# A Biomimetic Microfluidic Selection Platform Providing Improved Sperm Quality Metrics Compared to Swim-Up

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# Abstract

Sperm Selection is an essential component of all Assisted Reproductive Treatments (ART), and is by far and large the most neglected step in the ART workflow when it comes to technological innovation. Conventional sperm selection methodologies typically produce a higher total number of sperm with variable motilities, morphologies and levels of DNA integrity; Gold-standard techniques Density Gradient Centrifugation (DGC) and Swim Up (SU) have been proven to induce DNA fragmentation through the introduction of reactive oxygen species (ROS) during centrifugation. Here, we demonstrate a 3D printed, biologically inspired microfluidic sperm selection device (MSSP) that utilizes multiple methods to simulate a sperms journey towards selection. Sperm are first selected based on their motility and boundary following behavior, then on their expression of apoptotic markers, yielding over 68% more motile sperm than previously reported methods within a lower incidence of DNA fragmentation and apoptosis. Sperm from the MSSP also demonstrated higher motile sperm recovery after cryopreservation than SU or neat semen. Experiments were conducted side-by-side against conventional SU methods using human semen (n = 33) and showed over an 85% improvement in DNA integrity with an average 90% reduction in sperm apoptosis. These results demonstrate an easy-to-use platform for sperm selection mimicking the biological function of the female reproductive tract during conception.


# Capsule

Microfluidic sperm selection is capable of efficiently and consistently preparing sperm, resulting in significantly lower DNA fragmentation, lower apoptosis, better cryosurvival, and higher-grade motility compared with the SU method.

# 1 Introduction

Infertility is a growing global health issue with significant psychological, social, and economic implications, affecting over 185 million individuals worldwide [1]. In Australia, 1 out of each 6 couples experience infertility issues, with 1 in every 22 children now born via assisted reproduction. Male infertility solely contributes to ~ 30% of infertility cases globally [2], and to 40% of infertility cases in Australia [2,3]. Recent trends towards postponing first pregnancy demonstrate the limits of natural fertility and have accelerated the need for medical intervention and innovation in ART to overcome these limits. Therefore, to treat the increasing demand for infertility treatment effectively it is vital that the methods and technologies used in ART continue to improve, particularly where male factor infertility is concerned. Ensuring quality sperm selection is crucial to the success of assisted reproduction, since it influences many of the factors contributing to the success of assisted reproductive treatments (ARTs)[3–5]. Poor sperm quality correlates to an increased risk of birth defects, lower embryo fertilization rates, lower embryo quality, lower implantation rates, and has a negative association with live birth rates after In Vitro Fertilization (IVF)[6–9].

The quality of selected sperm is also heavily reliant upon the skill of the embryologist and unstandardized preparation protocols, often resulting in operator-to-operator variations in IVF success [4]. This is most prominent in the sperm processing stage which consists of gradient centrifugations, cell resuspensions and delicate aliquoting[10]. The most common clinical methods of sperm selection are DGC and SU. These conventional techniques circumvent natural sperm filters, neglecting important factors such as DNA integrity and sperm apoptosis. In fact, centrifugation-based selection approaches may induce sperm DNA fragmentation (sDF) in certain samples or will fail to reduce it [11–14]. Sperm cells presenting significant damage to their genetic material, in the form of DNA fragmentation, have been shown to increase the risk of miscarriage up to 3.94 times [15,16]. While motility-based sperm selection is inspired by natural sperm progression through the female reproductive tract (cervical crypts, uterine cavity, intratubal junction, fallopian tube etc.), there are a multitude of selective mechanisms that impact the migration of sperm before fertilization[17]. Natural sperm selection facilitates a reduction in sperm from 300 million upon ejaculation down to roughly 250[18], through a variety of mechanisms. Therefore, it stands to reason that using a combination of mechanisms for sperm selection may provide more fecund sperm, particularly for ICSI. One such mechanism is the tagging and removal of apoptotic sperm using Annexin V. The proportion of early apoptotic sperm cells in raw semen has been shown to reach over 20%[19], and it has been reported that eliminating apoptotic sperm using annexins correlated to an improvement in embryo quality[20]. Annexin A5 (AAV) is an example of one such apoptotic marker that binds to phosphatidylserine externalized in apoptotic cells[20]. This action reduces the impact of cells undergoing both spontaneous and testicular induced cell death via apoptosis during conception. Apoptosis is an essential mechanism occurring in both fertile and (to a larger extent) infertile men to eliminate unwanted cells, due to triggering stimuli (deprivation of intratesticular testosterone and gonadotrophins, Sertoli cell toxicants, chemotherapeutic drugs, and temperature imbalances)[21]. Apoptosis is important in the context of sperm because of the errors arising in the cells during their production and the resulting need for cell death to eliminate cells with genetic defects. The presence of apoptotic sperm in semen has been shown to be more prevalent in infertile men[22], especially those with unidentified male infertility[23]. Apoptotic sperm also present more frequently with DNA fragmentation which is a key performance indicator for sperm quality and future embryo quality[24]. Although technologies such as magnetic activated cell sorting (MACS) are available for the removal of apoptotic sperm cells through AAV binding, they only exist as an adjunct to conventional DGC or SU techniques. MACS on its own has been shown to provide little benefit to the overall quality of sorted sperm populations but has proven effective when used post DGC or SU[25,26]. However, this dramatically compounds the amount of time, equipment and human intervention required for sperm preparation, making it less appealing to clinics. Therefore, combining the use of Annexins with motile sperm selection in a single platform could further improve sperm quality without reducing clinical efficiency.

Here we report a hybrid microfluidic sperm selection platform (dubbed the hybrid MSSP) that can select sperm with considerably improved quality in 15 minutes; a quarter of the time required for SU, significantly reducing the time on task for embryologists and the amount of time sperm are required to

spend *in vitro*. The device itself allows for the selection of sperm via motility-based boundary following alone or in combination with apoptotic sperm trapping. 3D printing was used to maximize the yield of healthy sperm recovered from raw human semen by creating a pattern of layered ridges for sperm to follow, resulting in an average 68.4% concentration yield increase over previously reported straight channels[27]. Sperm populations isolated from the device demonstrate considerable improvements in motility (93.5% vs 74.1%), vitality (97.6% vs 86.0%), DNA integrity (1.4% vs 7.9%), cryosurvival (64.2% vs 52.8%), and apoptotic marker expression (5.66% vs 26.5%) than a conventional SU-based approach. Furthermore, our microfluidic platform achieves these results with a higher degree of consistency and with fewer steps, representing a clinically viable approach to sperm selection that may benefit the downstream process and overall success of ART.

## 2 Materials And Methods

# 2.1 Device Fabrication

Devices were fabricated using modified additive manufacturing techniques previously reported by our group for inertial microfluidic devices[28,29]. 3D printing was performed using a high-resolution Digital Light Processing (DLP) 3D printer (MiiCraft, Hsinchu Taiwan). The desired geometry was drawn in SolidWorks 2018 x64 Premium Edition and then exported as an STL file to Miicraft software (MiiCraft 125, Version 4.01, MiiCraft Inc), for pre-processing of the printing options. The printer projects a 405 nm UV wavelength through the resin (BV-007, MiiCraft Inc) to solidify the liquid photopolymer in a solid layered structure. Details of the resin structure have been previously reported and their effects on sperm cell vitality investigated [30,31]. The microfluidic device used a circular array of 184 microchannels, each with a height of 600 µm and a length of 7.5 mm. Each pair of channels congregates into a single channel as after 3mm and included a series of ridges running along the entire length of the channel walls to increase the number of surfaces and boundaries able to bear sperm while also allowing for an overall larger entry to each channel (supplementary Fig. 2). At the end of the channels as they converge on the center of the chip, a circular pattern of crescent moon shaped pillars was situated to help retain sperm in the center of the chip and prevent them from exiting the collection area. After printing, the top half of the chip was thoroughly washed with Isopropanol Alcohol (IPA) and DI water (three times). Between each wash, the part was blow-dried with a pressurized air gun, making sure all residual liquid resin was removed while not damaging the structures. The part was then cured under ultraviolet (UV) light for 120 seconds. Once the chip is ready, it was then attached to a Poly-methyl methacrylate (PMMA) sheet using a transparent double-sided pressure-sensitive adhesive tape (ARcare, Adhesive Research) coated with AS-110 acrylic medical grade adhesive. This approach effectively binds open 3D-printed microchannels with optically transparent acrylic sheets, producing a tightly sealed microchannel that allows on-chip microscopy.

# 2.2 Semen preparation

Human semen samples were obtained through ejaculation after 2–7 days of sexual abstinence, as recommended by the World Health Organization[10]. Raw semen samples (n = 33) were incubated at 37°C

for 20 minutes to allow for full liquefaction. All donors signed an informed consent. This study was approved by the ethics review board at UTS (ETH19-3677).

# 2.3 Device operation

Motility-based sperm selection.

The motility based MSSP was pre-filled with Sperm Rinse buffer from the center by injecting 1.5 mL through the central outlet using a 3 mL BD plastic syringe. A strip of the AS-110 acrylic medical grade adhesive tape was then used to seal the central outlet. 0.85 mL of liquified semen was then injected into the device using a 1 mL BD plastic syringe and the chip left undisturbed at 36°C (on a hotplate) for 5, 10, 15, and 20 minutes for concentration, motility, and vitality validation. Later, 15 minutes was used for DNA fragmentation, apoptotic and cryopreservation experiments as the optimal time for selection. After incubation, the tape was removed from the outlet port and 150 µL collected from the central outlet. In addition to the side-by-side comparisons between SU and microfluidic methods, an additional set of 5 devices was run for 15 minutes at room temperature (RT) to assess the impact of incubation temperature on the velocity profiles of sperm collected.

Combined Motility and Apoptotic Sperm Selection

The second iteration of the MSSP contained the same geometry of channels with an additional reservoir for holding superparamagnetic microbeads situated at the end of the microchannels between the end of the channels and the beginning of sperm retaining pillars (Supplementary Fig. 2). The microfluidic device was prepared by first injecting 1 mL of 180 µm iron paramagnetic microbeads (Thermo Fischer Scientific) coated with dextran and suspended in Annexin Binding Buffer (Thermo Fischer Scientific). This solution was injected using a 1 mL syringe tip through a dedicated inlet located next to the center outlet. This inlet hole was then sealed with tape. The device was then pre-filled with Annexin Binding Buffer in the same manner described above. The center outlet was covered with a strip of tape. 0.455 mL of the liquified semen was mixed with 0.425 mL of MACS ART Annexin V reagent (Miltenyi Biotec), incubated at room temperature for 15 minutes, and injected into the device from the semen inlet located at the outer ring of the device. The device was then placed between two opposing neodymium magnetic plates (AMF Magnetics) positioned above and below the device to create a magnetic field and left for 15 minutes. Once the incubation was over the tape was removed and 150 µL of the selected sperm immediately collected, while keeping the device between the magnets.

# 2.4 SU Method

The SU method used was appropriated from previous studies showing lower DNA fragmentation in SU than DGC[12,32]. 1 mL of the semen sample after liquefaction was gently layered with 1.5 mL pre-equilibrates Sperm Rinse media (Vitrolife) and placed in an incubator, at 37°C and 5% CO2. The tube was incubated for 45 min at inclined at an angled position, which allowed the motile sperms to swim into the overlaid medium. After incubation, 0.9 ml of upper layer was taken and transferred to a clean tube where 3 mL of Sperm Rinse media was added and mixed. Then the samples were washed by centrifugation at

500 g for 7 minutes, the supernatant discarded, and the pellet resuspended in 100 µL of G-IVF Plus media.

## 2.5 Sperm Cryopreservation

Sperm cryopreservation was performed by first aliquoting freezing medium (Vitrolife) and leaving to equilibrate to room temperature. Sperm to be frozen were then separated into different test tubes, one for each group (Raw, SU, and MSSP). Sperm samples were diluted with freezing medium 1:1 (v/v) by adding the freezing media to the sperm dropwise with a 1mL. Following this the mixture was left to equilibrate for 3 minutes and left at room temperature for 10 minutes. Mixtures were then transferred into 1 ml cryotubes suspended horizontally for 30 minutes, 5cm above the surface of the liquid nitrogen (LN2). Finally, the cryo-tubes were plunged into the LN2 (-196ºC) with the cryotube upside down. Samples were cryopreserved for 7 days before thawing for reassessment. To thaw the sperm, the cryotube was kept in a 37°C water bath for 1 minute. 7 second videos were then recorded for motility analysis of the recovered sperm.

## 2.6 Sperm DNA Analysis

Assessment of DNA fragmentation index (DFI) was performed by a modified sperm chromatin dispersion (SCD) test, using the HT-HSG2 kit (Halotech DNA Pty Ltd) as previously reported[31]. The DFI of sperm was obtained before and after each sperm selection method. To perform the SCD assay, 90 µL of sperm suspension was added to an Eppendorf tube and mixed with pre-warmed agarose. 10 µL of the semen-agarose mixture was pipetted onto super-coated slides and covered with a coverslip. The slides were placed on a cold plate at 4°C for 5 minutes to allow the agarose set. The coverslips were gently slid off the slides, and the slides immediately immersed horizontally in an acid solution (from the kit) and incubated for 7 minutes. The slides were then gently tilted vertically to allow the acid solution to run off the slides. The slides were horizontally immersed in 10 mL of the lysing solution for 20 minutes, then washed with distilled water for 5 minutes. The slides were then dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) for 2 minutes each, air-dried, and stored at room temperature in the dark. To add colour to the cells, slides were horizontally covered with a mixture of Wright's staining solution (Merck) and phosphate-buffered solution (1:1, Merck) for 5 minutes, and then were briefly washed in DI water. Sperm were counted under brightfield microscopy using an Olympus Ix73 inverted microscope with an Olympus DP80 camera at 20X magnification. A minimum of 300 spermatozoa per sample were scored. SCD analysis was performed by counting the number of sperm with and without visible halos as per the test manufacturer’s instructions. Sperm cells without a halo or with a weakly stained, small, or degraded halo were considered to have fragmented DNA, while sperm cells with medium to large halos were considered to have intact DNA. DFI is expressed as the percentage of sperm cells with fragmented DNA.

## 2.7 Sperm Concentration, Vitality, and Motility Analysis

Sperm concentration and progressive motility were assessed manually after collection for each selection method according to the World Health Organization manual (fifth edition). The assessment of sperm

vitality and additional motility characteristics were performed using previously reported methods [27,31]. Briefly, vitality was assessed using the fluorescent-based LIVE/DEAD sperm viability kit (ThermoFisher Scientific), by staining live and dead sperm according to the supplier manual. A hemocytometer was used for counting and observed through an Olympus IX73 inverted microscope equipped with an Olympus DP80 camera for fluorescent imaging. Sperm motility parameters including curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), and amplitude of lateral head displacement (ALH), were evaluated with OpenCASA plugin in ImageJ (version 1.80) using 5 second videos of sperm swimming in their collected medias before and after each selection method (each with a frame rate of 30 frames per second) [33]. CASA settings used were set to those from previously reported studies using the same CASA system[34].

## 2.8 Annexin V/PI binding assay and Flow Cytometry Analysis

The technique used for the Annexin V assay was appropriated from a previously reported method[19]. Spermatozoa were incubated in Annexin Binding Buffer (Biolegend) that contained FITC-labeled AAVV (0.1 mg/mL [w/v]) and PI (1 µg/mL [w/v]) (SigmaAldrich,). A negative control sample was suspended in HEPES A buffer only. After exactly 15 minutes at -20°C, the spermatozoa were analyzed in a CytoFLEX LX flow cytometer system and CytExpert software. A minimum of 8,000 spermatozoa were examined for each test. The sperm population was gated by using forward-angle light scatter; side-angle light scatter was used to exclude electronic noise and debris. All tests were run in triplicate.

## 2.9 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). The statistical significance of the differences between the values was assessed using Friedmans test for non-parametric data. $P < 0.05$ was considered statistically significant.

# 3 Results

### Device Geometry and Operation

Figure 1 illustrates the workflow of our device whereby sperm can migrate from a semen reservoir into microchannels preloaded with sperm buffer. Each microchannel contains layers of grooves and ridges to facilitate double the number of avenues for sperm to follow corners when compared to conventional straight channels. The channels themselves also provide a larger entry space compared to previous iterations of the device so more sperm find their way into the channels for selection. The geometry of the channels was optimized first by device stability and then by motile sperm yield. The width of the grooves and the height of each channel was incrementally increased to a point where the device consistently yielded over 60% more sperm than the original geometry without allowing semen to penetrate the channels during the semen injection step. Furthermore, we also present a second iteration of this microfluidic platform where sperm, having been pre-mixed with AAV coated magnetic nanoparticles, are

sorted based on motility before being introduced to a magnetic field amplified by paramagnetic microparticles, effectively trapping phosphatidylserine positive sperm. Lastly, sperm are collected from the center outlet.

### Sperm DFI and Conventional Quality Metrics

Sperm DFI, concentration, vitality, and motility values were compared before and after SU and microfluidic sperm selection methods. Samples greater than 2.1 mL were split, without dilution, between SU and microfluidic selection methods, while samples between 1.1 and 2.1 mL were split to give 0.1 mL of neat semen for raw sample analysis, and the remaining volume split 50/50 by volume and diluted up to 1 mL before selection. Samples less than 1.1 mL were not included in the study. Among the 33 volunteers who participated, 25 had semen volumes equal to or greater than 2.1 mL, with 5 of the donated classified as infertile according to WHO 5th Edition criteria. The average DFI value of all raw samples was 12.19% (± 5.59) and ranged from 3.0–25.12% (Fig. 2). The results show that DFI values of semen samples prepared through the microfluidic method (1.44% ± 1.4) were significantly lower than those prepared using the SU method (7.92% ± 4.72, P = 0.0176). This represents an average DFI reduction of 88.2% and 35.0% for MSSP and SU selected sperm, respectively.

For each experiment, 150 µL of isolated sperm were recovered from the microfluidic device. The microfluidic method represents a significant time improvement from conventional SU-based techniques. Sperm concentrations from the device increased with incubation time and ranged from an average of 0.61 million sperm/mL after 5 minutes of semen incubation to 1.54, 5.46, and 7.4 million sperm/mL after 10, 15, and 20 minutes, respectively. For 15 minutes of microfluidic device incubation, the average number of sperm collected from the device was 825,000 sperm, sufficient for droplet-based IVF and more than enough to select an individual sperm for intracytoplasmic sperm injection (ICSI). In addition, the vitality of sperm from the devices was assessed to confirm no adverse effects on the viability of cells was present from the materials being used in the device. Sperm vitality remained above 97% for all incubation times and was consistently greater than the raw semen (48.1% ±15.6) and SU (86% ±11.6) method.

Sperm motility was also assessed for each method and compared to the raw semen. This was done across four different incubation times for the device (5, 10, 15, and 20 minutes). The average progressive motility from the microfluidic device (93.5% at 15 minutes) saw a statistically significant increase when compared to raw semen (37%, P = 0.001) and SU method (74.1%, P = 0.0181) (Fig. 3A). Considering the high yield, vitality, and motility of sperm from the microfluidic device at 15 minutes, 15 minutes was chosen as the ideal time for DFI-based experiments, cryopreservation experiments, and apoptotic selection-based experiments. The OpenCASA plugin in ImageJ was used to quantify sperm motility parameters. The VSL, VCL, and VAP for sperm separated via microfluidics at 36ºC (44.2 ± 10.4, 73.3 ± 6.7, and 54.2 ± 15.2 µm/s) each showed a considerable average increase from the raw semen (28.17 ± 3.78, 59.47 ± 3.68, and 41.48 ± 4.76 µm/s) and from the SU method (30.2 ± 2.5, 73.8 ± 7.1, and 46.2 ± 3.6 µm/s) (Fig. 3B). Both room temperature (22°C) and body temperature were compared for microfluidic sperm

selection (at 15 min incubation) to compare the differences in sperm motility behavior upon collection, which may affect their level of hyperactivity, capacitation, and suitability for conventional IVF. The room temperature microfluidic sperm separation showed a similar increase in VSL (as the 36°C incubated device) but was comparable to the SU method in terms of VCL and had a higher range of values in VAP. Similarly, the LIN and WOB showed average increases with microfluidic sperm selection when compared to the SU method and the neat semen (Fig. 3C). However, ALH and BCF were largely unchanged between all groups. Interestingly, the SU method demonstrated a loss in LIN, WOB, and STR from the neat semen while the RT microfluidic separation gave the largest increases exceeding that of body temperature incubated microfluidic separation.

### Motile Sperm Recovery Following Sperm Cryopreservation

To investigate the effect of sorting on motile sperm recovery following sperm cryopreservation and the influence of different separation methods on sperm recovery, sperm cryopreservation was performed on raw semen and both SU and MSSP sorted sperm. Sperm selections were performed side-by-side and subsequently cryopreserved for 7 days before thawing alongside an aliquot of raw semen. As shown in Fig. 4A, sperm processed through microfluidics (on average) showed a significant 75% improvement in motile sperm recovery when compared to the raw conventionally from sperm (36.7–64.2%). SU processed sperm showed a non-significant average improvement of 43.9% (36.7–52.8%). The recovery of live sperm post-thawing on the other hand, showed no significant difference between each group with a higher averaged value for MSSP (Fig. 4B). Sperm frozen from a raw semen dilution had an average recovery of 66.5% while the SU and MSSP processed sperm had an average recovery of 49.4% and 67.0%, respectively. OpenCASA revealed differences in the post-thaw velocity parameter of sperm from different groups (Supplementary Fig. 3A and 3B). SU processed sperm showed the largest and most consistent decrease in velocity across VSL, VCL, and VAP, while unprocessed and MSSP sperm showed very minor changes in velocity in all except VAP where raw sperm showed an increase.

### Characterization of Apoptotic Sperm and Negative Selection of Apoptotic Sperm from the Hybrid MSSP (H-MSSP).

To investigate the incidence rate of sperm apoptosis caused by each selection method and to reduce the number of apoptotic sperm at collection, sperm apoptosis was measured for raw, SU, motility only and hybrid MSSPs. For the removal of apoptotic sperm from collected sperm samples a magnetic sperm selection approach was used to trap pre-labelled apoptotic sperm using a magnetic field amplified by super-paramagnetic microbeads (Fig. 5A). Raw, SU, MSSP only, and IVFM with magnetic separation were compared between 5 samples of larger volume (> 2.5 mL). The percentage of apoptosis was assessed via flow cytometry using PI and AAV-FITC double staining. The percentage of apoptotic sperm (Fig. 5B) showed a large average increase in SU sorted sperm when compared to the raw sperm (8.5–26.5%). While motile sperm selection via the MSSP only group showed no average reduction in apoptosis, the hybrid MSSP showed a near 50% reduction in apoptotic sperm versus the MSSP only sperm. Necrotic or late apoptotic sperm were reduced in all sperm selection methods (Table 1). Figure 5C shows example

distribution of the relative decrease in alive (AAV-/PI-), dead (AAV-/PI+), necrotic (AAV+/PI+) and apoptotic (AAV+/PI-) sperm populations between each method from the raw sample (with between 1% and 25% total apoptotic sperm).

Table 1
Sperm assessments before and after SU and Microfluidic Sperm Selection at 15 minutes

| Sperm Metric | Raw Semen | Swim Up | MSSP | Hybrid MSSP |
|---|---|---|---|---|
| Concentration (X10^6) | 87.7 (± 39.2) | 29.4 (6.6) | 5.5 (0.15) | 7.83 (0.23) |
| Total Motility (%) | 45.4 (± 14.3) | 82.2 (± 10.2) | 95.5 (± 3.1) | 97.6 (± 2.5) |
| Progressive Motility (X10^6) | 42.2 (± 15.0) | 74.1 (± 9.6) | 93.5 (± 4.5) | 97.0 (± 2.9) |
| Sperm Vitality (%) | 48.1 (± 15.6) | 86.0 (± 11.6) | 97 (± 2.1) | 98.5 (± 1.1) |
| DNA fragmentation (%) | 12.2 (± 5.6) | 7.9 (± 4.7) | 1.4 (± 1.4) | NA |
| Apoptotic Sperm (%) | 10.8 (± 9.9) | 26.5 (± 2.7) | 10.8 (± 2.8) | 5.66 (± 3.0) |
| Necrotic/Late Apoptotic Sperm | 23.6 (± 14.2) | 13.31(± 1.5) | 9.94 (± 3.5) | 5.85 (± 3.2) |
| Cryosurvival (% motile) | 36.7 (± 6.1) | 52.8 (± 14.3) | 64.2 (± 10.5) | NA |
| Cryosurvival (Vitality %) | 66.5 (± 9.0) | 49.4 (± 7.8) | 67.0 (± 8.4) | NA |

# 4 Discussion

# 4.1 Sperm Quality Metrics Before and After Selection

We have developed a 3D printed microfluidic sperm selection platform (dubbed the MSSP) for simple selection of high-quality sperm with significantly improved DNA integrity, motility, vitality, and cryo-survivability compared to a conventional SU-based approach. Comparative studies to conventional gold-standard methods are key to the translation of new techniques and until now remained uninvestigated in previous iterations of the MSSP [27,35]. SU was chosen as for this study because it is the most comparable to microfluidic motility-based selection and because studies report a more effective reduction in sperm DNA fragmentation compared to DGC [32,36]. Microfluidics has been increasingly involved in ART studies and several devices have been explored to sort motile or morphologically intact sperm from unprocessed semen[31,37–42]. However, the clinical translation and adoption of these technologies have been extremely limited, largely due to their complexity in operation, instability, and/or inconsistency, often a result of mechanisms such as gravity pumps, laminar flow, or charge-based selection. Without an intuitive user interface, many devices have not seen further side-by-side testing to evaluate their performance. A successful sperm selection platform must not only provide high quality sperm in a timely manner, but it

must also be simple to use and consistent in its performance. The platform we report here is a passive, easy-to-use, platform that selects sperm based on their preference to follow guidelines and turn corners, while simultaneously resuspending them in sperm nutrient buffer, without the need for centrifugation.

This newer geometry builds upon previous studies by using 3D printing to increase the number of boundaries (up to 3-fold) and size of channel openings for sperm to be guided out of seminal plasma and into fresh media for collection. This resulted in a 68.4% improvement in total sperm count (490,000 to 825,000) from the device for the same incubation time (15 minutes) when compared to Nosrati *et al* previously reported geometry. It is also worth noting that this was achieved using a lower average starting concentration from raw semen (87.7 million/mL compared to 120 million/mL), although testing with oligozoospermic samples is still required. 3D printing enabled the rapid iteration of complex geometries to maintain the stability of the device during sample loading and improve sperm yield. However, despite the fact that the 3D printed devices showed no adverse effects on sperm vitality, DNA fragmentation, or apoptosis, 3D printing is not a scalable manufacturing method. For the commercial application of this device, a layered microinjection molding approach in either COC or PMMA (both currently FDA, CE, and TGA certified materials for sperm selection) would be required. Although the MSSP does not reach the number of sperm isolated from the SU method it makes a significant improvement on previously reported results[27,35], bringing sperm concentrations to a clinically useful level. A strong correlation between high DNA integrity and the tendency for sperm to follow boundaries has been previously reported by Nosrati *et al* [35,43]. In these studies, boundary following demonstrated a near 80% improvement in DNA integrity compared to starting value in raw samples only[27]. However, to date, a side-by-side study comparing microfluidic sperm selection against the conventional motility-based selection method, SU, has not been performed. Furthermore, the effects of cryopreservation on selected sperm have not been investigated. Previous studies have claimed that both SU or DGC are not suitable preparation methods for high DFI samples, as they are often unable to reduce the DFI to within an acceptable range (< 15%) or in some cases may increase the incidence of DNA damage through ROS generation and iatrogenic damage [12–14]. In this study, microfluidic sperm sorting consistently made a significant reduction in sperm DFI in all samples and in several cases reduced the DFI by 100%. The average DFI reduction from MSSP sorted samples was 88.2% and 35.0% for SU-selected sperm which is consistent with previously reported studies on conventional SU-based approaches[32,37,44]. While SU did not increase the DFI in any samples it did have a wide variance of improvements which contrasts with the narrower range of DFI results from the MSSP, regardless of the DFI starting value. The highest raw DFI tested was 25.12%, which the MSSP reduced to less than 2%. While further testing with DFI values greater than 30% is still required, the low standard deviation of DFI values from MSSP sorted sperm indicates that (unlike SU) the initial level of DNA fragmentation is irrelevant. DFI values play an important role during blastocyst development and has been shown to play a prominent role in embryo implantation and miscarriage rates[6,45,46]. To reduce the risk of miscarriage after ICSI or IVF, a reliable and simple method to select sperm is needed. This study shows that the MSSP is effective for selecting sperm with little to no DNA damage in semen samples with motile sperm populations.

With many microfluidic methods, the yield of sperm is often only suitable for ICSI, with many devices collecting between 3,000 and 400,000 sperm per selection[39,47]. The microfluidic device in this study made use of a three-dimensional geometry to encourage sperm boundary following behavior and increase the number of sperm in the semen able to interface with the microchannels, without compromising the stability of the chip itself. The fabrication method used in this study relied upon 3D printing to create cantilever ridges, mimicking the folded lumens that form microgrooves within female reproductive tract. This allowed for total sperm counts of approximately 825,000 sperm when the device was incubated for 15 minutes, which is tunable to the quality of sample being used. Lower quality semen samples with lower motile sperm concentrations may require longer incubation times to achieve similar sperm yields or technicians may opt for shorter times in the case of ICSI patients where only a small population of high DNA integrity sperm are required. Considering the standard ratio for IVF in humans is 50,000 or more motile sperm per oocyte[48], the average yield of sperm from the MSSP represents a clinically useful platform towards both IVF and ICSI. However, it is worth noting that the lower limits of the sperm oocyte ratio for achieving fertilization heavily depend on sperm quality. Therefore, considering the high level of DFI reduction from microfluidic sperm selection, further investigation into the appropriate sperm oocyte ratio would be prudent. Furthermore, this study recovered a limited number of infertile donors' semen samples and a study dedicated to the processing of low motile sperm population is required.

To ensure there were no adverse toxic effects on the sperm vitality, staining was performed and showed no detrimental effects on sperm vitality after selection. However, SU yielded an average of 14% PI-stained sperm with a high variability compared to 3% from the MSSP, which, when compared with DFI and motility indicates a less standardized process prone to human error. SU inherently involves more manual interventions than microfluidic-based sperm selection and is provided a threefold improvement in sperm incubation time (45 minuets down to 15 minutes).

Sperm motility is an important metric for assessing the quality of sperm and their ability to fertilize an oocyte, particularly in IVF. Motility after SU typically lies within the range of 65–85% which is consistent with the average motility for SU prepared sperm in this study[32,37,49]. Although SU provided a general improvement in progressive sperm motility, it was significantly outperformed by the microfluidic sperm selection regardless of incubation time for the device. Furthermore, the grade of sperm motility may vary in terms of velocity profile (VCL, VSL, and VAP) and other motility characteristics (LIN, WOB, ALH, and BCF). All velocity parameters showed greater improvement in microfluidics selected sperm compared to SU sperm demonstrating a much higher proportion of grade A sperm, particularly when the device was incubated at 36ºC during selection. This is due to the boundary following behavior mechanism leveraged within the device which promotes the migration of highly motile sperm. Sperm selected at RT for the MSSP showed similar improvement in VSL but less so in VCL and VAP, which combined with a large level of LIN. However, none of the variations are indicative of sperm hyperactivation or capacitation where sperm swim at speeds two to three-fold faster and experience an increase in ALH and head oscillations.

# 4.2 Sperm Cryopreservation

Another important component of ART cycles is the use of cryopreserved semen and their post-thaw characteristics, particularly motility. Despite the well documented effects of sperm cryoinjury resulting from sperm cryopreservation, the cryopreservation of human sperm is becoming more commonplace, particularly for those undergoing treatment for cancers or for sperm donation due to the rising number of same sex and single parent cycles. During cryopreservation and thawing, sperm are subject to osmotic and oxidative stress as well as the formation of ice crystals, resulting in losses in motility and vitality typically between 40% and 50%[50,51]. Limited studies have reported the benefits of performing sperm sorting before cryopreservation[52,53]. However, here the cryopreservation and subsequent thawing of sperm from the MSSP (both incubated on a hotplate and at room temperature) showed a marked improvement in recovered motility. Although the post thaw recovery of living sperm from the MSSP was similar to sperm from diluted raw semen samples, a significantly higher percentage of motile sperm were recovered ($P = 0.0319$). SU processed sperm showed an average reduction in sperm vitality but a non-significant increase in average motile sperm recovery. The velocity parameters of MSSP processed sperm showed a greater variance in VSL and VCL compared to pre-freezing but retained their improvement in velocity when compared to SU which decreased post-thaw. This would indicate that sperm populations from microfluidic selection, possessing a higher percentage of grade A sperm, are more resistant to cryoinjury resulting in a reduction in overall motility.

# 4.3 Hybrid Sperm Selection

Recent reviews and studies have recognized the importance of moving beyond solely motility-based sperm selection methods towards combinational approach that use either negative or positive sperm selection based on the expression of certain biomarkers[17,54,55]. Conventional methods of selection (SU and DCG) omit the selection of sperm expressing any kind of biological moieties indicating abnormal sperm function or a lack of fecundity that may impact downstream processes in fertility treatment such as embryo quality or live-birth rate. One such marker is the externalized membrane protein phosphatidylserine, which is an indicator or early-stage apoptosis in sperm. It has already been proven that the proportion of early apoptotic sperm cells in raw semen can reach over 20%[19], and that eliminating apoptotic sperm using annexins correlates to an improvement in embryo quality[20] and a further decrease in sperm DNA fragmentation which is directly correlated to apoptosis[56]. High levels of early-stage sperm apoptosis have also been suggested to play a role in recurrent pregnancy loss[23]. It has also been demonstrated that the use of apoptotic sperm removal plays a significant role in improving fertilization rate and rates of clinical pregnancies when combined with the use of good quality donor oocytes[20]. The value of removing apoptotic sperm resides in the fact that they may still be able to fertilize an oocyte, particularly during an ICSI treatment, as they often present without morphological abnormalities. However, neither DGC nor SU methods can effectively remove apoptotic sperm populations and the only clinically viable method to achieve this has been the application of magnetic-activated cell sorting (MACS) [44]. However, the MACS process on its own compounds the time and cost of clinical sperm selection, bringing the total process to well over 2 hours per sample, which is not practical in a clinical setting.

To remedy this issue and provide a biologically inspired approach to sperm selection we have designed a microfluidic platform for dual-action sperm selection that allows the optional inclusion of apoptotic sperm removal for when it is clinically relevant. Our device first leverages sperm motility and boundary following behavior which is naturally observed in the cervix and uterotubal junction, then proceeds to trap sperm expressing apoptotic markers, which is thought to naturally occur in the male reproductive tract during spermatogenesis[17]. While the motility only MSSP did not increase the incidence rate of apoptosis in sperm post-selection, SU sorted sperm showed an average 3-fold increase in sperm apoptosis. This indicates that the SU method induces apoptosis in sperm. In comparison, the hybrid MSSP which retains apoptotic sperm in the device showed a marked reduction in AAV positive sperm. It also showed a similar reduction in what could be considered necrotic or late apoptotic sperm (AAV+, PI+) from the motility only microfluidic method. The benefit of microfluidic hybrid sperm selection as opposed to addon's such as MACS is the fact that no additional operation or sperm incubation time is required. Based on this, despite the limited number of samples tested, the use of biomarkers for sperm selection warrants further investigation and a larger number of experiments. The AAV-phosphatidylserine interaction is but one potential interaction that could be leveraged within this platform and may be appropriated to include more discerning sperm biomarkers in the future.

## 5 Conclusion

We have developed a simple biomimetic microfluidic sperm selection platform with the includable option for apoptotic sperm cell removal in a hybrid system. The device uses 3D printed ridges to select boundary following sperm with low DNA fragmentation and high progressive motility and better motile sperm recovery post-cryopreservation. The MSSP device is capable of efficiently and consistently preparing sperm, resulting in significantly lower DNA fragmentation, and higher-grade motility compared with the SU method. The device performs sperm washing and selection simultaneously whilst also significantly reducing the number of apoptotic sperm in the collected sample, providing clinically relevant sperm concentrations for IVF or ICSI within 15 minutes. By reducing the number of manual operations and time down to a third of conventional sperm sorting methods without the use of damaging centrifugal forces that risk iatrogenic injury on sperm, this platform has the potential as a technologically disruptive medical device for use in fertility treatments. Further research on the clinical use of the MSSP is needed to validate its usefulness in abnormal semen samples.

## Declarations

## Conflict of Interest

There are no conflicts to declare.

## Acknowledgment

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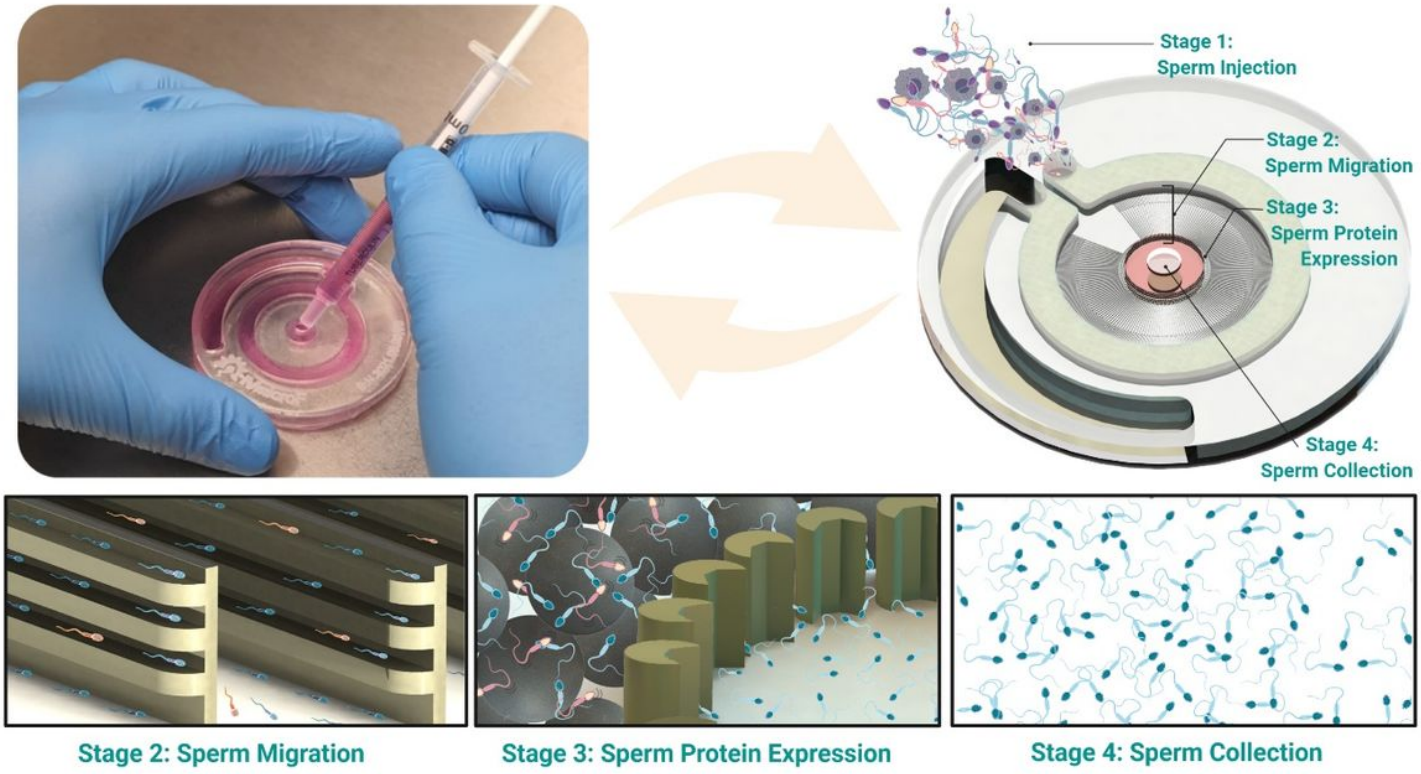
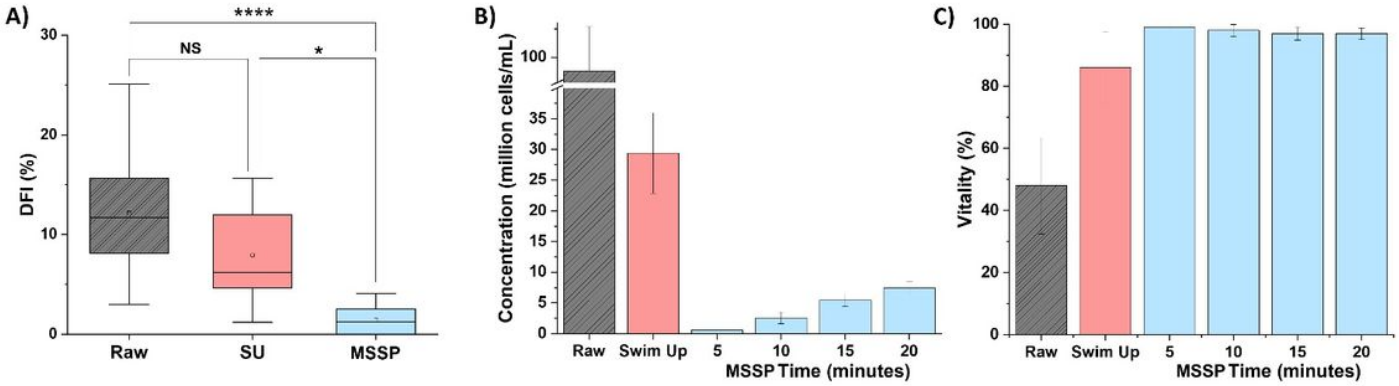
# Figures




## Figure 1

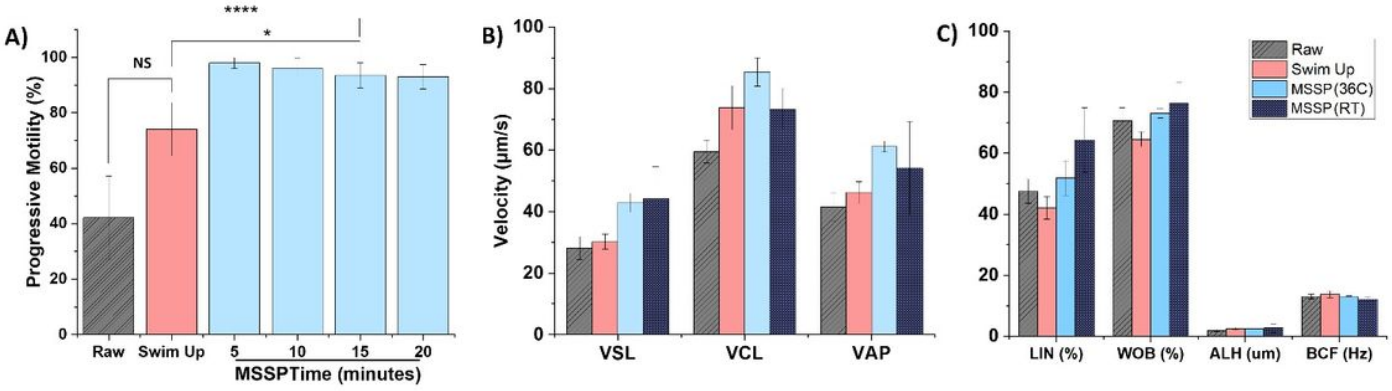
Overview of the sperm selection process within the microfluidic device and representative geometry, including image of actual device filled with food dye for visualization.




## Figure 2

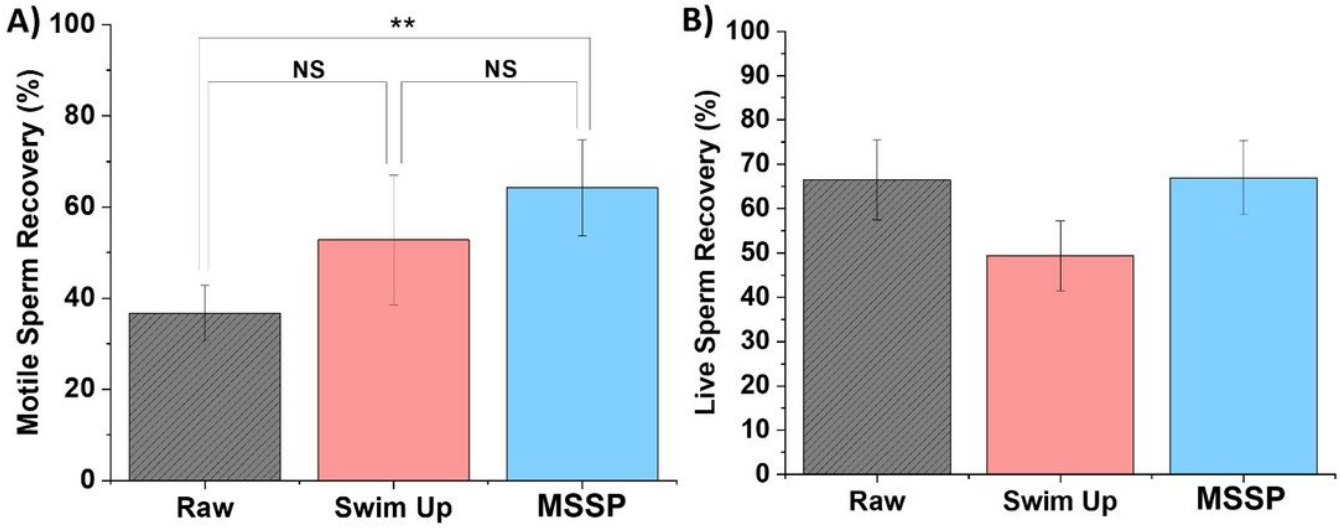
Sperm quality metrics comparing neat semen, SU, and microfluidic sperm selection methods. (A) DFI of side-by-side testing between SU and microfluidic selection methods where the microfluidic device was left to incubate for 15 minutes (N=19). (B) Concentration of sperm collected from the device across different time points compared to the SU method and raw semen. Bare graph is split to better illustrate the concentration of sperm from the device. Samples were the same as those used for DFI plus those used

for vitality staining (N=24). (C) Vitality of sperm from LIVE/DEAD staining between raw, SU and microfluidic sperm populations (N=5). Error bars represent standard deviation within the sample.




## Figure 3

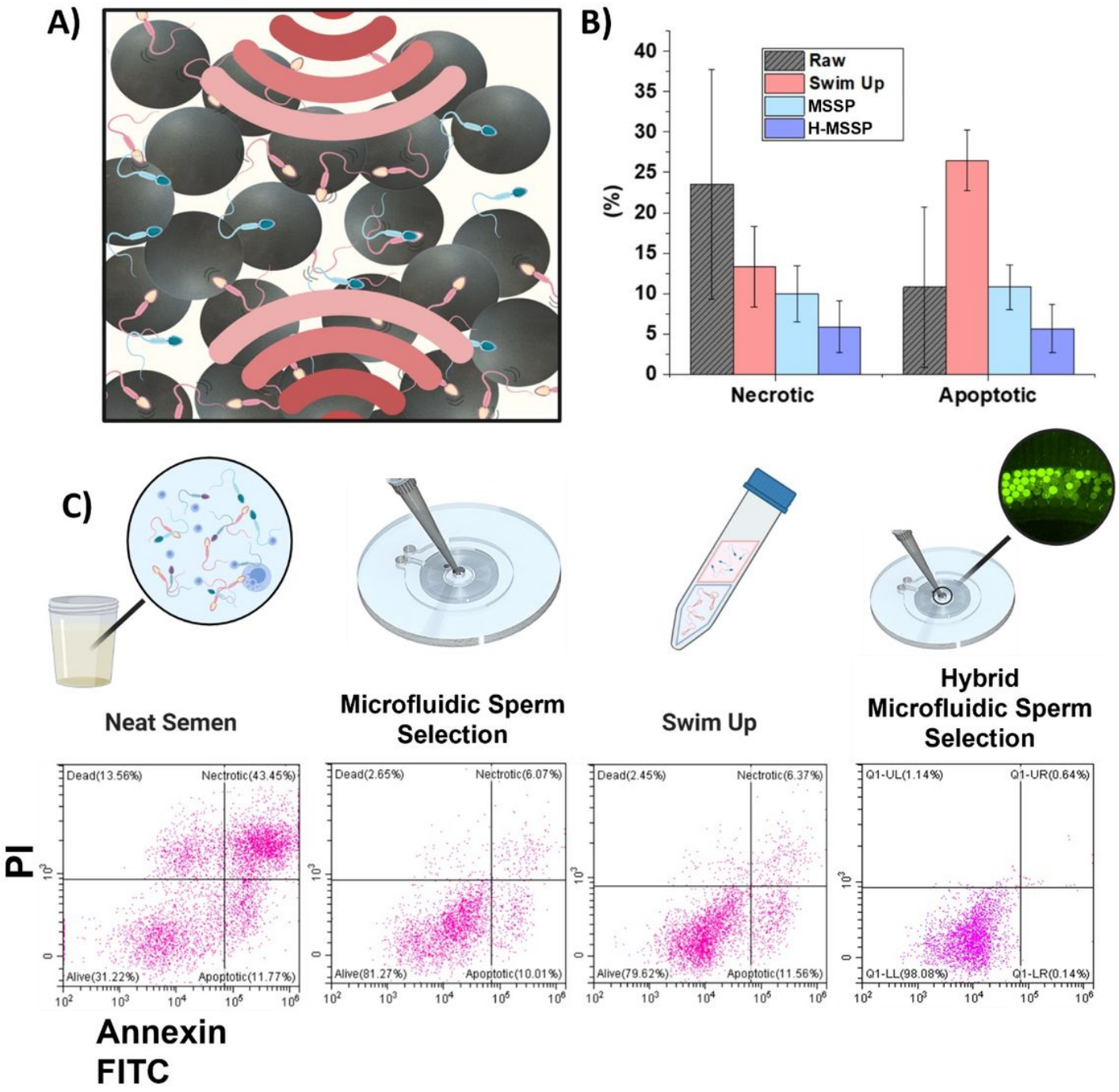
Motility parameters between raw semen, SU, and microfluidic separation methods. (A) Progressive motility of sperm processed by SU and microfluidic methods compared to neat semen samples. Microfluidic tests were split into 5-, 10-, 15-, and 20-minutes selections. (N=24) (B) Velocity parameters of sperm from the same aforementioned groups plus devices operated at room temperature, assessed by OpenCASA. (C) LIN, WOB, ALH, and BCF of sperm from the same aforementioned groups. (N=19 for Raw, SU and microfluidic (36C) and N=5 for microfluidic (RT)). Devices were incubated for 15 minutes each in both (B) and (C). Error bars represent standard deviation within the sample.




## Figure 4

Motility characteristics of sperm before and after cryopreservation from raw, SU and Microfluidic groups (N=4). (A) Percentage of motile sperm recovery following sperm cryopreservation. (B) Recovery

percentages of live sperm from Raw, SU and MSSP processed sperm. Error bars represent standard deviation within the sample.




## Figure 5

(A) Schematic representation of negative sperm selection through magnetic immobilization of apoptotic sperm in the MP MSSP. (B) Apoptotic and necrotic sperm percentages from raw, SU and microfluidics selected sperm. (C) Representative flow cytometry images from each group of stained (PI and AAV-FITC) sperm from the aforementioned groups showing the effective reduction of apoptotic sperm from hybrid MSSP. (N=5)

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ElectronicSupplementaryInformation.docx
- SupplemetaryVideo2SpermMigration.mp4
- SupplementaryVideo1RawsemenvsIVMF.mp4