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Abstract

Current methods of mixture separation in forensic DNA laboratories typically deconvolute the mixture after analysis using statistical analysis or probabilistic genotyping. To save time and effort of labs already backlogged, a method to separate mixtures on a cellular level before analysis needs to be developed. Optical trapping is a method that uses a focused 1064 nm laser to manipulate cells. Previous research has shown that approximately 50 spermatozoa or 15 leukocytes from a liquid sample are required to produce a full STR DNA profile. It was found that the number of spermatozoa required remains constant when the method of sample collection is changed to cotton swab mimicking sexual assault evidence. These spermatozoa can be collected at a rate of approximately two cells per minute and can retrieve nearly all spermatozoa within 400 nL if the surface of the coverslip upon which trapping occurs is modified with poloxamer 407. While leukocytes isolated from whole blood can be trapped, it has been found that leukocytes that have been reconstituted from a swab lose their morphology and are hard to identify and trap. To combat this issue, DAPI was used to stain the nucleus of leukocytes to identify them under microscopy for trapping. Stained cells were easily identified but were repelled by the trap. This research demonstrates that optical trapping is an efficient method to separate spermatozoa even in samples collected from sexual assault kits with low numbers of spermatozoa to generate full, single-source STR profiles. Further research should be conducted on the best method to separate reconstituted leukocytes of irregular morphology.

Keywords: Forensic science, optical trapping, spermatozoa, leukocytes, semen swabs, blood swabs, mixture

Introduction

The Impact of Mixtures on Analysis

The most common problem encountered by forensic biologists when analyzing forensic evidence is that of mixtures. A mixture occurs when two or more individuals' biological fluids or cells are deposited on evidence. This commonly occurs on sexual assault evidence as swabs collected from the victim are likely to contain that victim's cells as well as those of the perpetrator, and potentially consensual partners. Other possible mixtures could occur during physical assaults which could result in mixtures of blood, saliva, or both on skin, surfaces, or clothing [1].

Ordinarily, the presence of a mixture cannot be detected until the very end of the DNA analysis process when analyzing short tandem repeat (STR) profiles. This slows the process as mixtures with two contributors demand that the analyst deconvolute the mixture, carefully considering the makeup of alleles at each locus [2]. If the mixture has greater than two contributors, this often becomes infeasible to manually process. To address this problem, laboratories have recently begun implementing back-end bioinformatics solutions such as probabilistic genotyping software [3,4]. However, recent research has explored a more intuitive solution. Instead of deconvoluting mixture samples after they have already been processed, mixtures can be separated on a cellular level before they are amplified for STR analysis [5-10].

Differential Extractions

The oldest and most common method of front-end cell separation in the case of sexual assault samples is differential lysis prior to DNA purification [5]. In this approach, the mixed sample is treated with lysis reagents, such as sodium dodecyl sulfate (SDS) and proteinase K, and centrifuged. The lysis reagents lyse all non-sperm cells, leaving DNA free in the supernatant

and pelleting out unbroken spermatozoa heads and excess cellular debris. This allows the supernatant to be pipetted out, removing any epithelial DNA from the sample. The supernatant is referred to as the "non-sperm" fraction and can then be purified and the DNA eluted as normal. The pellet is then resuspended and treated with the same lysis reagents as well as dithiothreitol (DTT). DTT disrupts the disulfide bonds present in spermatozoa heads that otherwise prevent the cell from being lysed. The resulting solution is referred to as the "sperm fraction" and can then also be purified and the DNA eluted [5]. Unfortunately, not all male cells present during ejaculation are spermatozoa [11]. Male non-sperm cells will be lysed along with the victim's epithelial cells. Further, victim epithelial DNA may carry over into the sperm pellet using this method [12]. Thus, profiles developed from this technique can still contain low-level mixtures. As well, this method is time consuming and not conducive to automation as it contains two separate extraction incubation steps. Additionally, no current methods have been widely implemented in forensic labs to separate other common mixtures, such as blood/buccal cells or touch/buccal cells.

Fluorescent-Activated Cell Sorting

One endeavor to improve front-end cell separation has been to utilize fluorescence-activated cell sorting (FACS). FACS combines the common method of flow cytometry with a fluorescent probe to sort cells. Verdon et. al used this method to separate mixtures of blood and buccal cells by tagging them with anti-CD45 and anti-CD227 respectively. Two-person mixtures were combined at ratios ranging from 5:1 to 1:1000 blood to buccal cells as well as the inverse [6]. The percentage of blood and buccal cell contributor alleles seen in developed STR profiles were then compared before and after sorting. One hundred percent of alleles from both were observed in the 5:1 and 1:1 blood to buccal cell mixtures. However, alleles from the blood

sample contributor were detected in the isolated buccal sample of all but one of the mixtures. Likewise, alleles from the buccal cell contributor were detected in the blood fraction in 6 of the 14 mixtures [6]. Without full separation, probabilistic genotyping software was still needed to analyze the profiles on the back end of the DNA analysis process. Furthermore, the number of alleles from the blood sample contributor detected in the unsorted samples dropped to around 57% at a 1:10 ratio of blood to buccal cells and fell further to 25% at 1:100 and 8% at 1:1000 as the blood grew more dilute. Blood cells that were correctly sorted into the blood fraction produced profiles with more alleles than the unsorted mixtures, but not to the extent of a full profile [6]. This method may prove more useful in separating mixtures of a single cell type, as shown in Dean et. al [13]. In this study, two and four contributor mixtures were separated into clear major and minor contributors in mixtures containing only blood by sorting cells based on their relative fluorescent intensity governed by their uptake of HLA-A*02 antibody, but this method remains inefficient for the separation of multiple cell types [13].

Laser Capture Microdissection

Laser capture microdissection (LCM) is another method under investigation for integration into the DNA workflow. In this method, cells are adhered to a thermoplastic film and then placed on a glass microscope slide. This slide is then placed inverted under a microscope objective that focuses an ultraviolet (UV) laser. This laser can be used to cut the film around the cells, allowing them to either drop or be catapulted into a collection vessel for further analysis. Alternatively, the cells may be fixed to the slide with the film attached to a cap above the sample. In this method, the cap comes into contact with the sample and an infrared (IR) laser is used to cut out cells of interest which then adhere to the film [7,8]. Sanders et. al found in mixtures of semen and female buccal epithelial cells that no interference occurred from the female minor

contributor with full profiles being observed in samples of 75, 150, and 300 sperm cells with some dropout, however, Costa et. al observed contradictory results [14,15]. In their study, semen was combined with epithelial cells in 1:1, 1:4, 1:9, and 1:19 two-person mixtures. In all cases, mixtures were still observed in profiles with the 1:9 and 1:19 mixtures, including the presence of several stochastic effects, such as pull-up and allelic dropout [15]. This is likely due to non-target cells attaching to the film before it is cut [14]. This is most apparent in the IR type LCM where the thermoplastic film to which the cells adhere physically contacts the sample [7]. The cell fixation procedure is also cause for concern. Cells are fixed either with formalin, which is known to degrade DNA, or with xylene, which is highly toxic [8]. Additionally, the total time that would be added to the DNA analysis process for LCM would be approximately 2 hours and 20 minutes [15].

The DEPArray

A third potential method for cell separation is the DEPArray. The DEPArray is an instrument that uses the principle of dielectrophoresis to separate individual cells. Cells are distributed and held, suspended within 300,000 microelectrodes in a medium between two electrodes which generate a non-uniform electric field in what is referred to as "DEP cages" [9,16]. Dielectrophoresis allow cells to move through the instrument based on both fluorescence and cellular morphology directed by proprietary cell-sorting software [16]. Williamson, et. al investigated the use of this instrument in a forensic context [17]. Mixtures were created by combining buccal cells and dilutions of semen in 1:1, 1:10, 1:100, 1:1000, and 1:10000 ratios as well as buccal cells with blood and dilutions of semen in 1:1:1, 1:1:10, and 1:1:100 ratios. Single source male profiles were obtained from all of these ratios of semen to buccal cells up to 1:1000 and for all buccal cells, blood, and semen ratios [17]. However, the processing time per sample is

prohibitively long. According to the manufacturer, the processing time of the DEPArray v2 can take between 8 to 32 hours per sample with approximately two hours of hands-on time within that range [18]. The DEPArray NxT has a shorter instrument run time, but only reduces the total time per sample by approximately an hour and a half [19].

Optical Tweezers

This research explores the potential for optical tweezers to remedy the problems of specificity seen in FACS and LCM and processing time seen in LCM and the DEPArray. Optical tweezers operate by directing the momentum of light to impact an object of interest. This general principle is usually only seen on the astronomical scale where the sheer distance and intensity of light can affect the displacement of matter. However, it can be replicated on a smaller, practical scale by small particles and a laser. A laser pointed at an object will exert a gradient force on that object, drawing it closer towards its focal point until the gradient force overcomes the on-axis scattering force and the object is trapped, immobile [20]. In a conventional optical tweezer set-up, the laser is focused through a microscope objective with a high ratio of focal length to lens diameter (numerical aperture) (NA > 1.2) [21]. This microscope objective can be mounted on an inverted microscope with a charge-coupled device (CCD) camera set up to view the trap through the objective [22].

In 1987, Ashkin et. al applied this technology to living cells [23]. Previous work had shown the utility of the trap on non-living material, but this research aimed to see if the trap caused damage to living cells. A high-resolution optical microscope was set up with a 10.6 μm neodymium-doped yttrium aluminum garnet (ND:YAG) IR laser focused through a water-immersion objective with a numerical aperture of 1.25 [23]. The aim was to trap *Escherichia coli* within a three-dimensional container and observe if it maintained its life cycle and ability to

reproduce. An individual cell was lifted out of the bottom of the sample to a clear region within the container and observed. After 5 hours in the trap, the bacterium had progressed through 2.5 life cycles and the resulting 4 daughter cells had remained in the trap, indicating the bacterium had remained undamaged. The bacterium could also be moved throughout the space at 500 μm/s with no damage to the cell [23]. This suggested that optical tweezers could be used with other cells without damaging their inner workings.

Previous Work with Optical Tweezers at Virginia Commonwealth University

Auka et. al demonstrated the feasibility of using optical tweezers to separate spermatozoa from vaginal epithelial cells to develop single-source male profiles [10]. Semen and vaginal epithelial cells were mixed at a 1:1 ratio and 8- 55 spermatozoa were tweezed out of solution in individual samples. Percent recovery of spermatozoa ranged from 58-140% of Identifiler Plus loci (Applied Biosystems, Foster City, CA). DNA recovery exceeding 100% may be explained by more than the tweezed number of cells being extracted from the droplet. This preliminary study showed that at least 50 spermatozoa were needed to produce a profile with greater than 90% of the expected STR alleles [10].

More recent research has shown that leukocytes can also be tweezed using this method and less than 20 cells are needed to develop a full STR profile [24]. Leukocytes were prepared by performing an ammonium-chloride-potassium (ACK) lysis on 1 mL of venous blood, storing the resulting leukocytes and lysed erythrocytes in 100 μL of 1x phosphate-buffered saline (PBS) before cell separation. It was found that the most efficient method to lyse these cells was to eject collected cells from the tweezer directly into QIAamp Buffer ATL (QIAGEN™, Hilden, Germany) before DNA extraction.

Method Optimization

With the discovery that direct injection into lysis buffer improved the efficiency of DNA recovery with leukocytes, the procedure for spermatozoa might likewise be altered. Previously spermatozoa were ejected onto a glass coverslip. Once on the coverslip, the individual spermatozoa could be counted under microscopic magnification. The cells would then be extracted off the coverslip using a modified QIAamp extraction procedure [25]. In this new method, the coverslip was eschewed, eliminating the ability to count the recovered cells. However, quantification methods could be used to calculate an estimate of the number of cells injected after separation.

Cells were found to be most efficiently processed by direct injection likely due to the hydrophilic nature of the coverslip surface upon which trapping occurs. With samples of either spermatozoa or leukocytes, over time cells were settle to the bottom of the droplet and onto the glass surface of the coverslip. Since the coverslip is primarily composed of borosilicate, its surface is very hydrophilic, like the surface of a cell membrane. As a result, cells tend to adhere to this surface. It is possible that leukocytes ejected onto a coverslip and kept at -20°C were lysed, causing the DNA inside to adhere to the coverslip and the QIAamp DNA Investigator extraction method did not produce favorable conditions to unbind the DNA from the borosilicate. As well, cells that have settled and adhered to the surface cannot be moved by forces applied by the trapping laser. Thus, over time, less of the sample can be separated by an analyst. A possible solution to this problem is to alter the surface of the coverslip with the elastomer polydimethylsiloxane (PDMS) bonded to the copolymer poloxamer 407.

PDMS is an elastomer commonly used to alter surface chemistry without altering the other properties of a material. This polysiloxane is entirely hydrophobic but is amenable to modification by several processes such as the introduction of surface chemistry modifiers like

poloxamer 407. Poloxamer 407 is a triblock polymer consisting of repeating units of 65 hydrophobic polypropylene oxide (PPO) chains flanked by 100 hydrophilic polyethylene oxide (PEO) chains on either side [26]. When introduced to a PDMS-coated surface, the hydrophobic PPO chains interact with the polysiloxane on the surface, leaving the hydrophilic PEO chains free [27]. In this manner, adjusting the concentration of PDMS and poloxamer 407 allows dynamic control over surface chemistry polarity. With the reduction in hydrophilicity afforded by this surface treatment, the degree of cell to surface adhesion may be reduced.

Forensic-Type Samples

Following optimization with liquid samples, it is prudent to investigate the utility of this technique with samples resembling those received by case working labs. The most common method for serological collection at a crime scene or as part of a sexual assault kit is to swab the area of interest and then submit the swab to the laboratory. Therefore, cells were targeted and separated from reconstituted swabs of semen and vaginal fluid as well as blood to compare results from dried swabs to those obtained from liquid samples. Since it was unknown whether leukocytes remain intact upon drying onto a swab, a method was devised to stain exclusively nuclear material using DAPI to ascertain whether leukocytes remain intact and to identify their location within the sample.

Methods

Sample Collection

All samples used in this project were collected per a VCU IRB approved human subjects protocol (VCU HM20002931). One donor was used for each type of sample with mixtures made between types of samples with a minimal amount of STR alleles in common. Vaginal epithelial

cells were collected from multiple donors on cotton swabs. Semen and venous blood were collected from donors and stored at 4°C. Venous blood was collected with venipuncture and stored in EDTA Vacutainer® tubes to prevent coagulation.

Sample Preparation

Vaginal cells dried on cotton swabs were cut into 300 µL deionized H_2O and allowed to incubate at room temperature for five minutes to allow adequate time for the cells to elute off and become suspended in solution. Neat semen was diluted ten-fold in 4 mg/mL BSA in ddH_2O. Mixtures of semen and vaginal cells were prepared by creating a solution of 75 µL of 1:10 semen and 75 µL of vaginal cells in ddH_2O. Swabs with blood were prepared by inserting a sterile cotton swab into an EDTA Vacutainer® tube and allowing the swab to dry overnight. Semen and vaginal mixture swabs were then cut into 300 µL deionized H_2O and allowed to incubate at room temperature for five minutes with periodic vortexing to be reconstituted in solution.

DAPI Cell Staining

DAPI was prepared by modifying the ThermoFisher DAPI Counterstaining Protocol for flow cytometry [28]. Ten milligrams of DAPI dihydrochloride were added to 2 mL of dimethylformamide (DMF) to a final concentration of 14.3 M. Staining solution was prepared by diluting the 14.3 M DAPI in DMF in staining buffer [100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet P-40 substitute] to a final concentration of 3 µM. Leukocytes were eluted off a swab in one milliliter of 1x PBS for 15 minutes. The swab was then removed and the cells were pelleted by centrifugation at 10,000 rpm for two minutes. The supernatant was removed and the cells were resuspended in staining solution and 20 µL of 51 mM EDTA was added. The cells were incubated for 15 minutes at room temperature and then pelleted by centrifugation at 10,000 rpm for two minutes and resuspended in 10 µL of 1x PBS.

Coverslip Treatment

Coverslip treatment was performed by members of the Lemmon Group at Virginia Commonwealth University. To adjust the hydrophilicity of the trapping surface, standard borosilicate coverslips were first rinsed with 100% ethanol, then centered on a benchtop spin coater platform. Polydimethylsiloxane (PDMS) was slowly added to the coverslip while spinning with the following protocol: 500 rpm with an acceleration factor of 100 for five seconds, 2000 rpm with an acceleration factor of 200 for ten seconds, 3000 rpm with an acceleration factor of 500 for 35 seconds, and 2000 rpm with an acceleration factor of 200 for ten seconds. The coverslips were then incubated overnight at 110°C to cure and placed under UV radiation for 10 minutes to induce cross-linking. The resulting surface was placed in a 2% solution of poloxamer 407 for one hour.

Cell Separation

Before optical trapping, a glass coverslip was adhered to the bottom of a microscope slide with a hole bored through the center to form a well. Hexadecane was pipetted into the well to prevent evaporation of sample droplets. A droplet sized between 400 nL was created by pipetting the sample of interest into the hexadecane. A 100x magnification objective lens mounted to an AxioObserver D1 inverted microscope (Zeiss, Thornwood, NY) was used to focus a 700 mW (typically launched through an OD1 neutral density filter so the power at the trap focal spot was ca. 25 mW), 1064 nm continuous wave (CW) ND:YAG laser (CrystaLaser, Reno, NV) into the sample. Cells were trapped by being held at the focal point of the laser. The slide was then moved by the mechanical stage to transport the trapped cell to the interface between the droplet and the hexadecane. The specific cell to be targeted varied depending on the sample. In samples

of blood, leukocytes were targeted. In samples of semen and vaginal cells, spermatozoa were targeted. Approximately 50 spermatozoa were moved to the interface. After all the needed cells were relocated, a capillary was placed in 1x PBS briefly to collect a small amount of solution. This capillary was then quickly inserted into the interface where the trapped cells have been relocated, drawing the cells up via capillary action. The resulting solution was ejected into 300 µL of QIAamp Buffer ATL lysis buffer (QIAGEN™).

DNA Extraction

The QIAGEN™ QIAamp DNA Investigator Kit (QIAGEN™) was used for DNA extraction of each sample according to the "Isolation of Total DNA from Surface and Buccal Swabs" manufacturer protocol without the addition of carrier RNA at the lysis step. For samples containing spermatozoa, 20 µL of DTT were introduced at the lysis step. All samples were eluted in a volume of 30 µL [29].

DNA Quantification

The quantification of all samples took place on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) with the Quantifiler Trio Kit (Applied Biosystems) according to the manufacturer protocol modified for ½ reactions [30]. Standards were run in duplicate with the following concentrations: 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and 0.005 ng/µL to generate a standard curve. DNA concentration was determined by multiplying the quantity by the 30 µL elution volume and compared to the theoretical yield calculated by multiplying the number of tweezed cells by 3 pg per cell if haploid or 6 pg per cell if diploid [31]. A degradation index was also calculated by comparing the concentrations of the small autosomal (SA) and large autosomal (LA) targets [30].

STR Amplification

After quantification, DNA extracts were concentrated down to approximately 7.5 µL with a Savant DNA120 SpeedVac concentrator (ThermoFisher Scientific, Waltham, MA) at a low drying rate with no heat. The PowerPlex® Fusion 5C kit (Promega, Madison, WI) was used on an ABI ProFlex PCR System (Applied Biosystems) according to the manufacturer's protocol with a modification for ½ volume reactions [32]. The entire concentrated DNA extract was added to the PCR reaction to ensure the maximum amount of DNA was present. The following thermal cycler program was used: 96°C for 1 minute followed by 30 cycles of 94°C for 10 seconds, 59°C for 1 minute, and 72°C for 30 seconds, then 60°C for 45 minutes followed by a hold at 4°C.

Capillary Electrophoresis Analysis

Alleles from the STR PCR product were separated with an ABI Prism 3130 genetic analyzer (Applied Biosystems) according to the PowerPlex® Fusion System Technical Manual (Promega) with the following modifications: 0.3 µL of WEN ILS 500 and 9.7 µL Hi-Di Formamide were added along with either 1 µL of sample or allelic ladder to each well in a 96-well plate [32]. The plate was heated to 95°C in a thermocycler for three minutes followed by being snap-cooled on a freezer block for 5 minutes. The plate was run on the instrument with the following parameters: 3 kV injection for 5 seconds with 36 cm capillaries containing POP-4 polymer. Samples were analyzed with GeneMapper™ Software version 4.1x. An analytical threshold of 50 rfu was used and each electropherogram was assessed for overall quality. Electropherograms were evaluated by assigning contributors based on profiles from the male

sample, female sample, analyst who performed the workflow, and other individuals in potential contact with the sample.

Statistical Analysis

To determine the predictive capacity of the number of tweezed spermatozoa to the percentage of STR profile developed for both cell recovery methods, a non-linear regression growth curve model using Equation 1 was developed.

$$\hat{y} = \frac{A}{1 + e^{\frac{m-x}{s}}} \qquad \textit{Eq. 1}$$

Where $\hat{y}$ is the model estimate of the percent developed profile, x is the number of trapped spermatozoa, A is the asymptote, m is the inflection point of the curve, and s is a scaling factor. Since profile development is known to plateau at 100%, the variable A was set to 100. The Microsoft Excel SOLVER add-in was then used to optimize values of s and m to minimize the standard error of regression (how close the observed values fall to the regression line) (Equation 2) and the standard error of the residuals (the standard deviation of the residuals from the regression line) (Equation 3) [33].

The standard error of the regression was calculated using Equation 2.

$$\sqrt{\frac{\frac{\sum_i^n y_i - \hat{y_i}}{n-2}}{\sum_i^n (x_i - \bar{x})^2}} \qquad \textit{Eq. 2}$$

Where y_i is the observed percentage STR profile development, n is the number of samples, x_i is the number of trapped spermatozoa, and $\bar{x}$ is the mean of trapped spermatozoa across all samples.

The standard error of the residuals was calculated using Equation 3.

$$\sqrt{\frac{\sum_i^n y_i - \widehat{y_i}}{df}} \qquad \textit{Eq. 3}$$

Where df is the degrees of freedom calculated by subtracting the number of parameters of the regression equation from the number of samples.

A 95% confidence interval was used to calculate the standard error of prediction by multiplying the standard error of the residuals by 2 standard deviations. Plots of the resulting regression along with standard error clouds were generated using the statistical software R 3.6.1.

Results and Discussion

Method Optimization

Despite the improvement in yield and STR profile development seen in leukocytes, the number of spermatozoa required to produce a full profile remained unchanged when the method of cell recovery was altered to direct injection (Figure 1). However, it was found that the growth curve regression model for the direct injection recovery method was more accurate than the regression model for the coverslip recovery method as can be seen in the standard errors of regression (Table 1). It also produced more predictive power as can be seen with the standard error of prediction (Table 1). In the direct injection method, an analyst can predict the percent profile development based on the number of trapped spermatozoa with an accuracy of ±~19% compared to the coverslip method with an accuracy of ±~31%.

Notably, in samples of 26, 40, 50, 58, and 60 in the direct injection method, the observed yield is higher than the theoretical yield (Figure 2). However, although the samples of 26 and 50 cells did include the female contributor, in the samples of 40, 58, and 60, no drop-in or minor contributor was observed (Table 2). More cells than expected could have been collected in these

samples. The method of capillary action used to remove the cells has the possibility of collecting extraneous cells within the general area of the trapped cells. This uncertainty was visually estimated and reported, however this method is fallible as it is possible that more cells were collected than visually observed. Overall, samples of more than 23 cells showed greater than 80% profile development, but consistent full profiles were only developed in samples exceeding 50 cells (Figure 3). An electropherogram of 50 cells demonstrated 100% profile development with low levels of minor contributor alleles but no degradation (Figure 4).

Coverslip Surface Treatment

To reduce the amount of time needed to trap, the surface of the coverslip was altered with poloxamer 407. The mean ratio of cells trapped to time spent trapping for both methods was around two cells per minute (Table 3). The cells that were trapped on the untreated coverslips came from exclusively liquid spermatozoa and vaginal cell mixture samples while the cells that were trapped on the treated coverslips came from exclusively reconstituted swabs of spermatozoa and vaginal cell mixtures. The liquid samples were more densely populated with cells while the reconstituted samples were sparser. In the liquid samples, time was spent attempting to trap cells that were adhered to the surface which was time that could not be spent actively transporting cells to the interface to be recovered. In reconstituted samples, nearly all the cells within the droplet could be trapped and transported, but time had to be spent moving the trap to each cell. While the total time taken to trap the needed 50 cells appeared not to change, the treated coverslips allowed nearly all cells within the droplet to be trapped. Thus, if a sample has a low concentration of spermatozoa and a normal coverslip is used, not all spermatozoa can be recovered, making it necessary to use multiple droplets and increase overall time spent during cell isolation. However, if a treated coverslip is used, almost all spermatozoa may be recovered

from a single droplet, potentially eliminating the need for multiple droplets and leaving exclusively vaginal cells.

Reconstituted Semen and Vaginal Cell Mixture Swabs

Since the most common type of sample of spermatozoa received by a forensic laboratory is a swab, mixtures of spermatozoa and vaginal cells were dried on swabs and reconstituted for trapping. As mentioned previously, samples reconstituted from swabs had a much sparser population of spermatozoa per droplet. Additionally, most spermatozoa lacked tails after being dried onto a swab so spermatozoa heads were targeted for trapping. It had previously been found that 50 spermatozoa were needed to produce a full STR profile, so that number of cells or as many as possible were targeted for trapping. Quantification results showed general agreement between the observed and theoretical yields with the exception of the 50 cell sample likely due to the sparser population of cells within the droplet reducing the possibility of other cells being recovered with the trapped set (Figure 5). The sample of 50 cells had significant drop-in as well as alleles attributed to the female contributor (Table 3). As expected, a sample containing 50 cells produced a full STR profile (Figure 6). An electropherogram demonstrated full profile development and no degradation (Figure 7).

Reconstituted Blood Swabs

Unlike samples containing semen and vaginal cells, samples containing blood do not exclusively contain components with nuclei. Blood is composed of erythrocytes, leukocytes, plasma, and platelets. Valle previously found that attempting to trap leukocytes out of whole blood is infeasible. The conglomeration of erythrocytes prevents the movement of leukocytes

through a droplet [24]. Additionally, the 1064 nm trapping laser relies partially on a difference in refractive index between a cell and its environment. While spermatozoa have a sharp contrast between cell and background, leukocytes are harder to distinguish. To combat this issue, whole blood was enriched by lysing erythrocytes and removing all other components but leukocytes by use of an ammonium chloride potassium lysis method leaving a solution of leukocytes of regular morphology within 1x PBS [24]. This produced enough difference in refractive index difference between the leukocytes and their environment to permit trapping and removed physical barriers to the movement of cells to the interface. However, when blood is dried on swabs, erythrocytes may be lysed as far less are observed in a droplet from a reconstituted blood swab. Though even with a lack of erythrocytes, other components of blood are still present, thus the issue of refractive index similarity remains. Additionally, upon drying and reconstitution, leukocytes lose their characteristic morphology and become misshapen and irregular. This lack of identifiable morphological characteristics may cause confusion between nuclei-containing leukocytes and cellular debris. To ascertain whether an object in view is a leukocyte or other material, a DAPI cellular staining method for flow cytometry was used [28]. While leukocytes eluted off of blood swabs were found to uptake the fluorescent dye, it also produced significant cellular aggregation which could potentially be attributed to the presence of Mg^{2+} ions in the staining buffer due to the divalent cation being a known promoter of cellular adhesion [34]. Thus, EDTA was added to chelate excess divalent ions which did reduce cellular aggregation (Figure 9). Individual leukocytes of irregular morphology could be seen with the uptake of DAPI, positively identifying them as cells to be trapped (data not shown). However, upon interaction of the trapping laser with the stained cells, the cells were repelled by the trap possibly due to the properties of the nuclear stain indicating that DAPI may not be an optimal stain for use in

conjunction with optical trapping (data not shown). Cells with similar morphologies to those that integrated the DAPI stain were also difficult to trap. The size and irregular morphology appeared to create unfavorable conditions for the cell to remain in the trap.

Conclusions

The objectives of this research were to investigate the efficiency of the direct injection cell recovery method for spermatozoa compared to the previously developed coverslip method, improve trapping efficiency by reducing cellular adhesion, and applying the optical trapping technique to forensic-type dried samples. This project has demonstrated that the direct injection method developed for recovery of leukocytes works as well for the recovery of spermatozoa to an approximately equivalent degree to the previously utilized coverslip recovery method [24,25]. The number of cells needed for a full STR profile remaining unchanged from approximately 50 could be explained statistically using an equation derived by Lucy et. al for haploid cells [35]. Since one cell only possesses half of the genetic information of an individual, there must logically be some minimum number of cells required to be certain that all alleles are obtained from a sample. The equation is as follows:

$$n = \frac{\log(1 - \alpha^{\frac{1}{k}})}{\log(0.5)} + 1 \qquad \textit{Eq. 4}$$

Where n is the number of cells required to get a full STR profile, α is the certainty of obtaining a full profile, and k is the number of heterozygote loci of the individual [35]. So, with an individual with 16 heterozygote loci, it would take approximately 12 cells at 99% certainty to get a full profile. However, DNA extraction processes result in loss of sample. If 77% of the sample is lost during the extraction process, it would require approximately 50 cells to obtain enough DNA to achieve a full STR profile. This number is consistent with research into extraction efficiency

completed by Oorschot et al and Idris and Goodwin. Oorschot et al demonstrated between 20% and 76% loss in sample following a Chelex-100 extraction and Idris and Goodwin demonstrated that the QIAamp DNA Investigator Kit (QIAGEN™) is overall less efficient than a Chelex-100 extraction [36,37]. As well, a minimum input DNA of 125 pg required for both the Applied Biosystems Identifiler Plus kit and the Promega PowerPlex Fusion 5C kit [38,39]. This is mathematically approximately 42 haploid cells, thus drop-out from kit sensitivity may also play a factor [31]. A possible solution to lower the number of cells required to theoretical levels is to use direct amplification, rather than extract the sample after recovery [40]. While more drop-in was seen with samples that used the treated coverslips, this drop-in was consistent with the profiles of the analyst performing extraction and the female contributor (Table 3). It is possible that the curing process for PDMS acts to sterilize the coverslips by denaturation of DNA, but further research may be required to test drop-in rates. However, the surface treatment did make it possible to trap nearly all the spermatozoa in a sparsely populated droplet. Further research could be conducted using the treated coverslips and direct amplification to determine if this will develop a single-source full STR profile from a mixture sample with a low number of spermatozoa.

Additionally, research on the separation of leukocytes out of reconstituted blood swabs should continue. Treatment with DAPI demonstrated that leukocytes remain intact when dried onto a swab. A different dye could be used that does not have a repulsive interaction with the trapping laser such as thiazole orange homodimer. Alternatively, the morphology of cells that uptake DAPI could be noted and similar unstained cells could be trapped. A reconstituted solution of blood could be centrifuged and resuspended in 1x PBS to remove the unwanted plasma and lipids. A bead approximately the diameter of a sperm cell head could be coated with

a protein that binds leukocytes and used as a “handle” to ameliorate the difficulty in trapping large irregular cells. Lastly, trapping should take place on a slide treated with poloxamer 407 to lessen cell surface adhesion.

Optical trapping has now been demonstrated to provide a simple, time-efficient method to separate spermatozoa from mixture samples and isolated leukocytes from enriched whole blood to develop a full, single-source STR profile. With additional research into leukocytes, and touch cells, this method could be implemented into the front end of the DNA analysis workflow with analysts capable of separating cells at a rate of two cells per minute.

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Appendix

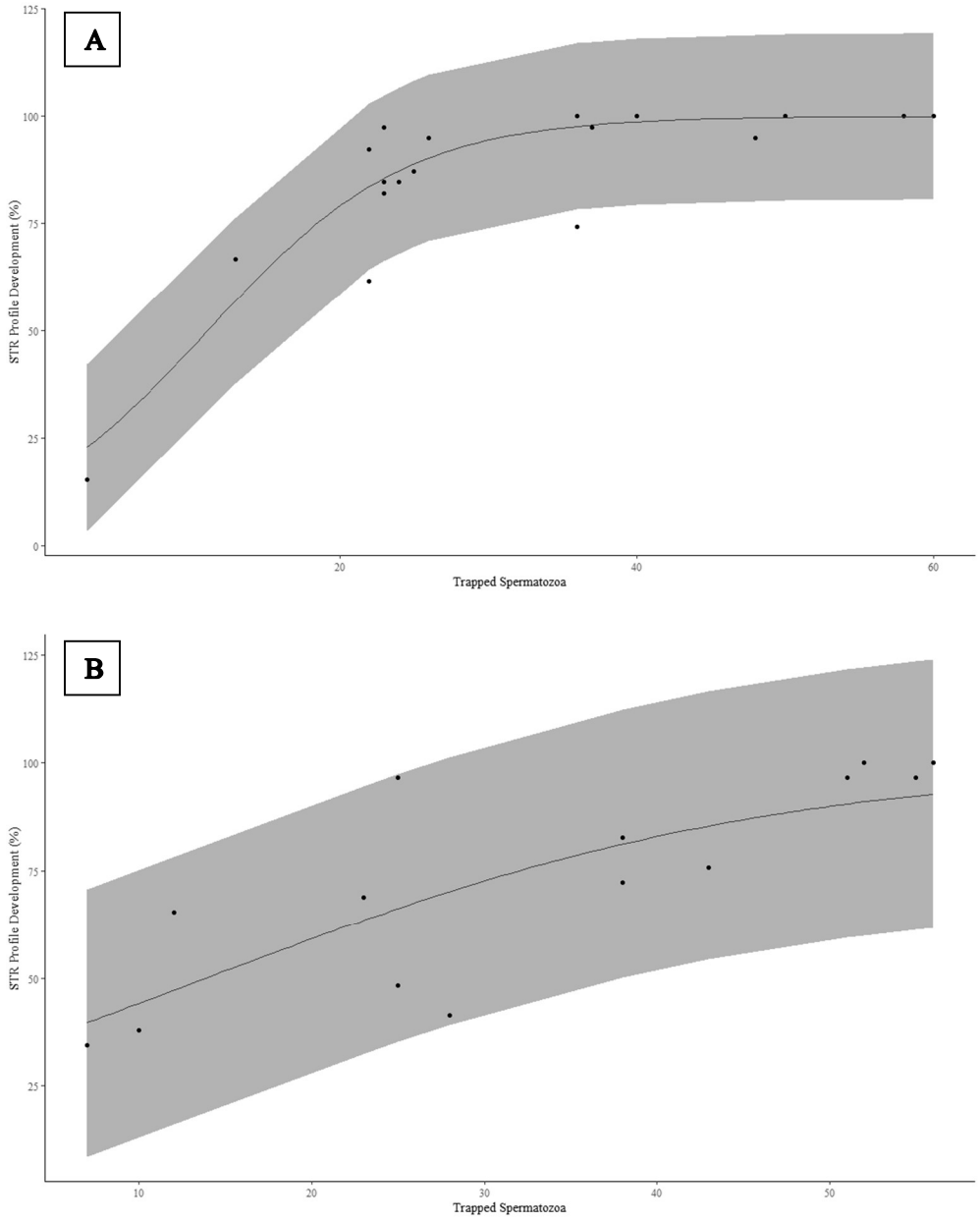

Figure 1 – Plots of percent STR profile development along with non-linear regression results for the coverslip and direct injection recovery methods. One hundred percent profile development was seen at ~50 trapped spermatozoa in both methods. Standard error of prediction plotted as gray ribbon around regression line. It was found the predictive power of the direct injection recovery method was stronger than the coverslip recovery method. **A)** Standard error of prediction of direct injection method was found to be ~±19% (In a profile of 39 alleles, this would be 7-8 alleles) **B)** Standard error of prediction of coverslip recovery method was found to be ~±31% (In a profile of 39 alleles, this would be 12-13 alleles).

Table 1 – Nonlinear regression results for the coverslip and direct injection recovery methods

Method	*A*	*m*	*s*	*Std Error of Regression*	*Std Error of Residuals*	*Std Error of Prediction*
Coverslip	100	13.87	16.47	0.244	15.5	±31.1
Direct Inject	100	11.11	6.69	0.177	9.65	±19.3

Table 2 – Results after STR profile development of isolated spermatozoa isolated from liquid semen/vaginal cell mixture samples

Trapped Spermatozoa	*Expected Alleles (%)*	*Drop-In Alleles*	*Female Contributor Alleles*
3	15	2	1
13	67	0	0
22	62	0	0
22	92	0	0
23	82	1	0
23	85	1	1
23	97	0	4
24	85	2	0
25	87	0	1
26	95	0	2
26	95	1	0
36	74	0	0
36	100	0	0
37	97	1	0
40	100	0	0
48	95	0	0
50	100	0	3
58	100	0	0
60	100	0	0

Table 3 – Efficiency of trapping in cells per minute for untreated and poloxamer 407 treated coverslips

Untreated Coverslips

Number of Cells	***Time (min)***	***Cells/Min***
20	8	2.50
20	24	0.83
20	33	0.61
35	20	1.75
35	28	1.25
35	27	1.30
25	14	1.79
25	31	0.81
50	18	2.78
50	13	3.85
		AVG 1.75 ± 1.03

Poloxamer 407 Treated Coverslips

Number of Cells	***Time (min)***	***Cells/Min***
10	17	0.59
15	7	2.14
22	22	1.00
25	7	3.57
25	6	4.17
25	23	1.09
25	16	1.56
25	22	1.14
30	25	1.20
		AVG 1.83 ± 1.24

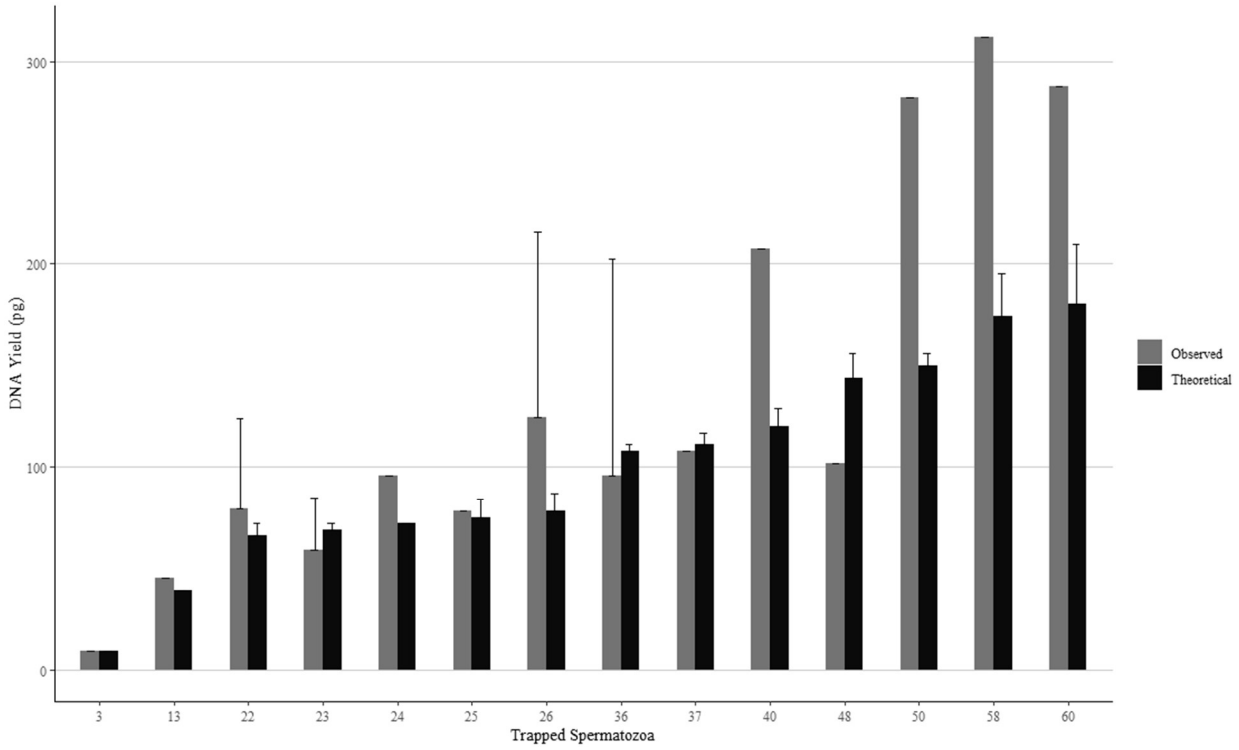

Figure 2 – DNA yield for cells isolated from liquid semen/vaginal fluid mixtures. n = 1 for all bars except for 22, 26, and 36 (n = 2) and 23 (n = 3). Error bars on observed yields represent the standard deviation from the mean for those samples with multiple replicate trapping events. Error bars on theoretical yields represent the error propagation of the number of spermatozoa visually estimated to have been recovered from a droplet. Samples tended to exceed the theoretical yield calculation based on 3 pg per cell.

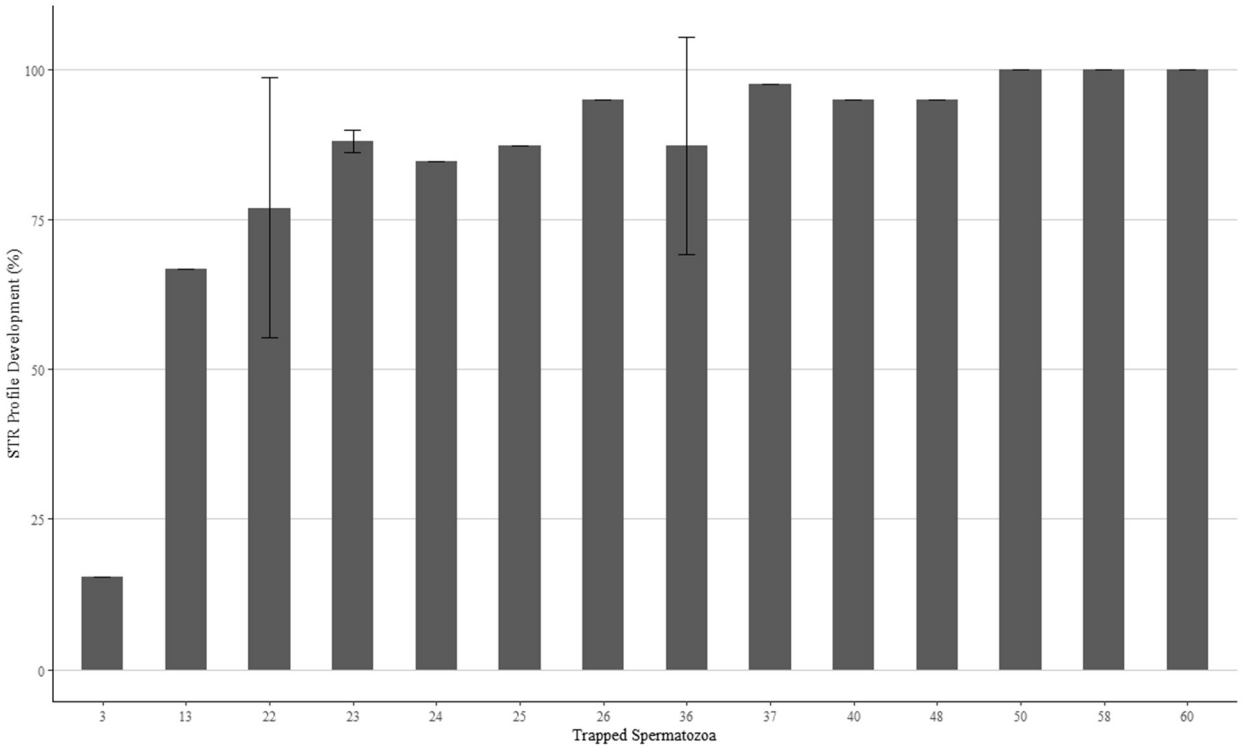

Figure 3 – Percentage of STR profile development for cells isolated from liquid semen/vaginal fluid mixtures and recovered using the direct injection method (n = 1 for all bars except for 22, 26, and 36 (n = 2) and 23 (n = 3)). Error bars represent the standard deviation from the mean for those samples with multiple replicate trapping events. Samples in excess of 23 cells showed >80% profile development, with samples exceeding 50 cells showing consistent full profile development.

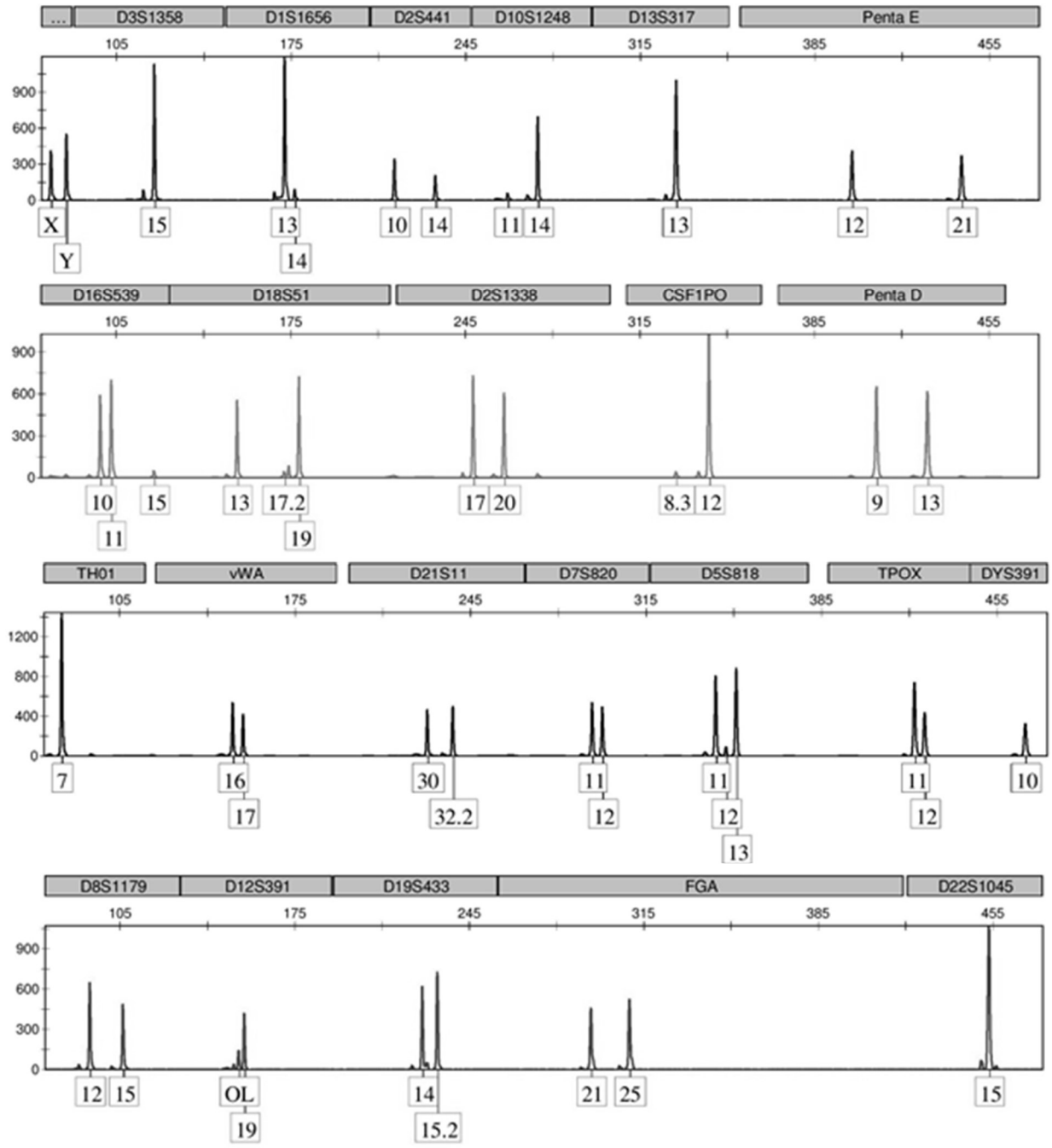

Figure 4 – Representative electropherogram for 50 cells isolated from a liquid semen/vaginal fluid mixture demonstrating full profile development with no degradation. Low levels of the minor contributor can be observed but no drop-in occurred. The "OL" allele called at locus D12S391 is the microvariant 18.2.

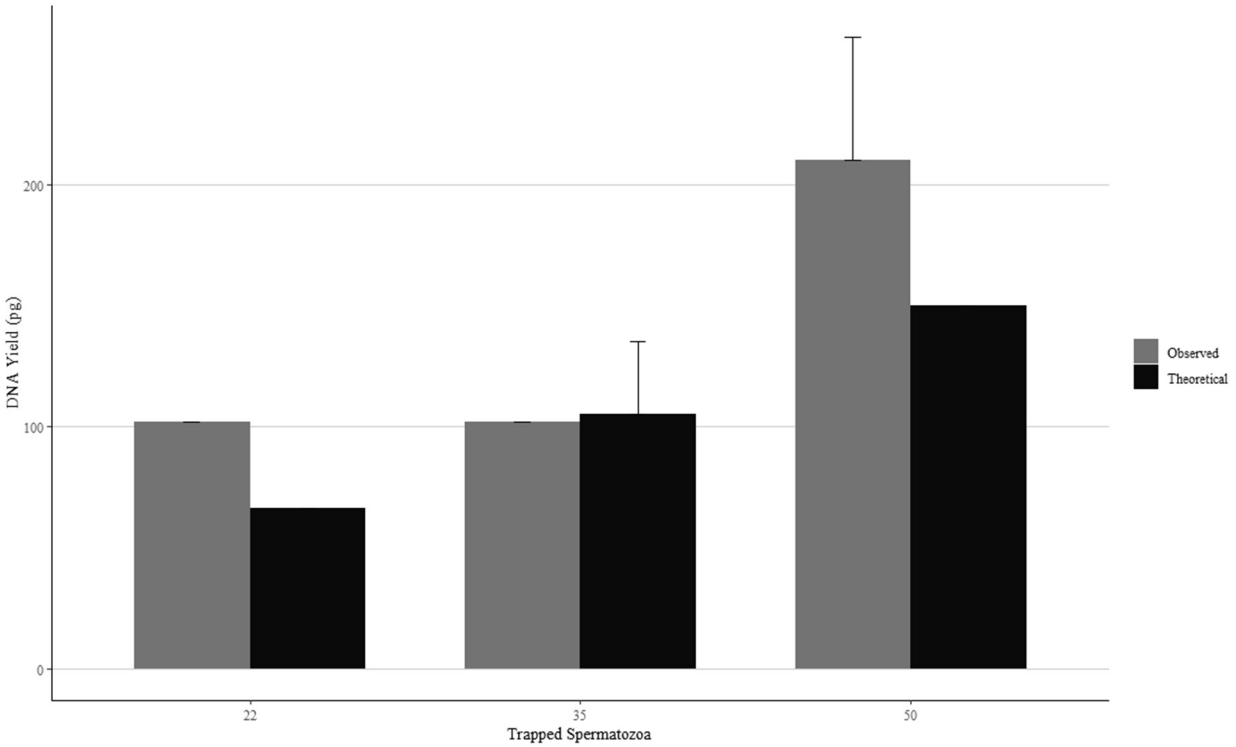

Figure 5 - DNA yield for cells isolated from reconstituted swab semen/vaginal fluid mixtures. n = 1 for each bar except for 50 (n = 2). Error bars represent the standard deviation from the mean for those samples with multiple replicate trapping events. Error bars on theoretical yields represent the error propagation of the number of spermatozoa visually estimated to have been recovered from a droplet. The samples of 22 cells and 50 cells show higher observed than theoretical yield calculated based on 3 pg of DNA per cell.

Table 4 – Results after STR profile development of isolated spermatozoa isolated from liquid semen/vaginal cell mixture samples

Trapped Spermatozoa	*Expected Alleles (%)*	*Drop-In Alleles*	*Female Contributor Alleles*
22	74	0	0
35	92	0	1
50	97	2	0
50	100	5*	3*

**** - One allele could be from the female contributor or drop-in***

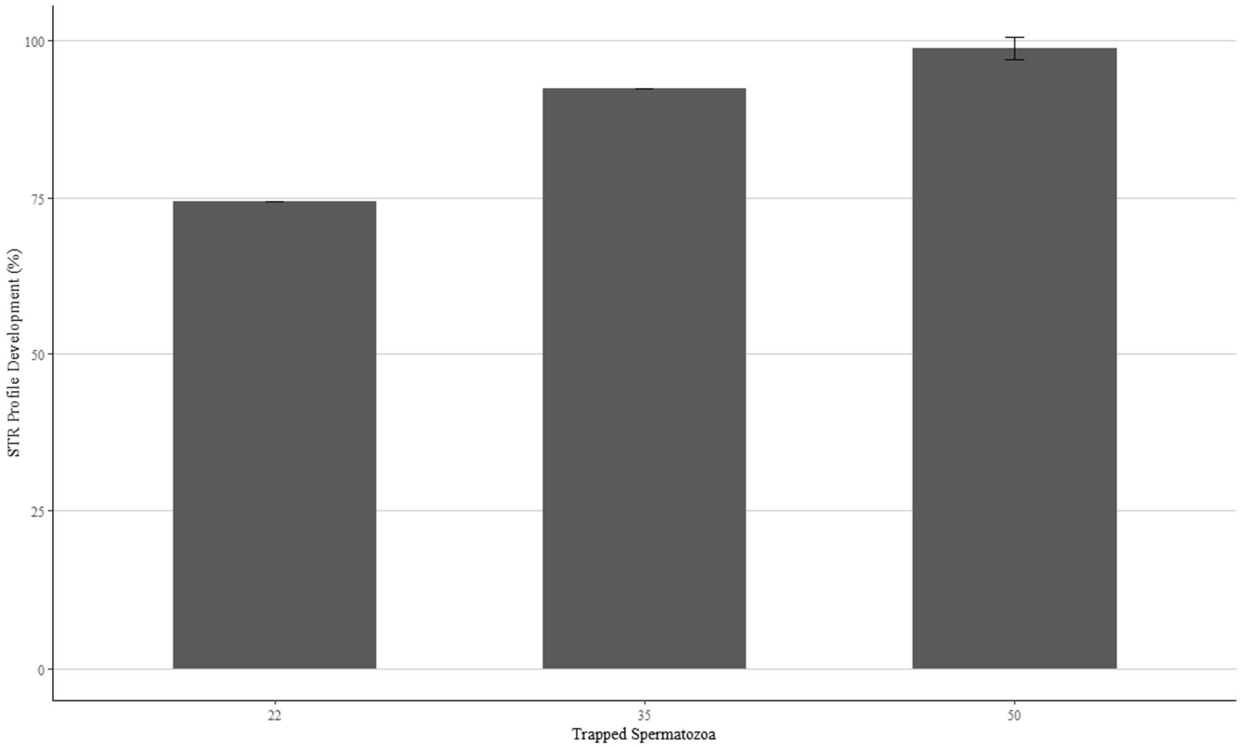

Figure 6 - Percentage of expected alleles for cells isolated from reconstituted swab semen/vaginal fluid mixtures and recovered using the direct injection method. n = 1 for each bar except for 50 (n = 2). Error bars represent the standard deviation from the mean for those samples with multiple replicate trapping events. Like the liquid samples, dried samples showed full profile development with 50 cells.

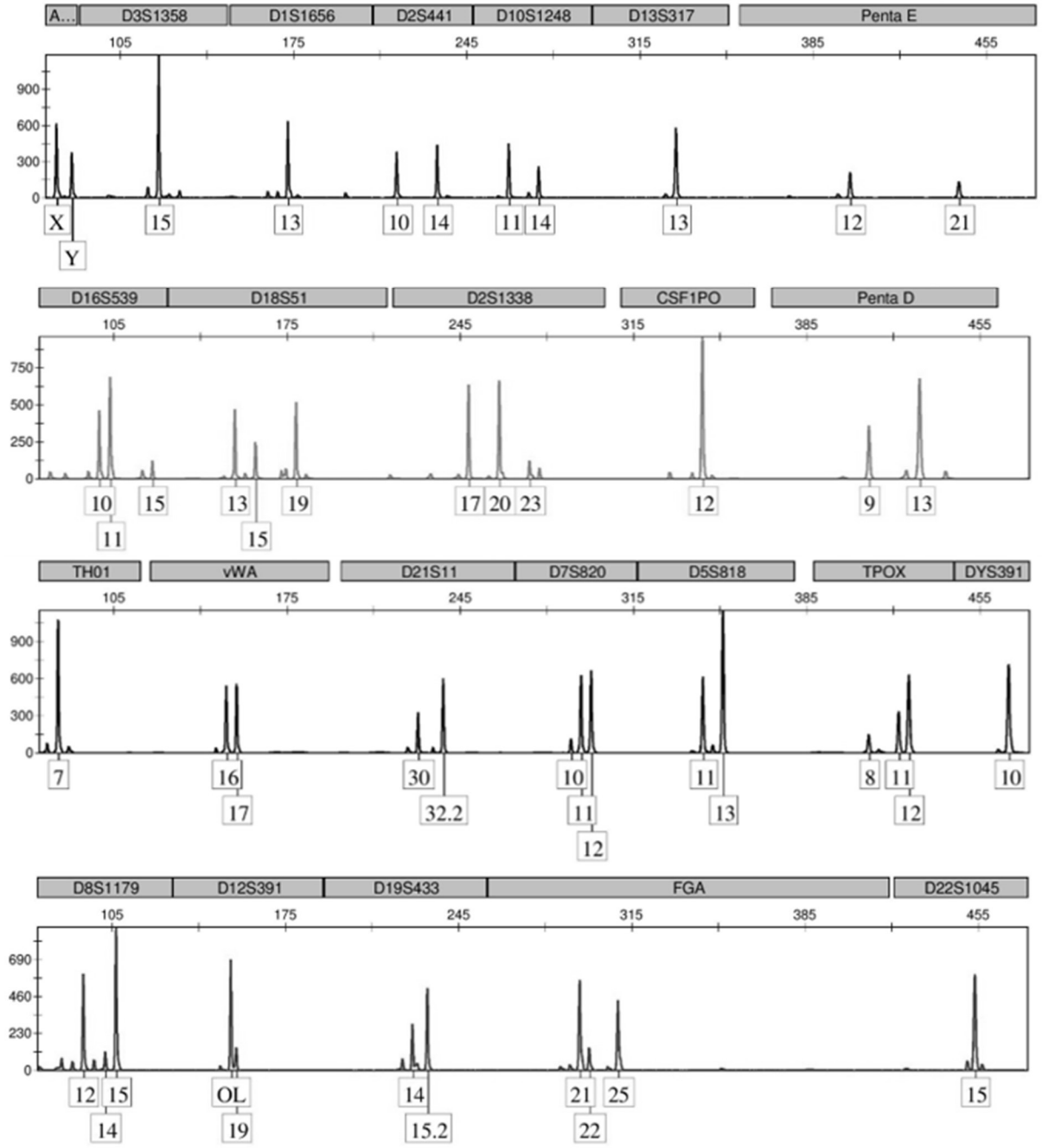

Figure 7 - Representative electropherogram for 50 cells isolated from a reconstituted swab semen/vaginal fluid mixture demonstrating full profile development with no degradation. Some drop-in is observed.

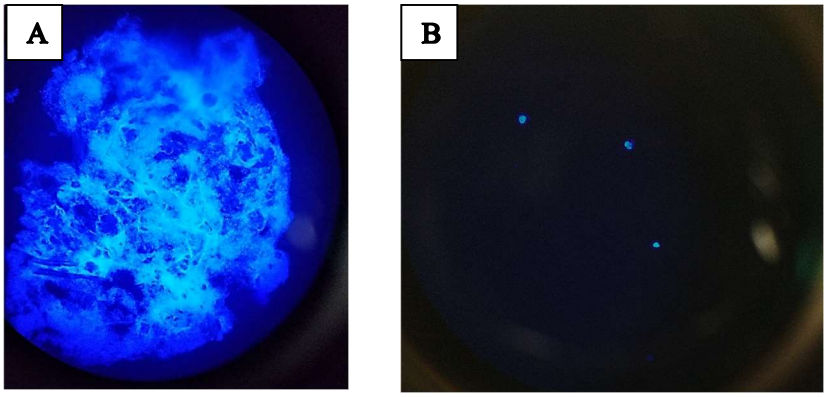

Figure 9 – Microscopic images of DAPI-treated leukocytes from reconstituted blood swabs. **A.** Significant cellular aggregation was observed with no addition of EDTA. **B.** Individual cells observed due to reduction of cellular aggregation after addition of EDTA.

Vita

Benjamin O'Brien was born in Indianapolis, IN on December 2, 1995. He graduated New Palestine High School in 2014. He attended Ball State University where he was a student of the Honors College and majored in Chemistry with a concentration in Biochemistry and minored in Cellular and Molecular Biology and Digital Forensics. Over the course of his undergraduate career, he pursued research in molecular biology and later nucleic acid nanotechnology. He attended the First Conference on Biomotors, Virus Assembly, and Nanobiotechnology Applications in August of 2017 where he presented a poster entitled "Investigation into the Properties of Hybrid Nucleic Acid Nanoparticles." He also presented a poster entitled "Programmable Nucleic Acid Based Polygons with Controlled Neuroimmunomodulatory Properties for Predictive QSAR Modeling" at the RNA Rustbelt Meeting in October of 2017. He authored an Honors thesis "The Ever-Expanding Field of Nucleic Acid Nanotechnology" before graduating with his Bachelor of Science in 2018. Benjamin began attending Virginia Commonwealth University in 2018 where he joined the Forensic Biology track of VCU's Department of Forensic Science graduate program. He pursued cell separation research in the Williams laboratory of the department from May of 2019 through April of 2020 and gave an oral presentation of his research "Application of Optical Trapping to Obtain Single-Source STR Profiles from Forensically Relevant Body Fluid Mixtures with Modified DNA Analysis Workflow" at the American Academy of Forensic Science annual conference in February of 2020. During his time at VCU, Benjamin served as Graduate Teaching Assistant where he aided instruction in undergraduate forensic science courses.