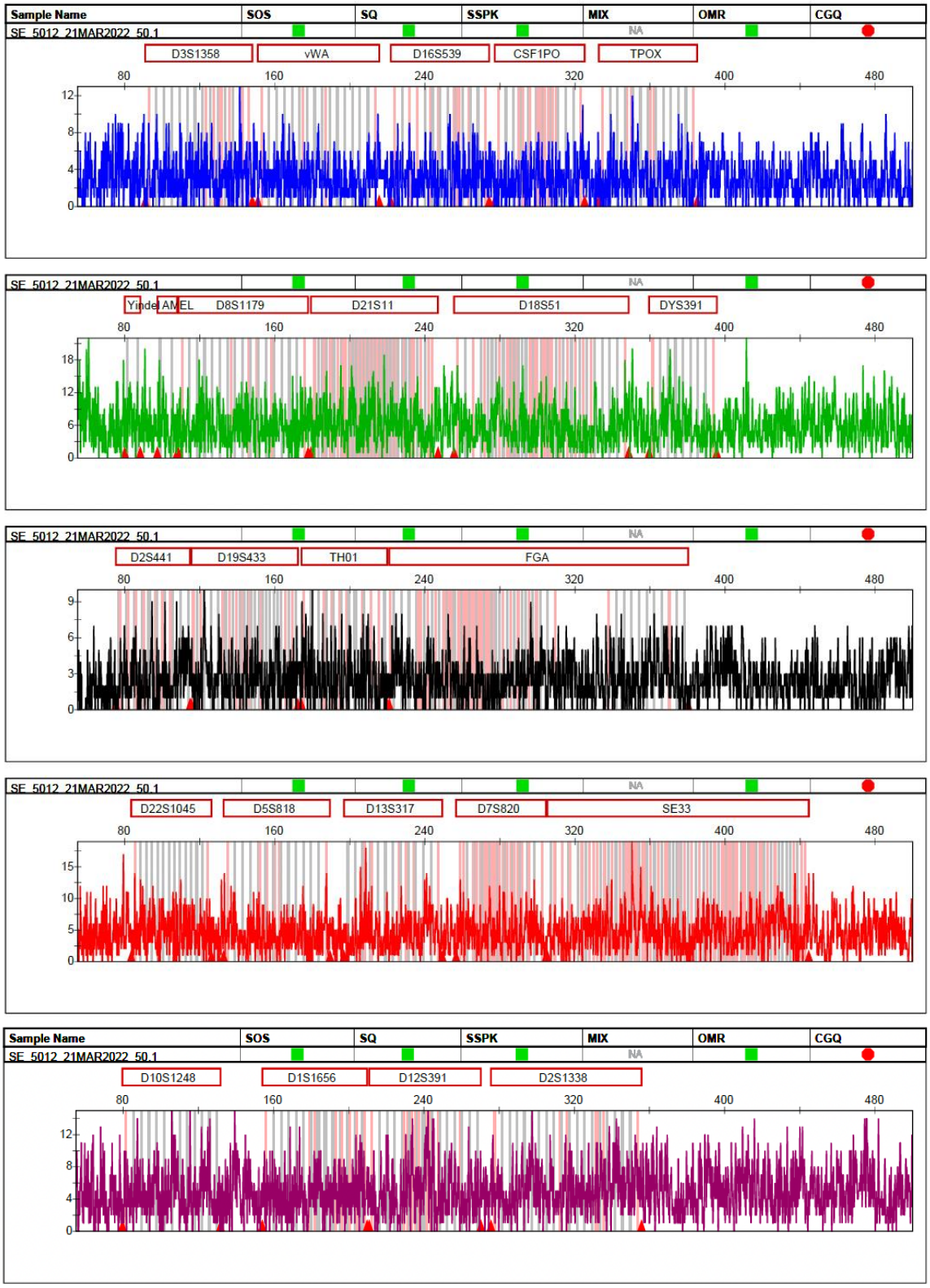

Figure 7: GlobalFiler STR profile of alkaline lysed optically trapped sample. Sample consists of 50 optically trapped sperm cells. No allele peaks above an analytical threshold of 100RFU were observed.

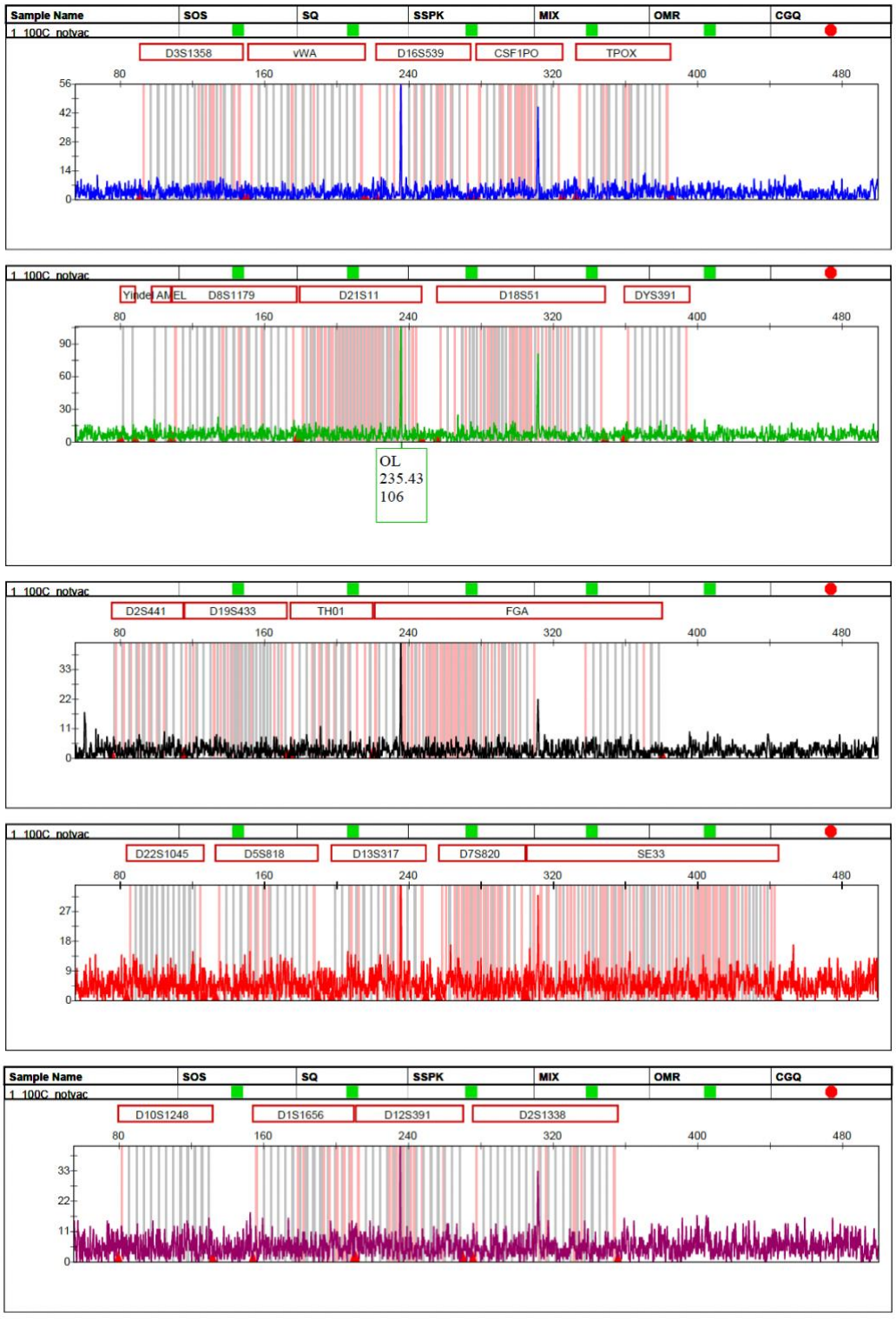

Figure 8: GlobalFiler STR profile of control alkaline lysis samples.
Electropherogram of a 1:100 dilution neat semen sample lysed via direct alkaline lysis, not vacuum centrifuged, and amplified with the GlobalFiler™ PCR Amplification Kit. Called off ladder peak by the software is a spike artifact. No true alleles above the analytical threshold were observed.

Table 7: 5W YLR-LP optically trapped 50 cell replicate quantitation and CE data.

Number of Cells Trapped	Total Yield (pg)	Theoretical Yield (pg)	Degradation Index	Expected Alleles (%)	Drop-in Alleles	Average Peak Height (RFU)
50	49.40	150	0.92	94.74%	0	502. 00 ± 263.74
50	115.42	150	1.13	100.00%	1	791.36 ± 268.49
50	90.17	150	1.25	97.37%	1	546.72 ± 225.72
Average*	**85.00 ± 33.31pg**	**150pg**	**1.10 ± 0.17**	**97.37 ± 2.63%**	-	-
19†	64.49	57	1.36	89.47%	1‡	303.80 ± 152.96

* Average calculated only using the 50 cell sample replicates.

† 19 sperm cell count sample was an off-target sample collected using the Harris Punch excision method and was not subjected to the alkaline lysis reaction chemistry.

‡ Drop-in Allele may be elevated n-4 stutter.

Table 8: Quantitation and CE data for hemocytometer control sample replicates.

Control Sample Name	Total Yield (pg)	Theoretical Yield (pg)	Theoretical Number of Cells Present	Degradation Index	Expected Alleles (%)	Drop-in Alleles	Average Peak Height (RFU)
1ul of 1:6 dilution 180 cells/µl Replicate A	18.33	90	6	1.20	23.68%	0	118.00 ± 32.53
1ul of 1:6 dilution 180 cells/µl Replicate B	36.96	90	12	0.62	97.37%	0	450.02 ± 272.05
1ul of 1:6 dilution 180 cells/µl Replicate C	10.05	90	3	0.40	44.74%	1	225.48 ± 104.71
Average	**21.78 ± 13.78pg**	-	**7 ± 5 cells**	**0.74 ± 0.41**	**55.26 ± 37.96%**	-	-
2ul of 1:6 dilution 180 cells/µl Replicate A	56.86	180	19	0.81	94.74%	1	407.10 ± 197.65
2ul of 1:6 dilution 180 cells/µl Replicate B	28.16	180	9	1.08	92.11%	0	548.80 ± 389.23
2ul of 1:6 dilution 180 cells/µl Replicate C	66.55	180	22	0.57	100.00%	1*	583.98 ± 218.72
Average	**50.52 ± 19.96pg**		**17 ± 7 cells**	**0.82 ± 0.25**	**95.61 ± 4.02%**	-	-
1ul of 180 cells/µl Replicate A	149.42	540	50	1.47	100.00%	1	1867.20 ± 642.26
1ul of 180 cells/µl Replicate B	57.96	540	19	0.99	92.11%	0	449.32 ± 241.84
1ul of 180 cells/µl Replicate C	421.66	540	141	0.54	92.11%	1	7124.34 ± 1991.15
Average	**209.68 ± 189.19pg**	-	**70 ± 63 cells**	**1.00 ± 0.47**	**94.74 ± 4.56%**	-	-

Table 9: Summary statistics of qPCR and CE analysis data for 50 sperm cell replicates versus the diluted control samples.

Sample Type	50 sperm cells	1ul of 1:6 dilution 180 cells/µl	2ul of 1:6 dilution 180 cells/µl	1ul of 180 cells/µl
Average Total DNA Yield (pg)	85.00 ± 33.31pg	21.78 ± 13.78pg	50.52 ± 19.96pg	209.68 ± 189.19pg
Average Degradation Index	1.10 ± 0.17	0.74 ± 0.41	0.82 ± 0.25	1.00 ± 0.47
Average Expected Alleles (%)	97.37 ± 2.63%	55.26 ± 37.96%	95.61 ± 4.02%	94.74 ± 4.56%

n = 3; ± Represents standard deviation

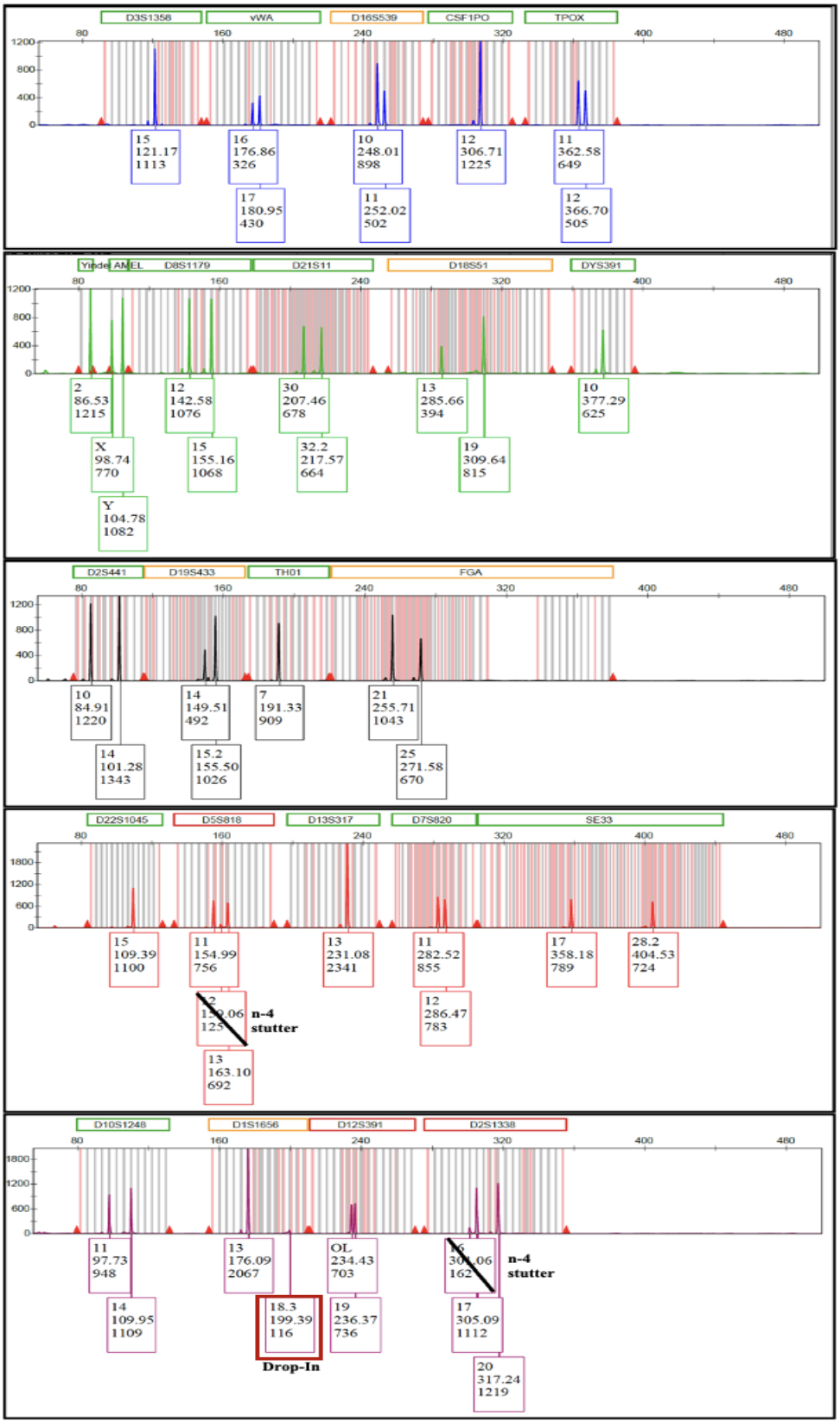

Figure 9: Representative full STR profile from 50 optically trapped sperm cells without ski sloping.

Vita

Samantha Pagel was born to parents Catherine and Raymond Scott Pagel in Cleveland, Ohio in 1998 and spent her entire childhood living in Strongsville, Ohio. She graduated from West Virginia University in May 2020 with a Bachelor of Science degree in Biology and Forensic & Investigative Science with an Area of Emphasis in Forensic Biology. During the summer of 2019 between her junior and senior year, Samantha completed an internship at the National Institute for Occupational Health and Safety in the Health Effects Laboratory Division under the supervision of Dr. Ann Hubbs and Kara Fluharty. Samantha started as a first-year graduate student in the Master of Science in Forensic Science program at Virginia Commonwealth University in August 2020 and pursued the forensic biology concentration. During her time at VCU, Samantha served as a Laboratory Teaching Assistant for the university's biology department teaching both the Biological Concepts Laboratory and Introduction to Biological Science Laboratory II. In the summer of 2021, Samantha started her directed research work in the Seashols-Williams laboratory investigating the use of a microfluidic device and optical trapping for the separation of sexual assault type samples.