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## Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species

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## Abstract

Fertilization and reproduction are central to the survival and propagation of a species. Couples who cannot reproduce naturally have to undergo *in vitro* clinical procedures. An integral part of these clinical procedures includes isolation of healthy sperm from raw semen. Existing sperm sorting methods are not efficient and isolate sperm having high DNA fragmentation and reactive oxygen species, and suffer from multiple manual steps and variations between embryologists. Inspired by *in vivo* natural sperm sorting mechanisms where vaginal mucus becomes less viscous to form microchannels to guide sperm towards egg, we present a chip that efficiently sorts healthy, motile and morphologically normal sperm without centrifugation. Higher percentage of sorted sperm show significantly lesser reactive oxygen species and DNA fragmentation than the conventional swim-up method. The presented chip is an easy-to-use high throughput sperm sorter that provides standardized sperm sorting assay with less reliance on embryologist's skills, facilitating reliable operational steps.

## Keywords

sperm sorting; DNA integrity; advance reproductive technologies; reproduction; reactive oxygen species

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## 1. Introduction

More than 70 million couples worldwide <sup>[1, 2]</sup> cannot reproduce naturally and have to go through assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and intrauterine insemination (IUI).<sup>[3, 4]</sup> With an increasing rate of male infertility due to environmental and physiological conditions, there is an ever growing need for the use of ARTs in reproductive clinics.<sup>[5, 6]</sup> Isolation of the motile and morphologically normal viable sperm is an integral process to commonly used IVF/ICSI/IUI procedures. Although current IVF/ICSI procedures result in successful pregnancy ~50% of the time, the output can be greatly compromised if the sperm being selected are abnormal.<sup>[7]</sup> There is a concern about the reported increased risk of birth defects in the offsprings from couples undergoing ART procedures.<sup>[8]</sup> These increased birth defects may be in part due to the inadequate selection parameters of healthy sperm which relies primarily on morphology and forward progression motility while neglecting DNA fragmentation and generation of reactive oxygen species (ROS) during the sorting procedures.<sup>[9]</sup> ROS exposure greatly harms seemingly motile and healthy sperm.<sup>[9, 10]</sup> In addition, sperm samples showing higher level of DNA fragmentation result in lower fertilization rates, impaired embryo progression and lower pregnancy rates.<sup>[11-14]</sup>

Currently, centrifugation based sperm swim-up, and density gradient separation are more commonly used sperm sorting methods.<sup>[4, 15-19]</sup> These methods reduce sperm quality during the repetitive centrifugation steps,<sup>[20, 21]</sup> as sperm concentration in solid pellets, often with incidental inflammatory cells shows higher ROS generation and DNA fragmentation.<sup>[20, 21]</sup>

Further, centrifugation-based sperm sorting techniques are labor intensive, time-consuming and outcomes can vary from technician to technician. Although various microfluidic based sperm sorting devices have been developed to eliminate centrifugation steps, these devices gave very low throughput and can only process small semen volumes, thereby limiting their application to reproductive medicine.<sup>[4, 19, 22, 23]</sup>

To address the limitations of conventional sperm sorting methods, we evaluate the feasibility of sorting functional human sperm with higher DNA integrity and fewer reactive oxygen species by creating an innovative centrifugation-free and flow-free microfluidic platform, where functional sperm are isolated from unprocessed semen sample with a high retrieval efficiency. Natural sperm selection in the female genital track is affected by a series of anatomic barriers, starting with the cervix and uterus, and ending in the oviduct where fertilization occurs.<sup>[16]</sup> Cervical mucus, endometrial and tubal fluids becomes less viscous to form tiny microchannels to guide sperm. The presented macrofluidic sperm sorter (MSS) chip is designed such that macro reservoirs are connected by micropores. The most motile and functional sperm pass selectively through the micropores against gravity leaving behind dead and less motile sperm.

## 2. Results

To develop a high throughput sperm sorting device, a two-chamber MSS separated by polycarbonate filter of various diameters, i.e. 3, 5, 8  $\mu\text{m}$  is designed (Figure 1). The sperm sample is injected into the bottom chamber and sorted motile sperm are collected from the top retrieval chamber (Figure 1b).

### 2.1 Sperm Motility, Viability, and Retrieval Efficiency

To investigate the motility of the sorted sperm, we analyzed the sperm collected from the top retrieval chamber of MSS. Results show that the sorted sperm have higher motility as compared to stock sperm (Figure 2a). Specifically, sperm sorted by 3, 5 and 8  $\mu\text{m}$  MSS show sperm motilities of  $95\% \pm 10$ ,  $90.4\% \pm 1.8$ , and  $85.9\% \pm 1.5\%$  respectively, which are significantly higher than the stock sperm motility of  $39.8\% \pm 1.9\%$ . The higher motility in sorted sperm is due to the presence of micropores between the two chambers that selectively allow the most motile sperm to swim through pores. To evaluate the effect of incubation time on motile sperm enrichment, sperm were collected after 15, 30, and 45 minutes. We observe that the sperm motility in the case of 45 min time point is highest whereas it is lowest for 15 minutes (Figure 2a). When human tubal fluid (HTF)+1% bovine serum albumin (BSA) media is pipetted to the top chamber of MSS, slight mixing of the two liquids (stock sperm sample and HTF+1%BSA media) occurs. This mixing in sperm sample is the possible reason for the lesser sperm motility at the start of the experiment (after 15 minutes) as compared to latter time points (after 30 and 45 minutes). In addition, we calculated the sperm retrieval efficiency, i.e. percentage (%) of healthy sperm retrieved out of the raw stock semen sample. Sperm retrieval efficiency was analyzed for 15, 30, and 45 min time points (Figure 2b). Sperm retrieval efficiency is saturated after 30 minutes ( $3.08\% \pm 0.42\%$ ,  $23.75\% \pm 3.96\%$ , and  $28.58\% \pm 2.81\%$  for 3, 5 and 8  $\mu\text{m}$  MSS respectively). To theoretically evaluate the sperm retrieval efficiency, we have created a coarse-grained sperm

persistent random walk model where  $p$  is the sperm probability of passing through the filter (Methods section). Simulations recapitulate the sperm retrieval efficiencies at 15 and 30 min time points, and predict higher retrieval at 45 min as shown in Figs. 2c and 2d. Saturation observed experimentally suggests that at longer incubation times the filter loses efficiency, potentially due to the sperm collecting near pores and blocking passage. To substantiate our findings that the sorted sperm are viable, we performed viability staining for sorted sperm. The viability of sorted sperm is significantly higher compared to stock sperm (Supporting Figure S2). Representative fluorescent images of live/dead sperm staining are shown in Figure 2e.

## 2.2 Effect of Sample Dilution on Sperm Motility and Retrieval Efficiency

To investigate the effect of sperm sample dilution on motility enrichment and retrieval efficiency, we diluted the stock sperm sample with HTF+1%BSA at the ratio of 1:4 before processing with MSS. Sorted sperm show higher motility than stock sperm sample. Specially sperm sorted by 3, 5, and 8  $\mu\text{m}$  MSS show sperm motilities of  $95.0\% \pm 5.0\%$ ,  $93.7\% \pm 4.7\%$ , and  $90.7\% \pm 2.5\%$  respectively, which are significantly higher than the stock sperm motility of  $45.8\% \pm 1.5\%$  (Figure 3a). Sperm retrieval efficiency increases if diluted sperm is used instead of undiluted stock sperm (Figure 2b and 3b). Maximum retrieval efficiency is  $52.68\% \pm 4.97\%$  for 8  $\mu\text{m}$  chip after 30 minutes incubation. This increased retrieval efficiency might be due to higher sperm velocities in low viscous solution.<sup>[24]</sup> Further, since the filter has fixed number of pores (<14% porosity), in the case of diluted semen sample, lesser number of sperm are trying to cross the filter pores at a time. It is more probable for each sperm to find an empty pore and pass through it. Simulations confirm this hypothesis and the probabilities of sperm passing through the filter increase from  $p=0.075$  to  $p=0.1$  and from  $p=0.15$  to  $p=0.4$  for 5  $\mu\text{m}$  and 8  $\mu\text{m}$  filter pore sizes, respectively. Simulation results recapitulate the sperm retrieval efficiencies as shown in (Figs. 3c and 3d).

## 2.3 Sperm Velocity Analysis

A representative image of sperm track showing various velocity parameters is shown in Supporting Figure S3 and Supporting Movie 1. The MSS sorted sperm show higher curvilinear velocities (VCL), straight line velocities (VSL), and average path velocity (VAP) compared to stock sperm sample (Figure 4a). Specifically, average sperm VCL values are  $52.7 \pm 6.0 \mu\text{m}/\text{sec}$ ,  $59.9 \pm 3.5 \mu\text{m}/\text{sec}$ ,  $75.3 \pm 3.1 \mu\text{m}/\text{sec}$ , and  $75.6 \pm 4.5 \mu\text{m}/\text{sec}$  for stock sample, 3, 5 and 8  $\mu\text{m}$  MSS respectively (Figure 4a). Average VSL values are  $44.4 \pm 5.6 \mu\text{m}/\text{sec}$ ,  $52.1 \pm 3.5 \mu\text{m}/\text{sec}$ ,  $63.4 \pm 3.5 \mu\text{m}/\text{sec}$ , and  $64.1 \pm 3.9 \mu\text{m}/\text{sec}$  for stock sample, 3, 5 and 8  $\mu\text{m}$  MSS respectively (Figure 4b). Average VAP values are  $48.4 \pm 5.8 \mu\text{m}/\text{sec}$ ,  $54.1 \pm 3.4 \mu\text{m}/\text{sec}$ ,  $68.0 \pm 2.9 \mu\text{m}/\text{sec}$ , and  $67.5 \pm 4.1 \mu\text{m}/\text{sec}$  for stock sample, 3, 5 and 8  $\mu\text{m}$  MSS respectively (Figure 4c). Higher sperm velocities indicate that the MSS sorted sperm are more motile than stock sample. We also observe that sperm sorted using 5 and 8  $\mu\text{m}$  MSS give higher VCL, VSL and VAP velocities than sorted by 3  $\mu\text{m}$  MSS. This is potentially because of mostly small sized abnormal but motile sperm having head sizes smaller than 3  $\mu\text{m}$  could pass through the 3  $\mu\text{m}$  micropores.

## 2.4 Sperm Morphological and Nuclear Maturity Analysis

Sperm morphology was assessed based on strict criteria defined by WHO (Figure 5b).<sup>[23]</sup> We observe that higher percentage of sperm sorted using 8  $\mu\text{m}$  MSS is morphologically normal compared to stock and sperm sorted using 5  $\mu\text{m}$  MSS (Figure 5b). Sperm sorted using 5  $\mu\text{m}$  MSS show similar morphology compared to unprocessed stock semen, though the sorted sperm are motile. To look at sperm nuclear compactness, sperm are analyzed for nuclear maturity. Representative images of sperm stained with aniline blue and their assessment criteria are shown in Figure 5c. We observe that higher percentage of sperm sorted using 8  $\mu\text{m}$  MSS is nuclear mature compared to stock and sperm sorted using 5  $\mu\text{m}$  MSS (Figure 5d). This percentage increase in morphological normal and mature sperm population in the case of 8  $\mu\text{m}$  chip might be due the larger diameter micropores which allow larger and mature sperm to swim through.

## 2.5 ROS Generation Analysis

Sperm sorted using MSS produce significantly lesser ROS than swim-up and washing methods (Figure 6a and Supporting Figure S4). Sperm washing and swim-up method produce ROS in  $10.1\% \pm 0.3\%$  and  $10.6\% \pm 1.1\%$  of the sperm respectively, whereas sperm sorted using MSS show ROS production in only  $0.8\% \pm 0.4\%$  (3  $\mu\text{m}$  MSS),  $0.7\% \pm 0.1\%$  (5  $\mu\text{m}$  MSS) and  $1.0\% \pm 0.1\%$  (8  $\mu\text{m}$  MMSS) of the sperm. Unsorted semen sample shows ROS generation in  $1.8\% \pm 0.6\%$  of the sperm, which indicate that the increased generation of ROS in swim-up and washing methods came from centrifugation steps.<sup>[20, 21]</sup>

## 2.6 DNA Fragmentation Analysis

Sperm sorted using MSS show significantly lower DNA fragmentation (%) than unsorted semen sample (Figure 6b and Supporting Figure S5). DNA fragmentation (%) is  $1.1\% \pm 0.3\%$  (8  $\mu\text{m}$  MSS),  $2.1\% \pm 0.7\%$  (5  $\mu\text{m}$  MSS),  $3.4\% \pm 0.8\%$  (3  $\mu\text{m}$  MSS),  $3.7\% \pm 1.2\%$  (swim-up method), and  $31.2\% \pm 1.2\%$  (unsorted semen) (Figure 6b). Sperm sorted using 8  $\mu\text{m}$  MSS show even lesser DNA fragmentation compared to sperm sorted using swim-up method ( $p$ -value = 0.06).

## 3. Discussion and Conclusion

The ideal sperm sorting technique should (i) be rapid and cost-effective, (ii) labor non-intensive, (iii) accommodate large sperm volumes, (iv) have high and efficient enrichment for motile sperm (v) isolate sperm with higher velocity, (vi) isolate sperm with normal morphologically and nuclear maturity, (vii) reduce ROS generation and morphological damage by eliminating centrifugation steps, and (viii) reduce the percentage of sperm DNA fragmentation. The MSS described here offers a simple platform meeting these requirements. The total material cost to fabricate a chip is less than a dollar (50 cents for filter, <50 cents for PMMA and DSA). The MSS rapidly (~30 minutes) isolates motile sperm from non-motile ones with the higher retrieval efficiency ( $28.6\% \pm 2.8\%$  retrieval from stock sperm) than swim-up technique (<20%).<sup>[23]</sup> For IUI procedure, sperm that have been washed are placed directly in the uterus around the time of ovulation. For such a procedure, a high throughput efficient sperm sorting technology increases the number of inseminated sperm which are capable of reaching the fallopian tube and fertilizing the egg. Currently,

swim-up method gives <20% sperm retrieval efficiency. We show retrieval efficiencies of  $52.7\% \pm 4.9\%$  ( $8 \mu\text{m}$  MSS) when starting with a diluted sample. Experimental results are also recapitulated using computer simulations based on persistent random walk model. Although sperm dilution gives higher retrieval of healthy sperm, it reduces the actual number of sperm that can be input into the device at a time. Conditions where stock sperm dilution may have advantages include (i) low volume ejaculates, and (ii) ejaculates with very low sperm count. The filters used in presented MSS have pores in <14% of the surface area, at higher sperm concentration, it would limit the number of sperm that can swim thorough at a time. The retrieval efficiency especially at higher sperm stock concentration can be further increased by using a filter with higher density of pores. It is important to highlight here that although the used polycarbonate filters show uniform pore diameters, in about 7% of filter area, two or more smaller pores are joined together to make up a larger pore, which would allow larger sized sperm to pass through small pore diameter filters (Figure 1c). Semiconductor fabrication processes and soft lithography methods can be utilized to make filters with high density, and non-overlapping micropores.<sup>[25, 26]</sup> Further, the MSS design can be easily scaled with filters of different diameters and multiple channels, thereby accommodating a variety of semen volumes (  $1.5 \text{ ml}$ ).<sup>[23]</sup>

ROS generation is an important measure to assess the sperm quality and its apoptotic status.<sup>[9, 10]</sup> There are various pathways and underlying mechanisms leading to sperm ROS generation such as poor differentiation during spermiogenesis,<sup>[27]</sup> poor chromatin compactness,<sup>[28]</sup> and presence of sperm in the vicinity ROS generating cells.<sup>[9, 20]</sup> Conventional techniques utilizing centrifugation steps to sort healthy sperm also lead to ROS generation as these techniques centrifuge sperm with ROS generating cells such as leukocytes. We observe that sperm sorted using MSS show significantly lower ROS generation compared to conventional swim-up method as sperm can swim away from the semen plasma into the fresh solution through sorting micropores. Additionally, sperm sorted using MSS show a significant improvement in DNA fragmentation compared to unsorted semen sample as shown in Figure 6b. Currently, sperm swim-up method is considered as a standard method to sort sperm with lower DNA fragmentation.<sup>[21, 29]</sup> We observe that sperm sorted using  $8 \mu\text{m}$  MSS show even lower DNA fragmentation than swim-up method.

Other functional parameter to consider for higher fertilization rates is the velocity of motile sperm.<sup>[30]</sup> In addition, higher percentages of mature and morphologically normal sperm, especially across a 4-10% threshold, increases the fertilization rate during IVF procedures.<sup>[31-33]</sup> Sperm sorted by MSS show enhanced velocity parameters (VCL, VSL and VAP) compared to stock unsorted sperm. Higher percentage of MSS sorted sperm are mature and enriches from  $17.6\% \pm 0.5\%$  to  $30.0\% \pm 7.6\%$  for morphologically normal sperm, which is a significant improvement over the stock sample (Figure 5).

Overall, the presented technology has practical advantages including, (i) decreased number of sample transfers, (ii) elimination of large equipment such as centrifuge, (iii) a simple closed system that decreases potential environmental contamination of samples, (iv) less complex operational protocols, (v) removal of seminal plasma without washing, (vi) concentration of motile sperm without spinning or gradients, (vii) improved recovery efficiency by minimizing losses due to transfers and repeated volume adjustments, and (viii)

reduced variations due to human error. The presented functional assay of sperm sorting using MSS is evident of qualitative, in addition to quantitative, improvements compared to conventional methods. Use of such a device in a routine therapeutic environment has operational advantages, and has potential to improve clinical outcomes.

## Experimental Section

### Chip Assembly

The poly (methyl methacrylate) (PMMA, 3 mm thick; McMaster Carr, Atlanta, GA) and double side adhesive (DSA, 120  $\mu\text{m}$  thick, St. Paul, MN) were cut using a laser cutter (Versa Laser™, Scottsdale, AZ). The design for the chip was generated on Coral Draw4 and implemented onto USLE Engrave software for cutting. Primary components of the MSS included one 3mm PMMA cut to an area of 50 mm  $\times$  30 mm (bottom chamber) and another cut to an area of 30 mm  $\times$  30 mm (top chamber). A 0.6 mm injection point was also cut into the bottom PMMA sheet at a 5 mm distance from the chambers. Cylinders of 20 mm diameter were cut into both PMMA components. The bottom PMMA chamber was first attached to glass slide using DSA. Top PMMA chamber was aligned and attached with bottom chamber using DSA. The Nuclepore™ track-etched polycarbonate membrane filters (Whatman Ltd, 25 mm diameter, 3  $\mu\text{m}$ , 5  $\mu\text{m}$ , 8  $\mu\text{m}$ ) were sandwiched between two PMMA chambers during chip assembly. Side view of the assembled chip is shown in Figure 1a. Side view of the chip along with the physical dimensions is shown in Supporting Figure S1. To make the whole assembly process sterilized, all components were washed with 70% ethanol, air dried and assembled inside cell culture hood.

### Human Sperm Preparation

Human Tubal Fluid (HTF) (InVitroCare®, Frederick, MD) supplemented with bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used for sperm preparation and sorting. Vials of cryopreserved anonymous human whole semen (1.0 cc each, Intracervical Insemination specimen vials) were purchased from California CryoBank following IRB 2012P000590 regulations and stored in liquid nitrogen. Sperm samples were thawed for 15 minutes in the water bath at 37°C before use.

### Sperm Sorting using MSS

Thawed, unprocessed semen sample (stock sperm) was injected into the inlet of MSS until it filled the bottom chamber (Figure 1b). The bottom chamber can hold upto 560  $\mu\text{l}$  of semen. In another set of experiments, the stock semen sample was diluted with 1% BSA in HTF at 1:4 ratio before injection into the MSS. Following injection, the upper portion of microfluidic chamber was topped off with 560  $\mu\text{l}$  of 1% BSA in HTF. Chips were then incubated at 37°C for 15, 30, and 45 min intervals before fluid from the top chamber was collected into eppendorf tubes for analysis.

### Concentration and Motility Analysis

A standard Makler counting chamber was used to manually analyze a 1 $\mu\text{l}$  sperm sample for concentration and motility according to the manufacturer's instructions.

## Viability Analysis

Sperm samples were analyzed for viability using LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes®). SYBR 14 dye was used to stain live whereas Propidium Iodide (PI) was used to stain dead sperm. Samples were stained according to manufacturer's protocol. Briefly, first SYBR 14 dye was added into sperm sample to the final concentration of 100 nM. The sample was incubated for 5 minutes at 37°C. To stain the dead sperm, PI dye was added to the sample to the final concentration of 10 µM and allowed to incubate for 5 additional minutes. The sperm samples were smeared on a glass cover slip and imaged at a fluorescent microscope using Green and red emission filters, respectively, for SYBR 14 and PI.

## Velocity Measurement

Viable sperm samples were analyzed by image analysis of a sample prepared using the method described by WHO laboratory manual for sperm analysis.<sup>[23]</sup> Briefly, sperm was retrieved from the MSS (3 µm, 5 µm, 8 µm) after 30 minutes. Slides were prepared by putting 6 µl of sperm sample onto a glass slide and covered by using a 18×18 mm cover slip to give the sample a depth of 20.7 µm.<sup>[3]</sup> To avoid drying up of samples, slides were made periodically, not simultaneously. Each slide was analyzed using light microscopy (Carl Zeiss) with live images of the sample being projected onto a computer monitor. Using a video capturing software (Snagit, TechSmith), movement of sperm samples were captured at random locations for 5 secs. Videos were converted to image sequences using VideotoJpeg (freeware, DVDVideoSoft) software at 100fps. The image sequence was input into ImageJ (National Institute of Health, <http://rsbweb.nih.gov/ij/>) for analysis using the CASA plugin to monitor sperm velocity parameters, i.e. straight line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP).<sup>[34]</sup>

## Sperm Morphology Assessment

Recovered sperm suspension from 5 µm, and 8 µm MSS were collected after 30 minutes. Sperm retrieved from 3 µm MSS were not analyzed for sperm morphology, as sperm concentration is too low for morphology analysis. A 10 µl sperm suspension was then taken and placed on a clean and sterile microscope slide and feathered smears were prepared. Smears were air dried and prepared for fixation. Sperm slides were stained for morphology assessments according to the manufacturer's instructions (Spermac, FertiPro).<sup>[17, 35]</sup> Briefly, dried smears were submerged into fixative solution (provided by manufacturer) for at least 5 minutes and then rinsed with DI water. Stain A was pipetted at one edge of the slides and allowed to flow over the smear. Slides were then placed on a flat surface and allowed to soak with stain for 1 minute. The slides were then rinsed with DI water twice. Next, stain B was applied similarly to Stain A and allowed to penetrate sperm for 1 minute. This was followed by a single rinse with DI water. Finally, stain C was pipetted over the smear and allowed to sit for 1 minute before rinsing with DI water. At this point, at least 100 sperm were imaged using oil immersion and 100X objective (N (no. of repeats) = 3). The sperm was considered morphologically normal if it falls within WHO strict criteria (Head: spherical head; acrosome covering 40-70% of head area; head length 3.7–4.7 µm; head width 2.5–3.2 µm; length-to-width ratio 1.3–1.8; no more than 2 small vacuoles; post-



acrosome region should not contain any vacuole. Midpiece: no residual cytoplasm in midpiece; length of midpiece should be approximately same as head length; no broken neck. Principal piece: no sharp angles or bends indicative of tail break; thinner than midpiece, length of principal piece should be approximately 10 times the head length.<sup>[23]</sup>

### **Sperm Nuclear Maturity Assessment**

Sperm were stained with aniline blue and analyzed for nuclear maturity.<sup>[33]</sup> Aniline blue staining can discriminate the lysine-rich nuclei of immature sperm and arginine/cysteine-rich nuclei of mature sperm.<sup>[36]</sup> Recovered sperm suspension from 5, and 8  $\mu\text{m}$  MSS were collected after 30 minutes. Sperm retrieved from 3  $\mu\text{m}$  MSS were not analyzed for nuclear maturity, as sperm concentration is too low for this analysis. Dried smears were fixed with the fixative solution for 5 minutes and subsequently rinsed with DI water. 5% aniline blue in 4% acetic acid solution was prepared and was poured over smears. Smears were soaked for 5 minutes in staining solution and then rinsed with DI water. At least 100 sperm were assessed using oil immersion 100X objective ( $N = 3$ ). Sperm heads that stained dark blue were declared immature, while those that remained unstained were considered mature.<sup>[17, 33, 36]</sup>

**ROS Detection**—Sorted sperm were analyzed for ROS generation. We have compared the ROS generation in the sperm after washing method, conventional swim-up method and MSS.

**Sperm Washing:** 1 ml of semen was removed from a cryopreservation tank and thawed for 15 minutes in a 37°C warm bath. Washed semen sample was prepared by adding 9 ml of HTF+1%BSA media to 1 ml of semen, centrifuging for 500Xg for 5 minutes and removing supernatant while leaving sperm pellet at the bottom of tube. This procedure was repeated three times. HTF media was added to sperm pellet and samples were stained with ROS studies.

**Swim-up Method:** The semen was diluted with 9 ml of HTF+1%BSA. The diluted sperm suspension was then centrifuged at 500Xg for 5 minutes. Then, the supernatant was removed and disposed. The remaining pellet was washed again by centrifuging sample at 500Xg for 5 minutes. The supernatant was removed and disposed again. Finally, 500  $\mu\text{l}$  of medium was added along the sidewall of centrifuge tube while avoiding the disruption of the pellet. The sample was then placed in the incubator and motile sperm were allowed to swim up out of pellet for 30 minutes. The motile sperm were collected by leaving pellet behind.<sup>[20]</sup> MSS chips were incubated for a 30 minutes period and sperm suspension was recovered for ROS studies.

### **Staining for ROS detection**

ROS generation was examined by using flow cytometry in conjunction with two fluorescent dyes, dyhydroethidium (DHE) and SYTOX green. DHE reacts with the superoxide anion which produces two fluorochromes which bind to sperm DNA and produces a red fluorescence. While SYTOX green is indicative of cell viability, it produces a green fluorescence when the cell is dead. For this experiment, four control samples were prepared

in which all consisted of 200  $\mu$ l of recovered sperm suspension mixed with 20  $\mu$ l of hydrogen peroxide. This was followed by an incubation at 37°C for 30 minutes. The dyes were added to the samples; no dye for negative control, DHE at 5  $\mu$ M was added to the second sample, SYTOX green at 50 nM was added to the third sample, and the fourth sample contained both DHE and SYTOX at 5  $\mu$ M and 50 nM respectively. Dyes were incubated for 15 minutes and then transferred to the flow cytometer for measurement 15 minutes prior to test samples. FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used during experiments. Argon laser excitation at 488 nm was coupled with emission measurements using 530/30 band pass (green) and 585/42 band pass (red) filters for FL1 and FL2, respectively. Nonsperm events were gated out, and at least 10,000 cells were examined. For test samples, 500  $\mu$ l sample from thawed semen, the swim up suspension, 3, 5, and 8  $\mu$ m filter pore size microchips were collected. DHE and SYTOX at 5  $\mu$ M and 50 nM respectively were added to each sample and allowed to incubate for 15 minutes. Samples were taken to the flow cytometer for measurement. Experiments are repeated for 4 times (N=4).

### DNA Fragmentation

Sperm sorted using MSS chips were analyzed for DNA fragmentation. TUNEL assay kit (*In Situ* Cell Death Detection Kit, Fluorescein by Roche Applied Science) was used to quantify DNA fragmentation for raw semen, swim-up, and sperm retrieved from microchip devices with filters of 3, 5 and 8  $\mu$ m pore size. All these samples were attained as previously mentioned in ROS Detection section. Initially, all the sperm suspensions were washed twice by centrifuging at 500Xg for 5 minutes with PBS and 1% BSA. Once washed, the concentrations of sperm cells were adjusted to  $2 \times 10^6$  cells/ml. Sperm suspensions were then fixed with 4% paraformaldehyde in PBS (200  $\mu$ l for every 100  $\mu$ l of cell suspension) for 30 minutes at room temperature. Sperm cells were washed twice at 500Xg for 6 minutes with PBS and 1 % BSA and permeabilized with 0.1% TritonX in 0.1% sodium citrate for 2 minutes on ice. Sperm were washed twice followed by 1 hour incubation at 37°C with 5 $\mu$ l of enzyme (TdT) solution and 45  $\mu$ l of label (dUTP-Flourescein) solution. Similarly, a negative and positive control sample was prepared. However, prior to staining, the positive control was incubated with DNase for 40 minutes at 37°C. During staining, the negative control was only incubated with label solution (without enzyme solution). After staining, samples were washed twice with PBS and 1% BSA and resuspended in PBS (Muratori et al, 2000). Fluorescence emission of DNA fragmented cells were assessed with flow cytometer and detected by the FL-1 detector (521 nm). A total of 5000 events were acquired. Sperm population was gated out from data to eliminate any signal from debris. Experiments are repeated 6 times (N=6).

### Statistical Analysis

Each experiment was repeated atleast three times and the results of sperm parameters were expressed as mean  $\pm$  SEM (standard error of the mean). Statistical analysis was performed by one-way analysis of variance (ANOVA). A value of  $P < 0.05$  was considered statically significant.

## Simulations of Sperm Motility through Filters

**Model:** We used a coarse-grained persistent random walk model<sup>[37]</sup> (PRW) to simulate sperm motility in the microfluidic device. In the simulations, sperm move on a straight-line with velocity  $S$ , for an average duration of persistence time  $P$ . Propagation directions of the sperm are chosen isotropically around a sphere with no memory of the previous direction. If a sperm collides with the walls, it will pick a new direction of travel. The sperm are non-interacting in this model, and further details of the model are described previously.<sup>[4]</sup> We also used a coarse-grained model for the filter. Once a sperm reaches the filter, it will pass through with a probability  $p$ , or with probability  $1-p$  choose a new direction to propagate in the lower chamber as if it interacted with a wall. VSL is measured by constraining the sperm to move in two dimensions, and calculating their displacement using a sampling time that matches experiments.

### Initial Conditions

To properly mimic the experimental setup, the sperm move in a cylindrical chamber with radius 10  $\mu\text{m}$  and height 6  $\mu\text{m}$ , and the filter is located at a height of 3  $\mu\text{m}$ . As the volume of injected sperm is comparable to the volume of the lower part of the channel, sperm start out uniformly distributed in the bottom half of the chamber, below the filter. Each Sperm are each given a velocity from a normal distribution with a mean of 60  $\mu\text{m/s}$  and a standard deviation of 30  $\mu\text{m/s}$ , giving an effective VSL of 52.29  $\mu\text{m/s}$ . Simulations are performed for 5  $\mu\text{m}$  and 8  $\mu\text{m}$  filter pore sizes.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

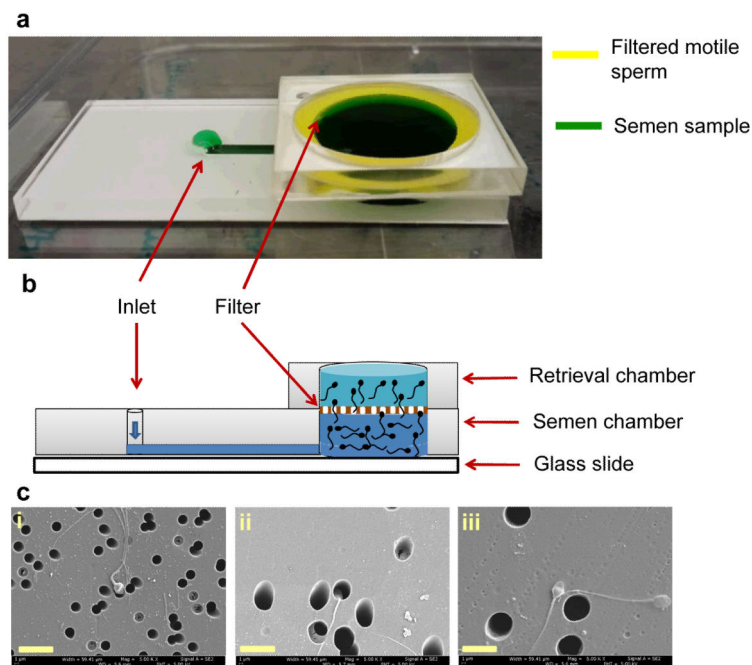
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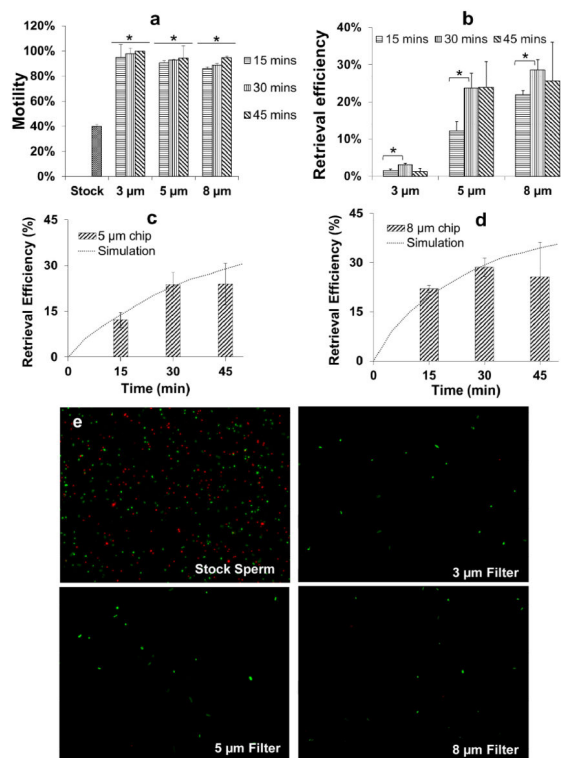
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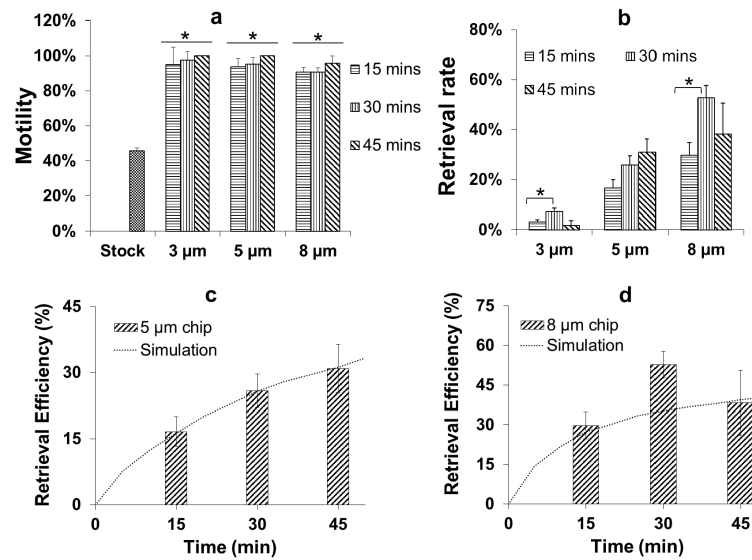


**Figure 1.** Macro- Microfluidic Sperm Sorter (MSS) for selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. (a) The photo of the MSS showing inlet, filter and two PMMA chambers. The MSS was filled with color dye to enhance contrast. (b) The illustration demonstrates the MSS design and working principle. The MSS consists of one inlet for the injection of raw unprocessed semen sample and two PMMA chambers separated by nuclepore track-etched membrane filter. The most healthy and motile sperm swim through the filter leaving unhealthy dead sperm in the bottom chamber. (c) SEM images of polycarbonate nuclepore track-etched membrane filters of different micropore diameters, i) 3  $\mu\text{m}$  ii) 5  $\mu\text{m}$  and iii) 8  $\mu\text{m}$ . These images shows the comparative size of various filter pores and sperm. The scale bar is 10  $\mu\text{m}$ .



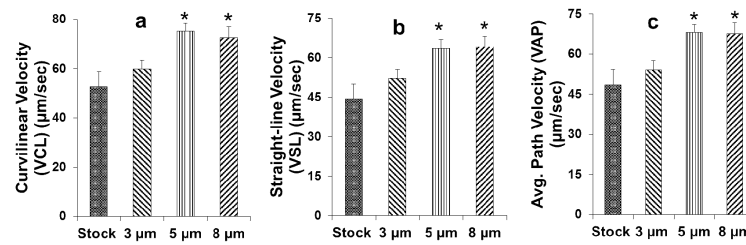
**Figure 2.**

Sperm sorting, motility and viability analysis. (a) % Motility of human sperm isolated using different pore diameter filters 3 μm, 5 μm and 8 μm. Sperm were retrieved at three time points, i.e 15, 30, and 45 min. Motility of the sperm sorting by using filters was significantly higher than stock sample motility ( $*p\text{-value} < 0.05$  between stock semen and sorted sperm,  $N=3$ ). (b) Retrieval efficiency of sorted sperm using 3 different chips. Retrieval efficiency was saturated after 30 min time point ( $*p\text{-value} < 0.05$  between time points,  $N=3$ ). Simulation results for (c) 5 μm chip and (d) 8 μm chip. Error bars represent standard error of the mean in (a-d). (e) Representative fluorescent live/dead (green/red) images of unsorted and sorted sperm using 3 different MSS chips.



**Figure 3.**

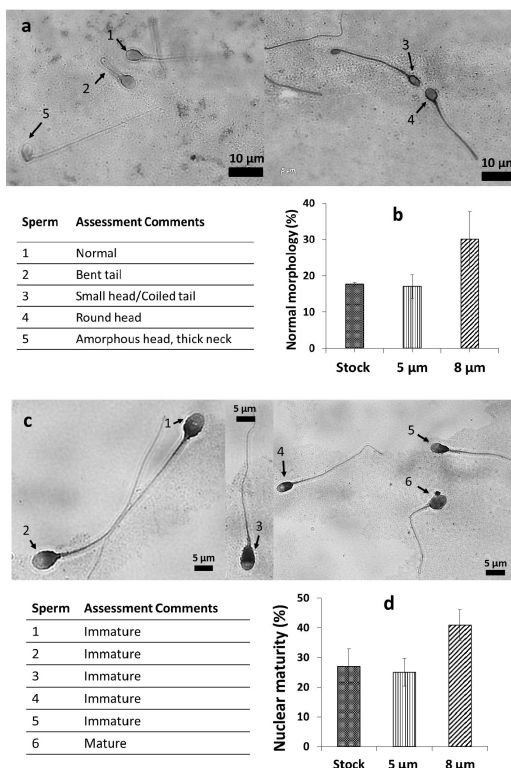
Diluted sperm sorting and motility analysis using MSS. (a) Semen sample was diluted with HTF+1% BSA buffer at 1:4 ratio. %Motility of diluted human sperm isolated using different pore diameter filters 3  $\mu\text{m}$ , 5  $\mu\text{m}$  and 8  $\mu\text{m}$ . Sperm were retrieved at three time points, i.e 15, 30, and 45 min. Motility of the sperm sorting by using filters was significantly higher than diluted stock motility (*\*p-value* < 0.05 between stock semen and sorted sperm, N=3). (b) Retrieval efficiency of sorted sperm using 3 different MSS chips (*\*p-value* < 0.05 between time points, N=3). Simulation results for (c) 5  $\mu\text{m}$  chip and (d) 8  $\mu\text{m}$  chip. Error bars represent standard error of the mean.



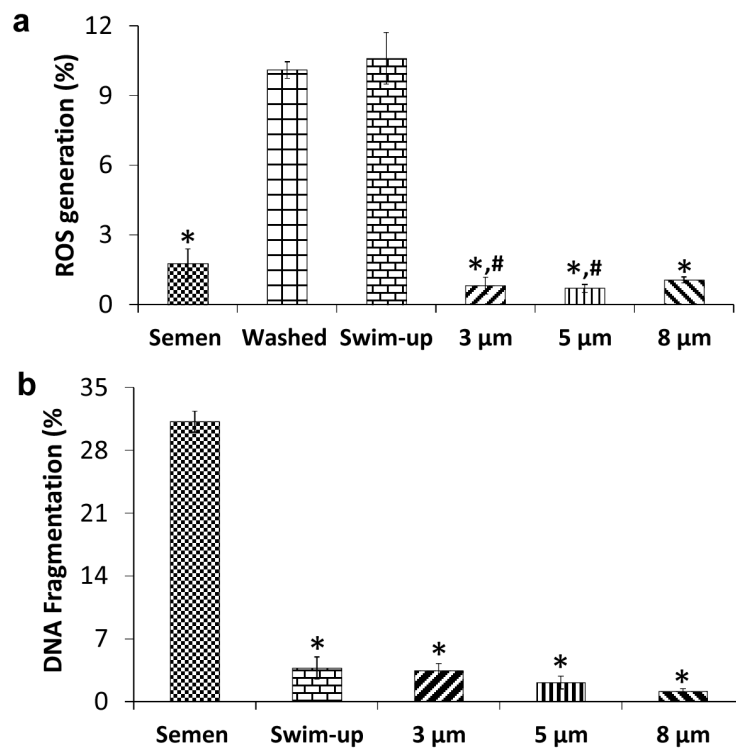
**Figure 4.**

Sperm velocity analysis. (a) Curvilinear velocity (VCL), (b) Straight line Velocity (VSL), and (c) Average path velocity (VAP) of stock and sorted sperm using 3, 5, and 8 μm MSS chips. The sperm analyzed for velocity parameters were collected from retrieval chamber of the chips after 30 min time point. The sperm sorted using 5 and 8 μm chips showed significantly improved velocity parameters compared to unprocessed stock semen (\**p-value* < 0.05 between stock semen and sorted sperm, N=10-20). Error bars represent standard error of the mean.





**Figure 5.** Morphological and nuclear maturity analysis of sperm. (a) Representative brightfield images of sperm using optical microscope and an oil immersed 100x objective. Total magnification was 1000x. The table summarizes the criteria and typical comments used to assess sperm morphology. Five sperm are shown in these images whereas only 1 is morphologically normal. (b) Plots shows normal morphology (%) for stock and sorted sperm. Sperm sorted using 8 μm MSS showed more percentage of normal sperm compared to stock and 5 μm MSS (N=3). (c) Representative grayscale images of sperm stained with aniline blue and a table to show condition of chromatin. (d) Mature sperm percentage calculated for sperm from stock, 5μm and 8μm MSS (N=3). Error bars represent standard error of the mean.



**Figure 6.** Analysis of reactive oxygen species (ROS) generation and DNA fragmentation in sorted sperm using flowcytometer. (a) Sperm sorted using 3, 5, and 8 μm chips showed significantly lesser ROS generation compared to swim-up method (\**p-value* < 0.05 between swim-up and other sperm samples, N=4). Sperm sorted using 3 and 5 μm chips showed significantly lesser ROS generation compared to washing method (#*p-value* < 0.05 between raw semen sample and others N=4). (b) Sperm sorted using 3, 5, 8 μm chips showed significantly lesser DNA fragmentation compared to unsorted semen sample (\**p-value* < 0.05 between swim-up and other sperm samples, N=6). Sperm sorted using 8 μm chips showed lesser DNA fragmentation compared to swim-up (*p-value* < 0.06). Error bars represent standard error of the mean.