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NON-MOTILE SPERM CELL SEPARATION USING A SPIRAL CHANNEL

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NON-MOTILE SPERM CELL SEPARATION USING A SPIRAL CHANNEL

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Abstract Microfluidic sperm sorting has historically relied on sperm motility. However, a motility-based sperm separation technology will not work when viable, non-motile sperm need to be separated from other tissues as occurs when performing testicular sperm extraction (TESE) and microdissection testicular sperm extraction (mTESE) techniques. This work demonstrates the use of inertial microfluidics technology using spiral channels to separate sperm from blood cells. The separation method, which is label-free, does not rely on sperm motility for sorting.

Basic principles of spiral channel separations were used to design a specific channel and flow parameters for separating non-motile sperm from blood. The spiral channels dimensions were: initial radius, 0.7cm; final radius, 0.899cm; channel width, 150µm; channel height, 50µm; turns of spiral, 4 turns; and space between channels, 310µm. If sperm are modeled as a 5 µm sphere, inertial microfluidics theory suggests that the sperm could be focused and separated from red blood cells (RBCs). Channels to implement these features were validated in a series of experiments. Mixed samples of RBCs and sperm were used to test the sperm separation capability of the device with the sample injection flow rate ranging from 0.1~0.52ml/min. After running the sample through the spiral channel, the samples were collected from four outlets and were inspected using microscopy.

The best results were obtained at a 0.52ml/min flow rate and generated a concentration ratio of 81%, representing the percent of sperm collected from the two outer outlets. For the same conditions, 99% of RBCs were collected from the two inner wall outlets. Using a high speed scanner, we were able to observe the focusing of the RBCs and general focusing of the sperm. As the sperm are not a uniform shape, they did not focus in a tight band, but were collected in a general region of the channel. Nevertheless, the purification ratio for these sperm was sufficient to greatly enhance the likelihood of finding rare sperm in TESE/mTESE samples containing millions of blood cells. Sequentially processing of the samples in the system proved to further improve the ratio of sperm to blood cells.

Introduction

Microfluidic techniques for cell manipulation and analysis have proven to be valuable tools for understanding molecular and cell biology. In addition, microfluidic technologies enable the development of diagnostics and treatments for human disease .[1] In the field of *assisted reproductive technology* (ART), microfluidics has become beneficial for gamete (sperm or egg) sorting and selection. In recent years, a number of microfluidic devices have been developed for sperm manipulation, allowing the sorting of healthy sperm to use for *in vitro* fertilization (IVF).[1] These technologies enable automation to replace tedious, manual approaches to viable sperm cell sorting from semen specimens, which requires hours of work by highly trained personnel.

One of the earliest microfluidic approaches for sperm manipulation contained multiple, straight microchannels connecting an input reservoir to a collection reservoir, enabling motile sperm to swim to the specific reservoir where they could be collected, while non-motile sperm and debris stayed behind in the inlet reservoir.[2] Today, the most popular microfluidics approach for sperm separation utilizes the introduction of parallel laminar streams of media through a straight microchannel. The parallel laminar streams are generated by introducing a dilute semen sample as well as media though two inlets. According to hydrodynamic principles[3], these two streams do not readily mix together, creating a boundary between the streams. Because motile sperm can swim across the boundary, they are able to enter the collection stream, while the non-motile sperm and debris are washed away to a waste collection area. Another notable sperm separation technique utilizes chemotaxis of sperm in addition to sperm mobility by inducing sperm to swim through microchannels toward chemo-attractants applied to the surface of outlet reservoirs.[4] Techniques have also been utilized in which a sample is inserted into an induced slow flow through a horizontal obstacle within a microchannel. This method screens out non-motile sperm and debris, which settle behind the inlet area, while motile sperm swim under/over the obstacle toward the outlet reservoir.[5] There have also been efforts to improve sperm quality with electrophoretic isolation methods, which require porous membrane filters to isolate the desired sperm cells.[6] [7]

Most existing conventional sperm separation approaches, such as those described above, utilize the motility of sperm to generate a

separation. However, these motility based techniques cannot be applied to sperm samples containing only non-motile sperm, such as those obtained from non-obstructive azoospermia (NOA) patients from which no sperm is found within the ejaculate. For these patients immature testicular sperm may be obtained by surgical procedures called testicular sperm extraction (TESE) and microdissection testicular sperm extraction (mTESE). TESE/mTESE specimens contain a combination of red blood cells (RBCs), white blood cells, Sertoli cells, and debris that must be distinguished from a limited number of non-motile spermatocytes (0~4,000ea/ml), making the search for and separation of sperm extremely difficult using current manual approaches (Fig. 1).[8] Even active sperm isolation techniques, such as electrophoretic sperm isolation,[6 7] will cause a loss of sperm and possible DNA damage.

Recently cell separations using inertial microfluidics have been demonstrated for collecting rare cells from blood such as circulating tumor cells. Without the use of any cell labelling, these studies show great potential for pure mechanical separation of other rare cells using inertial microfluidics. These studies suggest that a precisely designed spiral channel could generate flow focusing of sperm and be a suitable solution for precision sperm separations.[9 10 11 12]

In this work we demonstrate the use of inertial microfluidic technology to separate sperm from the major contaminant of TESE/mTESE samples, RBCs, by focusing the sperm and RBCs to different areas of the flow. Unlike other work on microfluidic sperm isolation, this separation method is not based on sperm motility and is label-free, which is required if the separated sperm are to be used clinically in a later step. The results show moderate non-motile sperm focusing and clear RBC focusing, which could be used for sperm separation from surgical samples derived using TESE/mTESE.

Design Principle/Theory

Substantial theory on the physics of flow focusing in spiral channels exists and was used to design a spiral channel for flow-focusing of non-motile sperm cells. The optimal dimensions of the spiral channel can be precisely calculated based on previously studied inertial microfluidics principles such as: the ratio of the inertial lift forces to the Dean drag force (R_f), the ratio between particle and channel dimension(λ), and the aspect ratio of the channel cross-section.[13 14 15 16 17] Flow focusing in spiral channels requires a balance between inertial lift forces (F_L), which push particles away from a wall, and Dean drag (F_D), a force generated by a lateral, secondary-vortex flow along a spiral channel. The balance can be established for a given particle type when certain physical parameters of the flow are in specified ranges. The inertial lift forces (F_L) and Dean drag (F_D) can be calculated by

$$F_D = 3\pi\mu U_{Dean} a_p \quad (1)$$

$$F_L = 0.05 \frac{a_p^4 \rho U_m^2}{D_h^2} \quad (2)$$

where μ is fluid viscosity, U_{Dean} is average Dean velocity, a_p is particle diameter, U_m maximum fluid velocity, and D_h is hydrodynamic diameter for a rectangular channel. For focusing, the ratio (R_f) between inertial lift forces, and Dean drag, is given by[16]

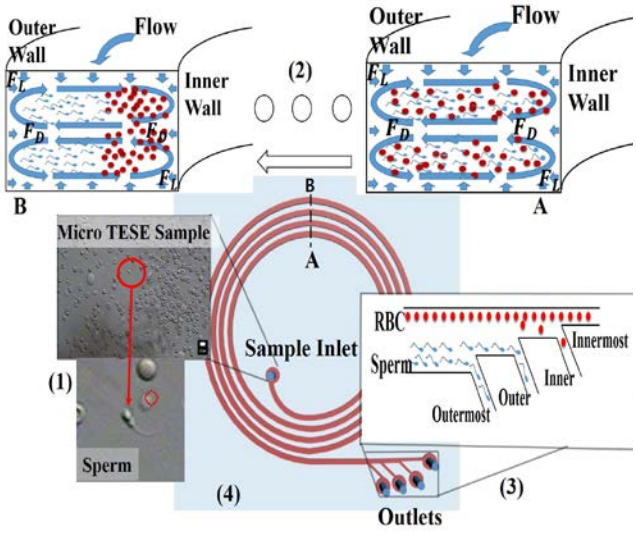

Figure 1. Overview of the device, (1) a sample(mTESE/TESE or simulated) injecting in the inlet of the channel, (2) flow focusing caused by lift forces (F_L) and Dean drag (F_D) from inertial microfluidics effect, (3) Separated sperm and RBC by flow-focusing.

$$R_f = \frac{F_L}{F_D} \geq \sim 0.08. \quad (3)$$

R_f should be greater than 0.08, which makes the Dean drag dominant (eq. 3). For strong focusing, the particle/channel dimension ratio (λ), given by

$$\lambda = \frac{a_p}{D_h} \geq 0.07\ , \quad (4)$$

should be greater than 0.07[17]. Based on previous experimental studies, the aspect ratio of the channel should be between ~ 1:2 and 1:4 (height:width).[18 19] Using these theoretical principles, calculations were performed to determine possible spiral channel designs that might effectively separate sperm. One challenge of this analysis was due to the irregular shape of sperm cells (approx. sperm head length: 4.79 ± 0.26 µm, width: 2.82 ± 0.23)[20], and the theory assumes spherical particles. As an initial estimate, the sperm were considered to be 5µm diameter spheres. For resolution estimates, RBCs were approximated as 9µm diameter spheres (measured RBC dimensions-diameters: 7.5~8.7µm, thickness: 1.7~2.2µm).[21] In our search for appropriate channel geometries, the sample injection flow rate was limited to be 0.1 to 7ml/min in 0.1ml/min increments.

Based on these calculations, a set of spiral channels with dimensions: initial radius, 0.7cm; final radius, 0.899cm; channel width, 150µm; channel height, 50µm; turns of spiral, 4 turns; and space between channels, 310µm; were manufactured and tested. These channels should generate the following metrics for a 0.55ml/min flow: R_f of 5µm diameter particle, 0.0806, and R_f of 9µm diameter particle, 0.4702; λ for 9µm diameter particle, 0.2, and λ for 5µm diameter particle, 0.0667. Four gradual-splitter-type outlets were designed to allow separate collection of the particles and to allow investigation of the location of the various particles in the flow. (Fig. 1)

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Methodology/Experimental

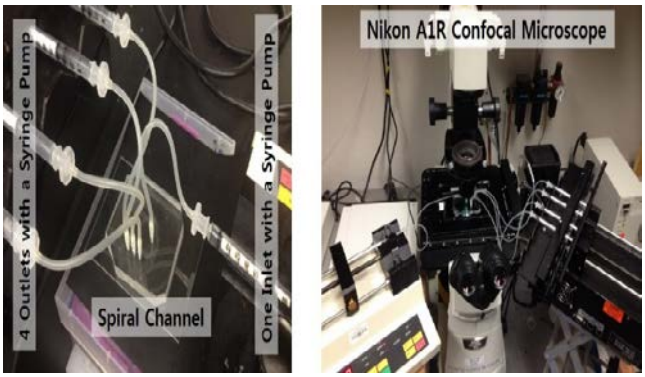

Figure 2. Experimental setup, (Left) Two syringe pump with a spiral channel device, (Right) stained sample run observation setup with Nikon A1R microscope and high speed scanner.

To demonstrate the capability of the designed spiral channel to focus and separate sperm cells from RBCs and other unwanted debris (such as white blood cells), multiple channels were built and tested.

Fabrication of the actual device was carried out using SU-8 (SU-8 3000, Microchem, MA, USA) as a mold for polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, MI, USA). The SU-8 mold was fabricated on a 100mm (4 inch) wafer according to the manufacturer's instructions in a clean room environment. 40ml of uncured PDMS at a 10:1 (polymer: curing agent) ratio was poured on the mold, and it was placed in an oven at 60°C for at least 6 hours. The molded PDMS was peeled off from the mold and any excess PDMS removed. Inlets and outlets were cored with a 1.5mm diameter coring tool. After cleaning the surface of the PDMS, a glass slide (70x50mm) was plasma bonded with the PDMS to form closed channels.

The experimental system utilized two syringe pumps (one dual syringe capable and one four syringe capable), five 1ml syringes and one spiral channel device. The dual syringe pump was used to inject sample through inlets of the channel, and the four channel syringe pump was arranged to pull sample from the four outlets. The removal flow rate was kept steady and slightly lower than the injection flow rate to obtain equal amounts of sample from each outlet.

All sperm and blood samples were acquired under an Institutional Review Board approved study, IRB00072239. Written informed consent was obtained from all participants for their tissues to be utilized for this study. Sperm samples were prepared from previously frozen semen specimens which were resuspended in water for 30s to immobilize the sperm. The sperm samples were then resuspended in either PBS or sperm media (Quinn's Advantage media with HEPES (Sage, CT, USA) and 3% of Serum protein substitute (Sage, CT, USA)). RBC samples were obtained from whole blood specimens within one week of collection. Collected blood samples were also resuspended in either PBS or sperm media. As required, the sperm and RBC samples were diluted or concentrated using PBS or sperm media. Samples were placed within two 1ml BD plastic syringes and connected to the spiral channel inputs using platinum-cured silicone tubing and 1/16inch barbs. 1mL syringes were connected to the outlets of the spiral channel and placed in the 4 channel syringe pump in withdrawal mode. (Fig. 2)

For the sperm-alone experiments, the base concentration of sperm samples was 25million/ml and sperm samples were injected into the spiral channel under flow rates from 0.1 to 0.22 ml/min. For mixed samples (sperm and RBC) characterization, the concentration of sperm was 1~2million/ml and the RBC concentration was 7~9 million/ml, which approximated a TESE/mTESE sample. The sperm concentration was selected because 1~2million/ml is the minimum concentration for use with a cell counting chamber. Mixed samples were injected into the spiral channel at flow rates from 0.1 to 0.52ml/min. The sperm and RBC concentrations in the collected samples from each of the four outlets were measured using a Mackler cell counting chamber under 20X magnification. 1ml of sperm sample was injected into the spiral channel and about 0.2ml of sample was collected in each of the outlet syringes, which means 0.2ml of sample might remain within the spiral channel and connecting tubing.To visualize focusing of the RBC and sperm cells, samples containing mixtures of sperm and whole blood were injected into the spiral channel, and observed with a Nikon AR1 confocal microscope under 4X magnification. Mixtures of sperm and whole blood samples were injected at flowrates from 0.1 to 0.52ml/min. The sperm samples were stained with DAPI and the RBCs stained with PKH26 (Sigma, MO, USA) to enhance their visual signal. Outlet channel concentrations were measured as described above. To achieve optimal flow-focusing of each cell type, the input sample total cell concentration was experimentally investigated to minimize inter-particle interaction effects.[13] Based on the results of these experiments, the input samples were diluted down to a total cell concentration of <~10million/ml.

A projection image of a section of the obtained high-speed (230fps) video was analysed to determine cell focusing patterns within a section of the spiral channel close to the outlets on the 4th ring. (Fig. 4 and Fig. 6). For initial sample runs, 10 s videos were recorded after the initial 30 second sample injection. Additional videos were recorded in several lengths (10, 30, 50sec, 1min, 10mins) after the initial 30 second sample injection. Videos were analysed using NIS-Element's analysis feature. A projection image from a selected section of the video file, was analysed to obtain intensity data for each wavelength for the different cell stains (DAPI and PKH26).

For cell streak visualization experiments, a second image recording technique was employed with CellTracker green (Invitrogen, USA) stained sperm samples, using an inverted microscope with a digital camera. The camera shutter exposure time was increased to 10s per image, which generates a streak image and shows a section of all four rings of the channel.

In order to both validate the separation mechanism and to investigate the possibility of serial purification using the spiral channel to increase the purification ratios, we performed a set of sequential sample runs (three steps) and analysed cell concentrations following each step. For these experiments, 1ml of sample from the previous step's collected outlet sample was used as the next step's input sample. After each step, collected samples were evaluated for

cell concentration. To eliminate sample remaining within the device and tubing from previous runs, a flushing run (1ml of sperm media) was executed after each run.

Results/Discussion

The collected samples from the outlets showed enrichment of sperm and blood cells at different outlets, suggesting that at least some focusing of these cells was occurring in the microchannel. Sperm cells, at the conditions tested, tended to accumulate near the outer walls, both when tested alone and when mixed with RBCs. Specifically, sperm concentrations were higher in samples collected from the outermost and outer outlets, which represent the outer wall of the channel, while sperm concentrations were lower in samples collected from inner and innermost outlets, which represent the inner wall of the channel (Fig. 3). In general, there was a trend of high sperm concentrations at the outer wall with the concentration decreasing towards the inner walls. The reverse occurred for the RBCs, and for the fastest flow rates there were almost no RBCs in the outermost exits (Fig. 5).

The flow-focusing trend became more distinct as flow rates increased up to 0.2ml/min, in which concentrations of the outer wall channel exits (outermost and outer) were 46.5 and 15.5million/ml. As a ratio, 71% of sperm eluted from the outer wall outlets (Outermost and outer)(Fig. 3). High speed projection images of a sample of stained sperm (Fig 4), supported the earlier sperm characterization results and indicated a improving flow-focusing trend for higher flow rates (0.3ml/min). The plot in Figure 4-(2) shows a comparison of the light intensity as a function of channel location generated by DAPI-labelled sperm for flow rates of 0.1 and 0.3ml/min. The concentration readings from Fig. 3, clearly indicate that sperm are move towards the outer wall for all flow rates; however, a sharp focus near the outer wall was not obtained for the sperm in contrast to previous work using micro beads,[13,15–19] most likely due to the fact that sperm are not spherical and their geometry as presented to the flow field may be somewhat random. Even though a sharp focus for the sperm was not obtained, the overall trend suggests that this method may be valuable for enriching sperm in complex samples.

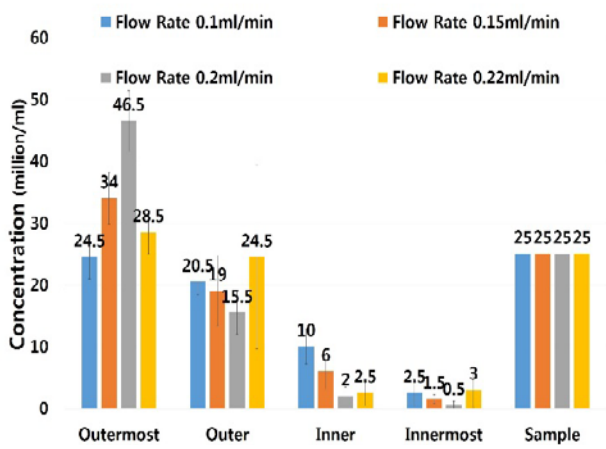

Figure 3. Sperm Characterization in various flowrate (0.1~0.22ml/min). The starting material of this experiment is human semen. Each bar represents concentration ratio among outlets, actual concentration are located on top of each bar.

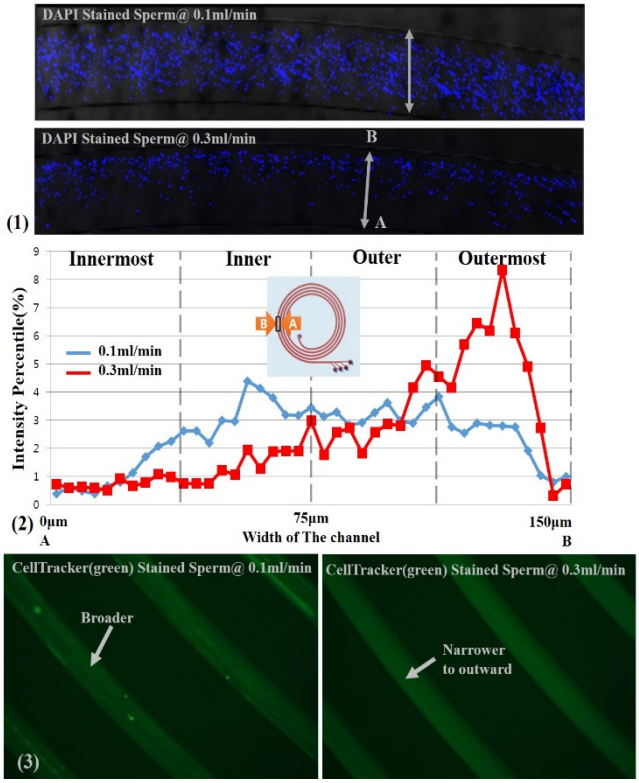

Figure 4. (1)High speed projection images of only stained sperm sample run with flow rate of 0.1ml/min and 0.3ml/min, (2) DAPI(blue) stained sperm population intensity analysis throughout width of the channel between two flow rates(0.1, 0.3ml/min), (3) CellTracker(green) stained sperm flow-focusing observation within a section of all four rings of channel (up 0.1ml/min, down 0.3ml/min)

In order to ascertain whether the spiral channels could facilitate sperm separation from other cell types beyond RBCs, outlet samples obtained after processing mixtures of whole blood and human sperm were analysed to determine sperm and RBC concentrations. RBCs (stained red) clearly focused toward the inner wall of the channel, while sperm (stained blue) focused more broadly toward the outer wall of the channel (Fig. 6). At a flow rate of 0.52ml/min, the sperm concentration is higher in outermost and outer outlets than in innermost and inner outlets, with measurements of 1.0, 0.6, 0.3 and 0.1million/ml respectively. The relative ratios of overall collected sperm at outlet outermost to innermost were 50%, 31.3%, 12.5%, and 6.3%, respectively. In contrast, the concentration of RBCs at a flow rate of 0.52ml/min was markedly higher at the innermost outlet than in outlets outermost, outer and inner with measurements (outlets outermost to innermost) of 0, 0.23, 1.1, and 22.6million/ml respectively. The concentration ratios of overall collected RBCs in outermost to innermost outlets were 0, 0.9, 4.6, and 94.4% respectively. As expected, based on previous inertial microfluidic studies, relatively small particles exited the outer outlets (sperm cells,

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81.2%) while larger cells mostly collected within the inner outlets (RBC, 99.0%) (Fig. 5).

Figure 6 shows a compilation of stained-cell intensity measurements of the stained sperm and RBCs acquired using high speed video for flow rates of 0.1ml/min and 0.52ml/min. Four minutes of video images were overlaid to obtain the images in Figure 6-(1,2), Figure 6-(3) is a "projection" of the images with the light intensity plotted as a function of channel position. For these experiments, the sperm and RBCs were flowed separately. At the lowest flow rates (i.e. 0.1 ml/min), there is essentially no difference between the positions of the sperm and RBCs(Fig. 6-(2,3)). At 0.52mi/min, there is a clear shift between the two cell populations, but they never quite separate, which is consistent with our earlier data showing enrichment, but not separation(Fig. 6-(1, 3)). The lack of tight focusing is thought to be due to the asymmetrical shape of sperm. On the other hand, better flow-focusing of RBCs occurred, likely because of the RBC's somewhat more symmetric geometry.

As noted previously, the shape of the sperm likely limits their ability to be tightly focused. Modeling may be helpful in determining a flow rate or condition that would help the sperm cells to align with some aspect of the flow, and then possibly be focused. Current inertial microfluidics models only work with symmetrical particles (spheres), so development of a more extensive model involving sperm would be valuable.

Since there was clear evidence that faster flow rates generate some separation and sperm enrichment, an effort was made to drive the flow faster. Unfortunately, it was generally difficult to go above 0.52ml/min, lower than calculated flow rate 0.55ml/min, as the pressures in the channel tended to cause leaks and ultimately ruin the devices. Even with the modest focusing, the substantial enrichment of sperm should make it much easier to find sperm in samples processed through the channel when compared to a raw TESE/mTESE sample.

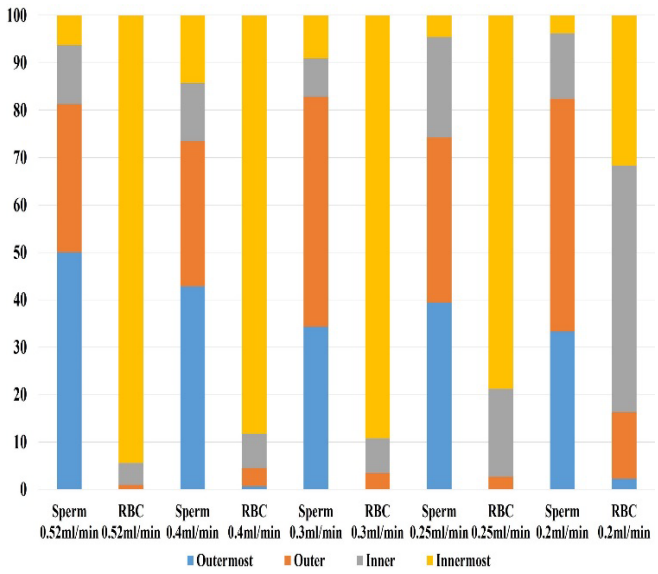

Figure 5. Mixed Sample (Sperm/RBC) Characterization under various flowrate (0.2~0.52ml/min), Each bar represents outlet collected ratio (in percentile) for each cell types(Sperm or RBC)

Sequential step runs were performed in order to determine whether the spiral channel can be used to improve purification of the samples through repeatedly treating the same sample (Fig. 7). Reprocessing the cells of outlet 1 and outlet 2 from step 1, which were at a concentration of: RBC, 0.3million/ml and sperm, 1.97million/ml, and outlet 2 of: RBC, 0.74million/ml and sperm, 1.6million/ml. Step 2 results with outlet sample 1 from step 1, were: RBC, 0million/ml and sperm, 1.55million/ml from outlet 1, and RBC, 0.05million/ml and sperm, 1.3million/ml from outlet 2.(Fig. 7-a) These results show that the RBC concentration of a

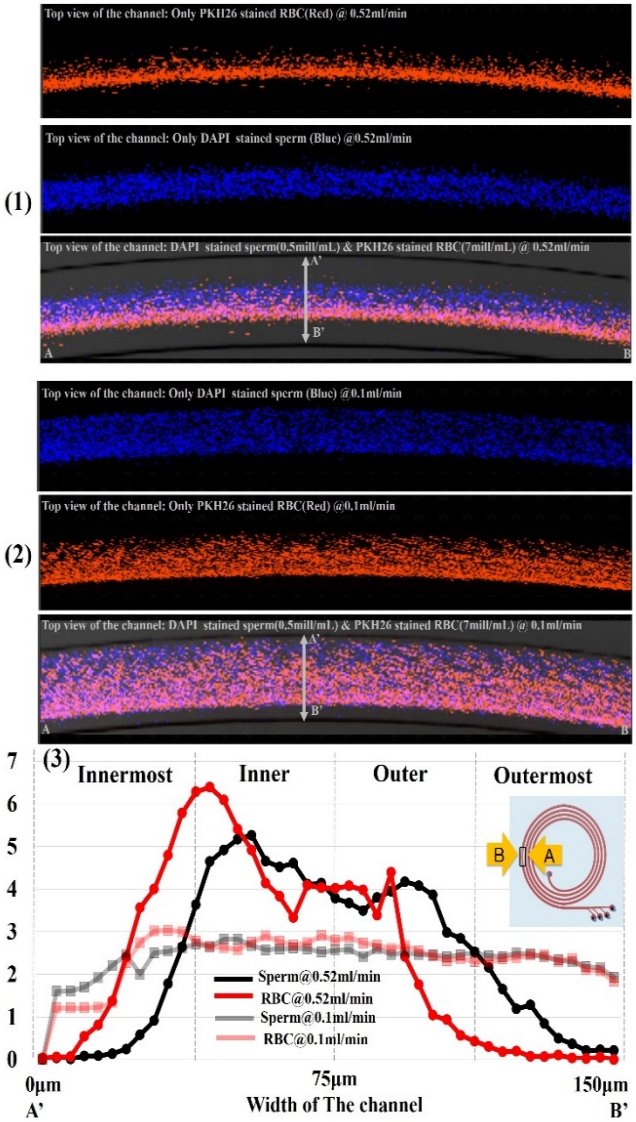

Figure 6. (1)high speed projection images of mixed stained sample (Sperm/RBC) with flow rate of 0.52ml/min, (2)high speed projection images of mixed stained sample (sperm/RBC) with flow rate of 0.1ml/min, (3) Stained cell population intensity analysis throughout width of the channel,

reprocessed sample exiting Outlet 1 is much less than for the original sample and that essentially all of the RBCs have been removed. The same trend is observed when the sample is reprocessed again (Step 3) results, which were: RBC, 0million/ml at outlet 1 and 2 of step 3 and sperm: 1.3million/ml(outlet 1) and 1.15million/ml(outlet 2). (Fig. 7-(a)) Based on the cell concentration results from step 1 to step 3, the sample is clearly collecting more sperm cells from outlet 1 while rejecting all of the RBCs, suggesting that this method could be used to eliminate RBCs from the sample. In this case, it is 100% filtering the RBCs from the original sample which went from 9.95million RBCs/ml to 0 RBCs/ml.(Fig. 7-(a)) Similar results were obtained for the later levels of processing, where all RBCs could be eliminated from nearly all of the outlets. In addition, the sperm cells that were lost through Outlet 3 in step 1 could be recovered by further processing, as shown in Fig 7-(b) and 7-(c). Thus, sequential processing has significant promise for allowing the collection of all sperm in a sample, which is critical in these mTESE samples that may have only 10s or 100s of sperm.

The overall result of step runs suggest that even though the spiral channel was only able to generate a weak focus with sperm cells towards the outer walls, the sharply focused RBC flow can purify the RBCs away from the sperm. In each step run, the concentration of sperm in outlets 1 and 2 was increased compared to the previous step. Despite the sequential step purification results achieved, there is still a possibility of sperm loss, measured to be about 19% with the current spiral design, which occurs with every stepwise run, so it is unlikely that these steps can be repeated indefinitely, though reprocessing of samples can be used to recover some portion of the lost cells.

A close observation of the data also indicates that cells that were focused to outlet 1 originally do not always return to Outlet 1 in the next separation step, suggesting that there is either some randomness to the focusing, which would be expected due to the non-spherical sperm and RBCs used here, or some interactions at high concentration that limit focusing. The results suggest that there is some randomness associated with the shape factors, and less related to concentration effects, as there were no clear changes at the lower concentrations.

Conclusions

In conclusion, we successfully demonstrated the use of inertial microfluidic technology to purify sperm by focusing particles in a spiral channel flow. Unlike conventional sperm separation techniques, the technique presented here was not dependent upon sperm motility, nor do they require any labels. Modelling of the sperm and RBCs as 5µm and 9µm diameter spheres respectively, a set of spiral channel dimensions was selected that adequately separated these cells, though further modelling may suggest better channel geometries for these

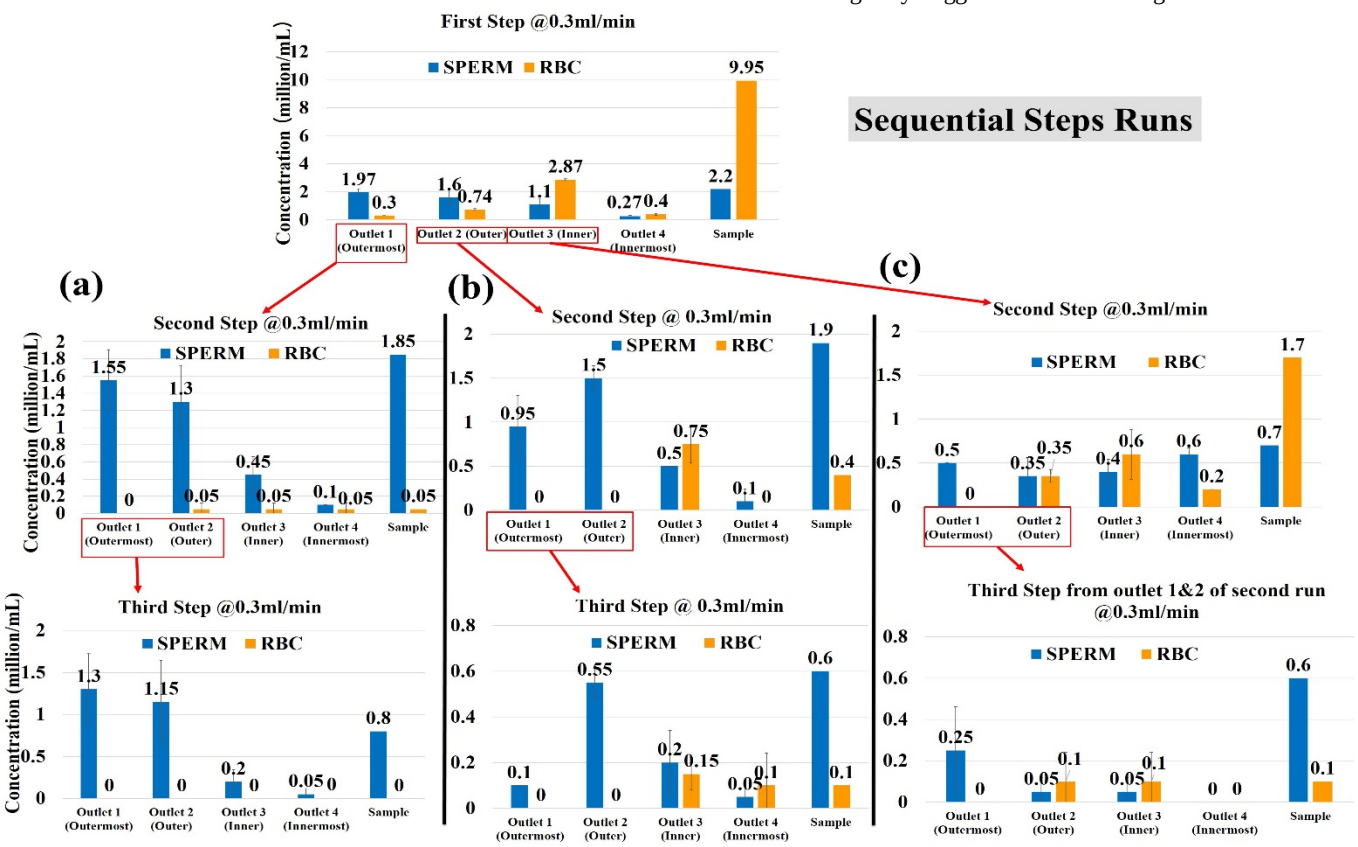

Figure 7. Sequential step runs to confirm purification of sample use of the spiral channel a) step runs of 2, 3 with outlet 1 sample of step 1, b) step runs of 2, 3 with outlet 2 sample of step 1, c) step runs of 2, 3 with outlet 3 sample of step 1. Note: name of outlet are selected for convenience, Outlet 1: Outermost, Outlet 2: Outer, Outlet 3: Inner, and Outlet 4: Innermost.

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asymmetric particles. The results corresponded well with previous publications which suggested that inertial microfluidics should be able to generate focused-flow of 5μm diameter spheres at the outer wall area and focused-flow of 9μm diameter spheres near the inner wall of the channel.

With sample injection flow rates up to the calculated optimal flow rate (0.52ml/min), sperm only and mixed (sperm/RBC) samples run through the spiral channel demonstrated moderate flow focusing of sperm toward the outer wall of the channel and sharper flow-focusing of the RBCs toward the inner wall of channel. 81% of non-motile sperm were recovered at outer wall exits and 99% of RBCs could be recovered at the inner wall outlets for an injection flow rate of 0.52ml/min. These results were verified through visualization by acquiring high speed video of cells transiting the channel for a small section of the outer rim of the channel confirming two different focused-flow lines of stained sperm and RBCs within the channel.

Reprocessing of samples showed that 100% removal of RBCs was possible while still collecting a high percentage of sperm. Sperm that were lost in earlier processing runs could also be recovered while removing all of the RBCs using a reprocessing approach. Even with the possibility of losing of sperm cells through multiple step runs, this study showed the possible usefulness of spiral channels in purifying sperm from background cell debris. Application of this approach, which only takes a few minutes, could significantly improve the current processing time of mTESE samples significantly, because 99% of mTESE samples are unwanted cells and debris, and technicians currently spend significant time looking through these samples for sperm. Presenting the technicians with a sample where 99% of the debris has been removed would be a significant improvement.

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