

Separation of Sperm and Epithelial Cells in a Microfabricated Device: Potential Application to Forensic Analysis of Sexual Assault Evidence

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Forensic DNA analysis of sexual assault evidence requires separation of DNA from epithelial (victim) and sperm (perpetrator) cells. The conventional method used by crime laboratories, which is termed “differential extraction”, is a time-consuming process. To supplant the conventional process, separation of sperm from a biological mixture containing epithelial cells has been demonstrated on a microfluidic device. This separation utilizes the differential physical properties of the cells that result in settling of the epithelial cells to the bottom of the inlet reservoir and subsequent adherence to the glass substrate. As a result, low flow rates can be used to separate the sperm cells from the epithelial cell-containing biological mixture. Following cell separation on the microdevice, DNA extraction, amplification, and separation were performed using conventional laboratory methods, showing that the cell separation product in the outlet reservoir was of male origin. The reported cell separation has the potential to impact the forensic DNA analysis backlog of sexual assault cases by circumventing the time-consuming conventional differential extraction procedure.

DNA analysis has proven to be a valuable technique for identifying the perpetrators of crimes, particularly those crimes involving sexual assault. However, because of the increasing utility of DNA analysis and the lack of sufficient funding, there currently exists a major backlog of cases waiting to be analyzed. As a result of this backlog, it is not uncommon for evidence to be stored for 6–9 months before analysis, if analyzed at all. The backlog of rape kit evidence was estimated at ~500 000 cases nationwide in 1999 and is growing continually.¹ Forensic science laboratory directors fault the time and cost requirements of these analyses as the bottleneck to DNA analysis.² The most time-consuming step in DNA analysis of sexual assault evidence is the conventional differential extraction process that is used to obtain DNA from a sample.³

Forensic DNA analysis of sexual assault evidence requires extraction of the DNA in a differential manner to obtain separate male and female fractions of DNA that can be used to establish the presence of the victim’s DNA and to identify the perpetrator. DNA extraction is followed by polymerase chain reaction (PCR) amplification of genetic markers, separation of the PCR products, and data analysis. To date, efforts have been directed toward improving the speed and efficiency of sample processing for these later steps, namely PCR,^{4–8} DNA separation,^{9,10} and data analysis.¹¹ However, little improvement has been made in the differential extraction process, which is the most time-consuming step of the DNA analysis. In particular, robotic automation of the extensive differential extraction process has been shown to improve sample processing efficiency and throughput.¹² Because of the costs associated with the robotic system, such implementation may not be practical for many forensic laboratories. The work presented here describes an alternative to, rather than automation of, the current method as a means of reducing analysis time. Any reduction in the processing time must be completed without compromising the extraction efficiency, purity of product, sensitivity, or the selectivity of the sample preparation process, because effective DNA analysis of evidence in sexual assault cases demands reliable separation of perpetrator DNA from that of the victim.

Perpetrator DNA is most easily obtained from sperm cells collected on vaginal swabs, taken in the routine collection of sexual assault evidence. The majority of genetic material collected on such swabs is from the victim,¹³ however, normally in the form of epithelial cells that are collected from the vaginal lining. These

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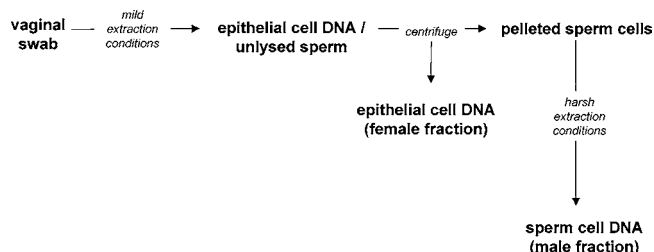


Figure 1. Differential extraction method currently used by forensic laboratories to obtain separate male and female DNA fractions. (See description in text.)

cells, or DNA from these cells, must be separated from the sperm cells before sperm DNA is recovered and amplified for analysis by capillary electrophoresis. Currently, this is performed by chemical means, involving differential lysis of the cells collected on the vaginal swab and exploiting the differential stability of the cell membranes.¹⁴ This multistep procedure (illustrated in Figure 1) begins by lysing the epithelial cells using mild conditions while adsorbed on the cotton swab. The intact sperm cells (predominately heads because tails are often degraded), desorbed from the cotton swab at the same time, are pelleted by centrifugation, allowing the soluble DNA from the epithelial cells to be removed in the supernatant. The pelleted sperm cells are then resuspended and lysed in a buffer that contains dithiothreitol (DTT) for reduction of disulfide bonds, and the DNA is extracted using phenol/chloroform/isoamyl alcohol. This differential extraction procedure requires more than 3 h and is often allowed to incubate overnight.³

A reasonable alternative to the current method involves the separation of the sperm and epithelial cells before DNA extraction. Eisenberg¹⁵ has addressed the separation of sperm and epithelial cells through the development of antibody-based separation schemes, using magnetic beads with covalently bound sperm-specific antibodies to selectively retain the sperm heads. There are potential problems associated with this approach, most notably clogging of the separation column by the large numbers of epithelial cells in casework samples. In addition to clogging, drawbacks of this technique include the cost of the materials required for the antibody/bead separation method, combined with the numerous steps required to yield PCR-ready DNA. A second method for the selection of sperm cells was reported by Elliott et al., who prepared slides from swabs and then selectively captured the sperm cells from the slide, using laser capture microdissection.¹⁶ This method is also capable of isolating the sperm cells selectively; however, it is time-consuming, labor-intensive (to identify the sperm cells in the sample), and not likely to be amenable to high-throughput applications. In a similar vein, Schoell et al.¹⁷ demonstrated a fluorescence-activated cell sorting method for the separation of sperm and vaginal cells. However,

the authors indicate that the use of this method would require altering the collection of evidentiary samples from vaginal swabs to vaginal lavages. Chen et al.¹⁸ demonstrated a separation of sperm cells from a mixture of sperm and epithelial cells using an 8- μm nylon mesh membrane, which retains the larger epithelial cells but allows the sperm cells to pass through it. However, the presence of female DNA in the sperm cell fraction is a factor in this method, because epithelial cells easily lyse, allowing free DNA or epithelial cell nuclei to pass through the membrane. Following the separation in all of these techniques, the purified sperm undergo normal forensic DNA analysis for genetic identification. With some of the advances and improvements in forensic DNA analysis relying on microfabricated devices, it is of interest to perform the cell separation on microfabricated devices as well.

Microdevices have been used previously for cell transport and manipulation, including clinically relevant assays with sperm cells, such as infertility testing,¹⁹ isolation of viable sperm,²⁰ and in vitro fertilization techniques.²¹ Li and Harrison²² showed yeast and *Escherichia coli* cell transport, as well as on-chip erythrocyte cell lysis in silicon microdevices. Microscale cell separations have also been accomplished by various means recently; however, these methods do not fulfill the needs of the forensic community for the processing of sexual assault evidence. Most commonly, previously reported methods are too extensive and costly to be implemented into our nation's underfunded crime laboratories. For example, Quake and co-workers^{23,24} have developed a microfabricated fluorescence-activated cell sorter. Although this method results in high-specificity separations, there are numerous drawbacks to its implementation for this specific cell separation. Namely, the cells must be fluorescently tagged, and, for interrogation of the cell fluorescence, the technique requires that the channels narrow significantly ($\sim 6 \mu\text{m}$) at the detector. This channel narrowing would result in clogging of the channel by the significantly larger (40–60 μm in diameter) vaginal epithelial cells in the forensic separation. Dielectrophoretic sorting of live and heat-treated *Listeria* cells on microdevices has been shown by Li and Bashir.²⁵ However, the intricacy of the engineering—specifically, the electrode array and microchannel footprint—will likely prevent this technique from being adopted in criminal laboratories. A much simpler method of cell sorting on microdevices is needed if forensic laboratories are to adopt cell sorting as an alternative to conventional differential extraction.

The work presented here addresses and successfully overcomes the inability of the differential extraction process to be integrated on a microdevice and provides a microchip alternative to the conventional method. In contrast to other microdevice cell separation techniques, a rapid microchip cell separation is shown that is ideal for the separation of sperm and vaginal epithelial cells

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for DNA analysis of sexual assault evidence. Clogging of the microchannel by epithelial cells, which is a limitation of alternate microchip cell separation methods, is not encountered here. This is primarily because the method does not result in mobilization of the epithelial cells, thereby limiting the possibility of an aggregate of cells obstructing the channel. Overall, the demonstrated microchip separation of sperm and vaginal epithelial cells for forensic analysis is a simpler method for rapid, cost-effective cell separation.

EXPERIMENTAL SECTION

Mixed cell samples were prepared by adding 1 μL of semen to a buccal swab and then eluting the biological material in 0.4 mL of pH 7.4 phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) for 1 min with agitation. Because of the nature of forensic samples, the number of sperm cells on a vaginal swab will vary widely. Therefore, 1 μL of semen was used for consistency in these experiments. Buccal swabs and semen samples were obtained by voluntary donation from healthy males and females (HIC Approval No. 10896). All human sample handling was completed following the guidelines of the University of Virginia Office of Environmental Health and Safety (Biosafety Level 2).

Microchip Preparation. Microchips were prepared from borofloat glass (1.1 mm) using standard photolithography and wet chemical etching techniques. The photomask was designed on AutoCAD and printed as a negative on transparent film with 3600 dpi resolution. Ultraviolet (UV) exposure of the positive photoresist resulted in transfer of the design to the glass wafer (Nanofilm, Westlake Village, CA). The design was etched with concentrated hydrofluoric acid solution ($\text{HF}/\text{HNO}_3/\text{H}_2\text{O} = 20:14:66$). Reservoirs were drilled (1.1 mm in diameter) into a coverplate, using a diamond-tipped drill bit (Crystalite Corporation, Lewis Center, OH). The etched plate and the coverplate were bonded using standard thermal bonding techniques. Each microchip contained a straight channel (2.5 cm long, $\sim 50 \mu\text{m}$ deep, and $\sim 90 \mu\text{m}$ wide at half-height). Channel dimensions were chosen to facilitate visualization with light microscopy.

Microscope/DVD Setup. Microchip separations were conducted on a microscope setup that was used to monitor the movement of cells through the channel during the separation. The magnified region of the separation channel was imaged onto a CCD camera (model KP-D20BU, Hitachi, Tokyo, Japan), using a Leitz orthoplan microscope with a 20 \times objective. Continuous recording of the CCD output was performed using a DVD-video recorder (model DMR T2020, Panasonic, Secaucus, NJ); this allowed further analysis of cell movement through the field of view following the experiment. The final magnification of the field of view was $\sim 8000\times$.

Fluorescence Experiments. For fluorescent visualization of the cells, a LIVE/DEAD Viability Kit (Molecular Probes, Eugene, OR) was utilized, according to the manufacturer's specifications. The Leitz orthoplan microscope was used, in combination with a complementary xenon arc lamp, to view and record fluorescent images. For visualization of the DEAD red fluorescence, the excitation band-pass filter was 490 nm and emission band-pass filter was 620 nm.

Microchip Cell Separations. A microchannel was rinsed with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) prior to use. For

gravity-driven separations, both the inlet and outlet reservoirs were evacuated of buffer prior to sample addition, leaving only the separation channel filled with buffer. Approximately 20 μL of buffer was added to the outlet reservoir, to prevent gravity-induced hydrodynamic flow from the inlet reservoir upon addition of the sample. A sperm/epithelial cell mixture (1 μL) was added to the sample reservoir, followed by 10 μL of sample buffer. The cell mixture contained ~ 5000 epithelial cells and 8000 sperm cells per microliter, as determined via a hemacytometer. After a minimum of 5 min of "settling time", gravity-induced flow was initiated by removing 5 μL of buffer from the outlet reservoir. Throughout the entire separation, including the 5 min reverse-flow settling time, the microchannel was imaged to the CCD and digital video recorder. This served to ensure proper flow magnitude and direction were attained as anticipated. Following the desired separation time, product was removed from the outlet reservoir via pipet and processed as described below.

Syringe-pump-driven flow was initiated in both the infusion and withdrawal modes, using a model SP120-300 syringe (World Precision Instruments, Sarasota, FL) and a 1-mL Becton-Dickinson disposable syringe. The syringe was connected to the microchip using 0.75-mm inner diameter (i.d.) (PEEK) tubing and minitight fittings (Upchurch Scientific, Oak Harbor, WA) with a plexiglass microchip interface that was constructed in-house. Infusion separations were accomplished by the addition of 1 μL of the sample mixture to the inlet reservoir and subsequent connection to the macro-to-micro interfacing described previously. Withdrawal separations were initiated by interfacing the syringe pump in a manner similar to the outlet reservoir. A temporary plastic reservoir was affixed to the inlet, using epoxy, to increase the sample size that the microdevice could accommodate (on the order of 1-50 μL) in the withdrawal mode.

Post-Separation Sample Processing. DNA was extracted from the separated cells in the outlet reservoir, using a QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA), following the manufacturer's suggested "Blood and Body Fluid Spin Protocol". The sample was eluted in 70 μL AE buffer that was supplied with the kit.

PCR amplification was performed on a GeneAmp PCR System 2400 Thermocycler (Perkin-Elmer, Wellesley, MA). For β -globin amplifications, 30 cycles of 60 s of denaturation at 94 $^\circ\text{C}$, 45 s of annealing at 60 $^\circ\text{C}$, and 60 s of extension at 72 $^\circ\text{C}$ were completed. A first cycle included 5 min of denaturation time at 95 $^\circ\text{C}$ and the final cycle included a 10-min extension time at 72 $^\circ\text{C}$. The Applied Biosystems Amp/STR COfiler (Applied Biosystems, Foster City, CA) multiplex amplification was performed in a reaction volume of 25 μL . The COfiler kit contains primers to simultaneously amplify seven loci: Amelogenin, D3S1358, THO1, TPOX, D16S539, D7S820, and CSF1PO. The amelogenin amplification (112-bp Y chromosome fragment) is male-specific. In addition, the short-tandem repeat (STR) profile, when compared to a reference sample, is used to infer the source. Thermocycling consisted of 28 cycles of 60 s of denaturation at 94 $^\circ\text{C}$, 60 s of annealing at 59 $^\circ\text{C}$, and 60 s of extension at 72 $^\circ\text{C}$. The amplification was begun with a 60 s 95 $^\circ\text{C}$ initial denaturation and ended with 45 min at 60 $^\circ\text{C}$. PCR products were separated using an Applied Biosystems (Foster City, CA) PRISM 310 Genetic Analyzer, following the manufacturer's protocols.

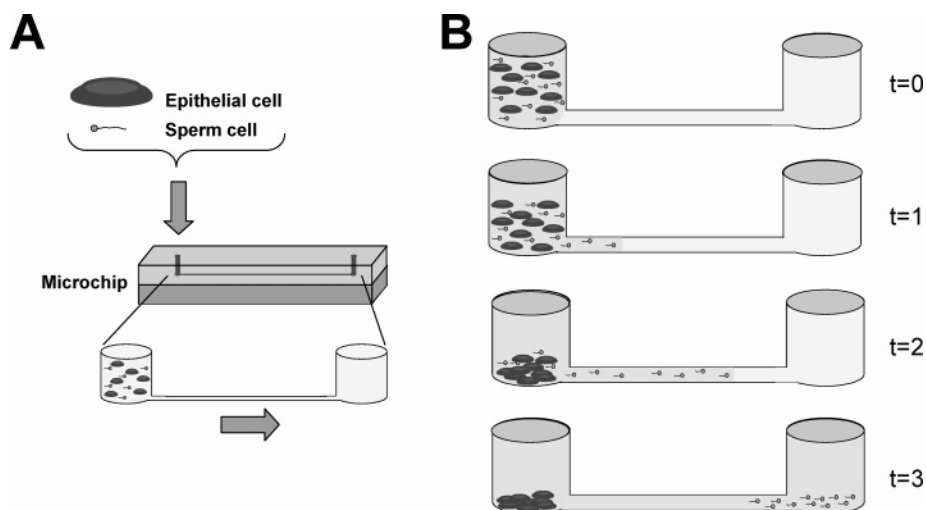


Figure 2. Mechanism of microchip-based sperm and epithelial cell separation. A mixture of sperm and epithelial cells is added to the inlet reservoir of the microdevice; the epithelial cells are shown settling to the bottom of the inlet reservoir ($t = 0$). After 5 min ($t = 1$), flow is induced ($t = 2$) to mobilize the sperm cells, while the epithelial cells remain in the inlet reservoir. At $t = 3$, the sperm cells are collected in the outlet reservoir, from which further sample processing (DNA extraction, PCR amplification, and DNA separation) can occur. Note that shading (in the microchannel) is used simply to illustrate flow from the inlet to the outlet.

RESULTS AND DISCUSSION

Replacement of the differential extraction process with a microdevice-based technique that incorporates a cell sorting step requires that several criteria be met by the new technique. These include high sensitivity detection (to separate and detect fewer than 100 sperm from a vaginal swab), high selectivity (undetectable female DNA in the male fraction), robustness (must be compatible with dried/re-hydrated cells and work with samples that are predominantly female cells), and cost-effectiveness (such that these devices can be made disposable). In addition, acceptance and implementation of this technique by the forensic community will require that certain requisites regarding speed and simplicity also be met.

Cell Separation Mechanism. Separation science is based on exploiting the physicochemical differences (primarily charge and mass) of the species involved—the separation of cells is no different, relying on many of the same principal characteristics, albeit on a more macroscopic scale. If the morphological differences that distinguish sperm from epithelial cells are significant, it is reasonable to expect that they can be leveraged for a microchip-based separation of these cells. Comparing the two cell types, the differences are clear, with respect to size, specific gravity, surface proteins, proclivity for adsorption, and possibly charge. With respect to cell size, sperm heads have an average diameter of 4–6 μm , whereas epithelial cells are an order of magnitude larger, at 40–60 μm . Specific gravity differences are small but perceptible, with sperm cell density on the order of 1.12 g/mL ²⁶ and epithelial cell densities in the range of 1.04–1.08 g/mL .²⁷ These values characterize differences in the rate at which cells would settle to the bottom of the inlet reservoir. Theoretical calculations were performed to determine if this could be used to differentially remove one cell type from solution. By approximating the cell as a sphere, the settling time of each cell was calculated using the relation

$$U_o = \frac{gD_p^2(\rho_p - \rho_f)}{18\mu} \quad (1)$$

where U_o is the sedimentation rate, D_p the cell diameter, ρ_p the cell density, ρ_f the fluid density, and μ the viscosity of the fluid. The difference in the calculated sedimentation rates (>0.1 $\mu\text{m}/\text{min}$ for sperm cells and 2–4 $\mu\text{m}/\text{min}$ for epithelial cells) is sufficient to selectively sediment the epithelial cells (and not the sperm cells). However, this differential settling may occur over a lengthy time frame and may include trapping of sperm cells as the epithelial cells settle.

A difference that could also be exploited for the separation is the greater proclivity of the epithelial cells for adsorption; the larger surface area of the epithelial cells and their high concentration of cell surface binding proteins, which are capable of specific interaction with surfaces, present the possibility for binding to glass substrates. This property has previously been exploited for separation of viable white blood cells by Wilding and co-workers,^{28,29} who were able to use this property to selectively trap white cells on a series of physical weirs in a microfabricated channel. Because the cells in sexual assault evidence are rarely viable, biological adhesion is less likely, but adsorption to the glass is possible and could be used to selectively isolate the epithelial cells from solution, either alone or in conjunction with the sedimentation rate differences. In fact, aggregation of the epithelial cells, binding to each other, can further increase the sedimentation rate and, thus, contribute to faster settling. Note that the term “adhesion” is not used extensively in this paper, to minimize confusion with protein-mediated adhesion phenomena in viable cells. Aggregation and adsorption refer to those processes due to hydrophobic or other general interactions, but not those which require cell viability.

Figure 2 is a schematic of the separation process that is based on differential settling and adsorption as it occurs in the micro-

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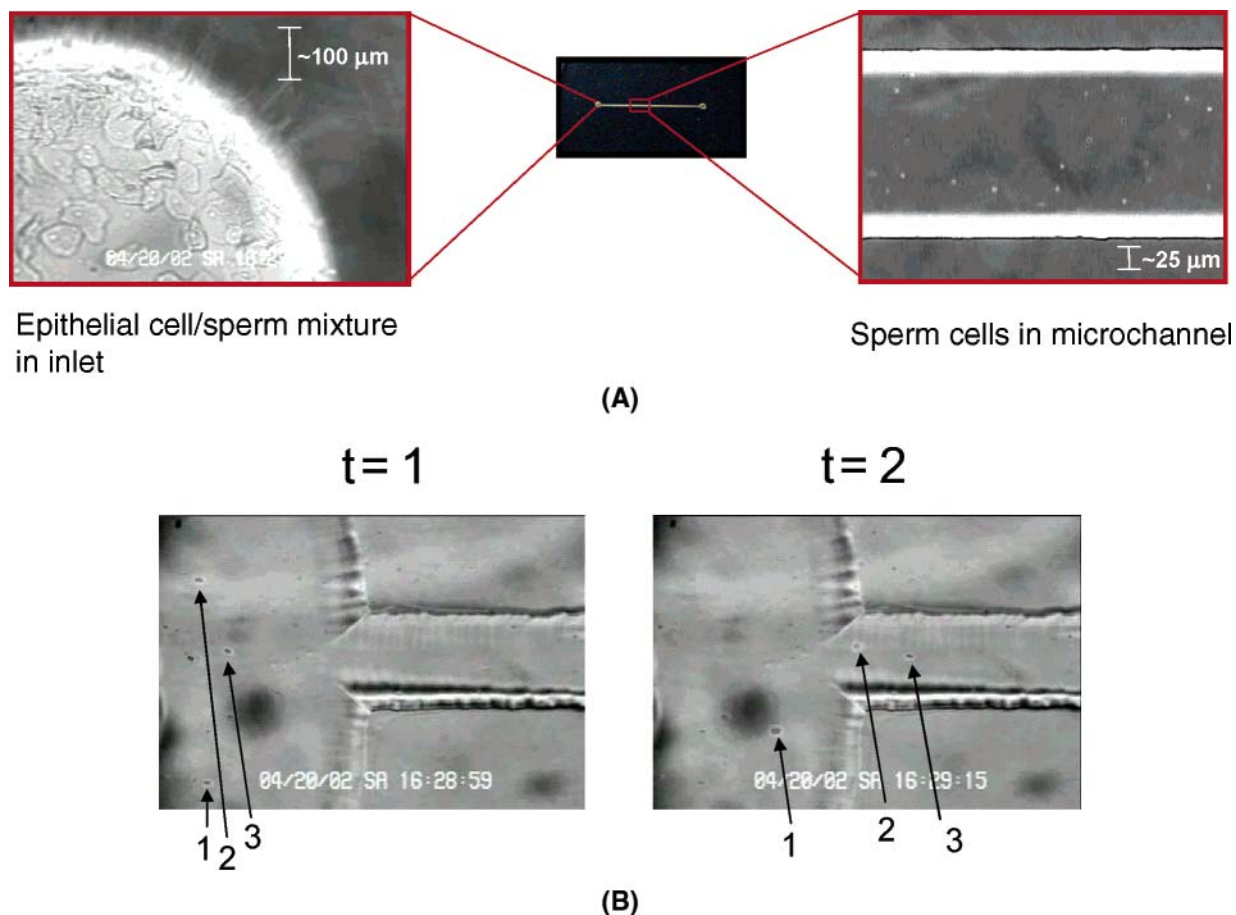


Figure 3. Cell separation in a microfabricated device. Sperm cells were mobilized after 5 min of settling time. Flow ($1 \mu\text{L/h}$) was induced by a mismatch in volume (removal of $5 \mu\text{L}$ of phosphate-buffered saline (PBS) from the outlet reservoir). Sample composition is as described in the text. The etched microchannel is 2.5 cm long, $\sim 50 \mu\text{m}$ deep, and $\sim 90 \mu\text{m}$ wide at half-height. Panel A shows micrographs of the cell separation process: (left) epithelial cell and sperm mixture in the inlet reservoir before separation and (right) sperm cells flowing down the microchannel during cell separation. Panel B shows time-lapse photomicrographs of a cell separation; sperm cells (indicated by the arrows) are shown migrating out of the inlet reservoir and into the microchannel, toward the outlet reservoir, and epithelial cells, although present in the inlet reservoir, are outside of the field of view.

fabricated device. In this mechanism, the epithelial cells are believed to adsorb to the glass substrate of the inlet reservoir and to each other in multilayer formations. The number of epithelial cells on the samples used in this work is thought to be representative of vaginal swabs. However, one could simply increase the surface area of the inlet reservoir to effectively capture additional epithelial cells. The trapping of sperm cells is possible in the multiple layers of epithelial cells, which could also be reduced by increasing the size of the inlet reservoir.

Cell Separation Under Gravity-Induced Flow. The previous discussion suggests that allowing for cell settling, combined with appropriate flow rates to mobilize sperm cells through a channel without disrupting the settled epithelial cells, should provide selective separation of sperm cells from a mixture. To determine the time required for complete epithelial cell settling, settling times of 0–20 min were investigated. In conjunction with a $1 \mu\text{L/h}$ flow rate (generated by a mismatch of volumes in the two reservoirs), no epithelial cell movement was observed in the channel when settling times of 5 min or more were used. Settling times of < 5 min resulted in mobilization of the epithelial cells with the sperm cell fraction, potentially occluding the separation channel. Figure 3A shows the epithelial and sperm cell mixture in the inlet reservoir prior to separation (left micrograph) and the sperm cells

in the microchannel during the separation (right micrograph). Time-lapse photos from a separation process are shown in Figure 3B, with sperm cells progressing in the direction of fluid flow; no epithelial cells were observed in the field of view during this separation.

To improve the visualization and detection of sperm cell movement, as well as epithelial cell retention in the inlet, fluorescent staining of DNA in the cells was performed. The fluorescent staining method served two purposes: (i) it allowed for unequivocal distinction between sperm and epithelial cells by microscopy, and (ii) it ascertained that only sperm cells (and not epithelial cells) were mobilized down the channel under flow (data not shown). However, it was also apparent that fluorescent detection of sperm cells, for counting purposes during separation, was a less viable option, because detection of the fluorescent cells outside the focal plane was more difficult than with light microscopy.

The average linear velocity of sperm cells in gravity-induced hydrodynamic flow experiments was determined by timing cells as they passed through the field of view during a separation. A value of $\sim 56 \mu\text{m/s}$ was determined, which correlated to a flow rate of $\sim 1 \mu\text{L/h}$. With gravity-driven flow, flow is induced by a height difference between the inlet and outlet reservoirs; thus,

Table 1. Results from a Typical Microchip-Based Cell Separation Using Gravity-Driven Flow

	No. of Cells		
	original mixture	20 min	70 min
sperm	~8400	~380	~2140
epithelial	~3600	0	0
	sperm/epithelial cell ratio	enhancement factor	
pre-separation	8400/3600		
post-separation	380/1 ^a (20 min)	162× (20 min)	
post-separation	2140/1 ^a (70 min)	917× (70 min)	

^a For statistical purposes, the epithelial cell count following separation must be reported as >0.

the flow rate range was limited using this method. Results from a typical cell separation using this flow rate and a settling time of 5 min are shown in Table 1, where the sperm cells were counted as they passed through the field of view. No epithelial cells were observed passing through the channel over the course of a 70-min separation. Comparison of results with conventional differential extraction is difficult, because both sperm and epithelial cells are lysed in the process and results are reported in terms of DNA concentration, not numbers of cells. To our knowledge, the minimum number of sperm cells required for further analysis, to result in a complete STR profile, has not been studied. Typically, 0.5–1 ng of DNA is required for genetic identification, although a partial STR profile can be obtained from much smaller amounts (on the order of 100 pg of DNA), but stochastic effects may result.^{30,31} Translation of the minimum DNA requirements to the number of sperm cells needed requires assumptions about the efficiencies of differential extraction and DNA extraction from sperm cells. As a result, comparing the separation ability reported herein to the conventional procedure is not truly meaningful at this point.

The gravity-induced flow mechanism illustrates the feasibility of sperm and epithelial cell separation, based on their different physical properties. A 20-min separation (~380 sperm cells) provided sufficient material for DNA extraction and PCR amplification of a 380-bp fragment of β -globin, which is a representative human gene. However, the gravity-induced flow was determined to be inconsistent, primarily because of the dynamic change in the fluid volumes in the microchannel reservoirs over the course of the separation. Takayama et al.²⁰ developed a chip design for obtaining reproducible gravity-driven flow; however, this was not readily compatible with our current microfabrication methods. Therefore, an alternative flow mechanism (pressure-driven flow induced via a syringe pump) was explored to make the flow more easily controllable and consistent. Under these conditions, further improvement to the separation speed and efficiency could be addressed.

Cell Separation under Pressure-Driven Flow. The flow rates achievable with gravity were slow, not easily controlled, and

influenced over time by several parameters, including changing reservoir fluid levels. To determine whether it was possible to achieve a faster separation by increasing the flow rate, but without dislodging the epithelial cells from the inlet reservoir, a syringe pump was used to generate hydrodynamic flow through the microchip channel. As anticipated, a flow rate on the order of several microliters per hour in these channels resulted in laminar rather than turbulent flow. Higher flow rates (~10 $\mu\text{L}/\text{h}$) could be used for separation of cells, although visual sperm counting was no longer possible, because of the rapid transport of the sperm cells. It was also not possible to calculate flow rates based on sperm motion through the field of view; thus, flow rates are reported as the programmed speed of the syringe pump. Movement of epithelial cells in the channel was still easily detected at these flow speeds, because the epithelial cells are much larger and move significantly slower than the sperm cells.

Syringe-pump-driven flow can be created in the microchip in two ways: (i) positive pressure flow generated by having the syringe force fluid in through the inlet (where the sample is loaded) or (ii) negative pressure (vacuum) flow created by withdrawing fluid from the outlet. With the former, several engineering difficulties arose, in regard to sample introduction, including creation of a significant head pressure and trapping of air bubbles that often negatively affected the separation efficiency, reliability, and robustness. Using positive pressure, flow rates as low as 2 $\mu\text{L}/\text{h}$ occasionally resulted in mobilization of epithelial cells with the sperm cell fraction, eventually clogging the microchannel, even with settling times of >5 min. This may have resulted from disruptions caused when the tubing connecting the syringe pump to the microchip was attached at the inlet reservoir after sample addition.

As a result of the engineering difficulties encountered with the positive pressure syringe-pump-driven flow, negative-pressure (vacuum) flow was invoked, using the syringe pump to withdraw fluid from the outlet. Not only did this configuration enhance flow reproducibility dramatically, but macro-to-micro interfacing with the microchip was also simplified, because any air bubbles created in the system during connection of the tubing could be easily expelled prior to the addition of the sample. After allowing the sample to settle in the inlet reservoir, fluid was withdrawn from the outlet at a flow rate of 30 $\mu\text{L}/\text{h}$. Higher flow rates were tested, although a concomitant increase in the number of epithelial cells that were dislodged was observed. Because of the importance of obtaining a pure sperm cell fraction (i.e., no epithelial cells) for forensic identification purposes, the highest flow rate that could be used without disruption of epithelial cells was selected. The ability to use substantially higher flow rates without movement of epithelial cells suggests that settling alone may not be preventing movement of the epithelial cells from the inlet reservoir; instead, the adsorption of epithelial cells to the microchannel surface is likely playing a role. Moreover, the higher flow rate results in a greater flux of sperm cells through the microchannel and into the outlet reservoir; this significantly reduced the separation time necessary to yield a sufficient number of sperm cells for DNA processing. Therefore, the use of negative pressure flow was the preferred method for obtaining fast separations with limited engineering concerns.

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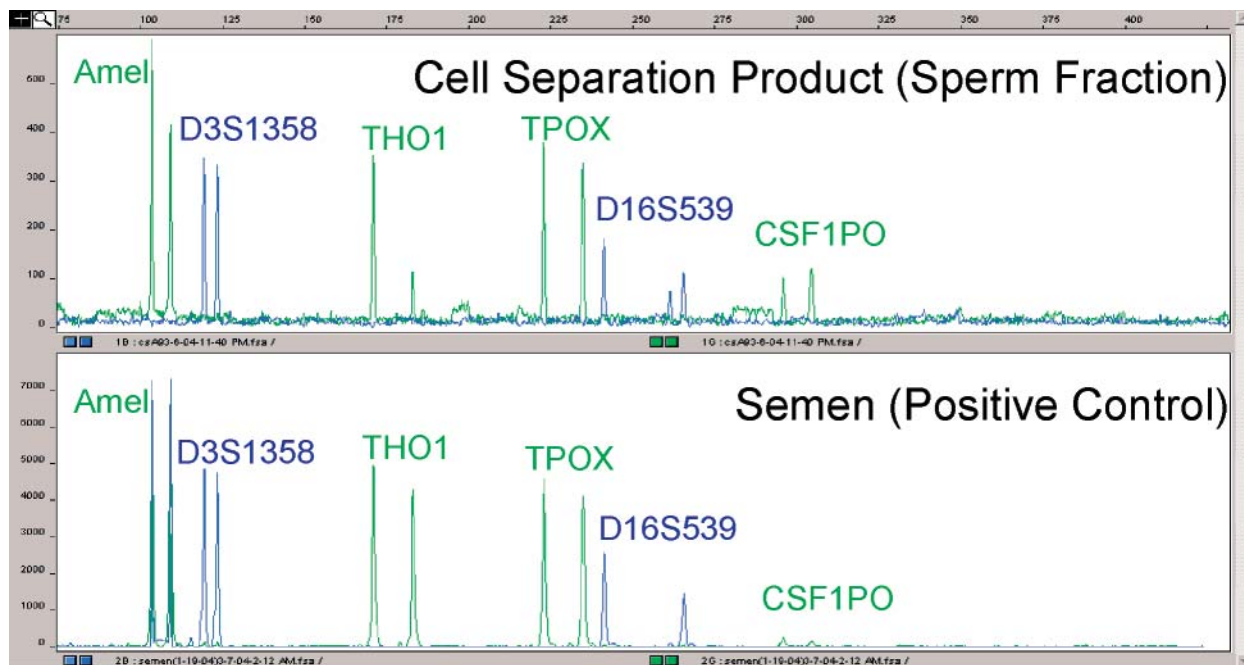


Figure 4. Short tandem repeat (STR) analysis of the sorted cell product from the pressure-driven microchip separation. Five of the common STR loci were amplified using a COfiler kit. The resulting STR profile illustrates the purity of the cell separation product. Cell separation was performed with syringe-pump-driven withdrawal flow. Buffer containing the product from the outlet was removed from the syringe and tubing. Conventional QIAGEN DNA extraction, PCR amplification, and DNA separation was performed off-chip.

A representative sample of the sperm cell fraction collected at the microchannel outlet was processed through conventional DNA purification, using a QIAGEN DNA extraction kit prior to COfiler STR amplification, with the PCR-amplified products being analyzed by capillary electrophoresis, using multicolor laser-induced fluorescence detection. Figure 4 shows the resulting STR profile for the sperm fraction, as well as that of the semen donor. Although small, the presence of female DNA in the sperm fraction is evident in the electropherogram, as indicated by the presence of an extra D16S539 allele that is not present in the STR profile of the semen donor. This is also supported by the uneven distribution for the amelogenin peaks. DNA from a male donor results in a roughly even distribution of alleles, because of the equal presence of the X and Y chromosomes, whereas an amelogenin profile from a female yields only one peak, corresponding to the X chromosome. The most likely source of female DNA was due to the presence of free DNA in the sample. This is further addressed in the following section.

Control of Free DNA in the Cell Separation. The nuclear membrane of epithelial cells is known to be relatively weak, in comparison to that of sperm cells; this is a characteristic that is utilized in the conventional differential extraction method in forensic laboratories. With that method, the number of epithelial cells lysed upon elution of cells from the swab is maximized through the use of a mild lysis buffer that contains sodium dodecyl sulfate and other detergents, to selectively lyse the epithelial cells. Sperm cells require the use of dithiothreitol (DTT) in this method to break the cross-linked thiol-rich proteins of the nuclear membrane and release the sperm DNA.¹⁴ However, the microchip method proposed in the current work to obtain pure male and female DNA fractions involves sorting of the nonlysed cells, followed by DNA extraction from each cell type. Consequently, any pre-existing free DNA, resulting from already lysed cells or

cells lysed during extraction from the swab (not surprising in their dehydrated state on the swab), is expected to be from epithelial cells and could potentially taint the sperm cell fraction.

Although it is known that epithelial cells easily lyse, the literature is not clear on what percentage of epithelial cells dried onto cotton swabs are expected to break open over time.¹⁸ With the conventional method, in which the epithelial cells are immediately lysed, this issue is not important, thus research in this area has not been formally reported. However, with the fragile nature of epithelial cell membranes, it was expected that the fraction lysed would not be insignificant. Fresh epithelial cells from buccal swabs were obtained and desorbed from the cotton swab, using phosphate-buffered saline. The free DNA in solution was assessed using a human-specific real-time quantitative PCR assay (Quantifiler, Applied Biosystems). Experimental data indicate that ~7 to 12 ng of free DNA (data not shown) may be present in the material desorbed from a dried buccal swab when eluting with phosphate-buffered saline. Because this amount of epithelial cell DNA would be significant if it were to co-migrate with the sperm cells, the movement of free DNA in the cell separation method was assessed. At the same time, experiments were performed to minimize (or eliminate) DNA co-migrating with sperm cells; these investigations used various means to alter the surface chemistry of the microchannel to enhance adsorption of DNA to the microchannel surface.

A multitude of approaches can be entertained for minimizing the amount of DNA migrating to the outlet during the cell separation process. The most obvious approach is to exploit the microchannel surface in a manner that allows it to do what is typically avoided in most separations—that is, have analyte–wall interaction that leads to the adsorption of free DNA. DNA is known to adsorb to silica surfaces in the presence of high salt or chaotropic agents;³² thus, a change in the type of buffer (or its

composition) could enhance the adsorption of free DNA to the microchannel walls. Preliminary quantitative experiments were performed to determine the amount of free DNA in the outlet before and after microdevice cell sorting. Using a fluorescence DNA quantitation reagent (PicoGreen, Molecular Probes, Eugene, OR), both a phosphate-buffered saline (PBS) solution and a tris-EDTA buffer were investigated. It was interesting to observe that, following cell sorting, the amount of free DNA detectable in the outlet was 2-fold lower when using a PBS solution (~15% of the DNA loaded at the inlet) instead of a tris-EDTA buffer (~29%) for the cell sorting. Although these results are preliminary and still under investigation, if free DNA in the samples is determined to be problematic for further genetic analysis (leading to observable female DNA in the sperm fraction), simply using the appropriate buffer (e.g., PBS) may circumvent the problem. Alternately, giving the channel surface a cationic character would allow for DNA "trapping" via ion exchange. This would be a reasonable approach for the cell sorting described here, because we have shown that the cell-sorting process continues unabated with poly(diallyl dimethylammonium chloride) as the cationic polymeric coating (data not shown). This is interesting because the number of sperm cells observed to adsorb to the cationic surface was relatively low, which is surprising, in light of the net negative charge of most cells at physiological pH values.

Considerations for Real Sample Analysis. A novel method of separating sperm from epithelial cells has been demonstrated on a microfabricated device for potential application to forensic analysis of sexual assault evidence. Separation mediated by either gravity-driven or syringe-pump-induced flow is simple and effective, with higher flow rates and faster separations being attainable with the syringe-pump-driven withdrawal mode. With total separation times on the order of 30 min, this compares favorably with conventional methods and promises a potentially faster method that is amenable to high-throughput automation. The material recovered following the cell separation was shown to be amenable to further downstream DNA analysis processing by conventional means. Because of the variability in forensic casework, little work has been done to assess the purity of the sperm cell fraction following conventional differential extraction. The majority of cases result in a clean sperm fraction, although co-amplification of the female's alleles in the sperm cell fraction has been suggested to

occur in ~40% of the samples.³³ This new method should effectively prevent any vaginal cells or free female DNA from migrating to the outlet reservoir, thus outperforming the traditional differential extraction process in selectivity.

Application of this method to actual sexual assault evidence will require ancillary investigations focused around the removal of cells from stored swabs. For example, little is known about samples that are rehydrated following storage for various periods of time—any age-dependent effects on cell adsorption (and/or aggregation) will need to be characterized to determine whether this adversely affects settling and dislodging during the separation process. In addition, the age effects on the amount of free DNA in the sample that must be adsorbed will have to be investigated. Although it is not anticipated that the presence of other cell types (such as bacteria) will affect the separation mechanism, this should be evaluated and the location of bacteria defined following the separation. This is potentially a complicating issue, not with the STR analysis (because the STR amplification is specific for amplification of human DNA, i.e., primers do not recognize bacterial DNA), but bacterial migration with the sperm could interfere with effective counting of sperm cells using optical means if this is applied.

It is clear that the cell separation method described here, once fully developed and validated, has numerous distinct advantages over the conventional, time-consuming, differential lysis method, providing the possibility for reducing the backlog of forensic DNA casework that currently exists in criminal laboratories.

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