

SYSTEM AND METHOD FOR SPERM SELECTION FROM SEMEN SAMPLES**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/515,174, filed July 24, 2023, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] The present disclosure relates generally to sperm selection systems and methods. Conventional sperm selection methods involve a high centrifugal force that sediment healthy sperm along with dying sperm that can generate excessive reactive oxygen species (ROS) and cause DNA fragmentation. Therefore, there is a need to develop a method that can select functional and healthy sperm in the sorting process while mimicking natural selection.

SUMMARY

[0003] In one aspect, a microfluidic device configured for filtering out non-functional sperm and isolating motile sperm is disclosed, the microfluidic device including: a main channel having a flow direction from a first end towards a second end of the main channel; a buffer inlet in fluid communication with the first end of the main channel; a collection chamber in fluid communication with the second end of the main channel; a secondary channel in fluid communication with the collection chamber on a downstream side; and a waste chamber in fluid communication with the secondary channel on a downstream side.

[0004] In some implementations, a cross-sectional shape of the main channel is circular. In some implementations, a cross-sectional shape of the main channel is rectangular. In some implementations, a height of the collection chamber is in a range of 0.5 mm to 50.0 mm (e.g., 1.5 to 10.0 mm).

[0005] In some implementations, a microfluidic device, further including a pump or syringe coupled to the buffer inlet and configured to create a flow within the microfluidic device in the flow direction. In some implementations, a microfluidic device, wherein a steady flow rate through the microfluidic device is in the range of 1 to 2000 $\mu\text{l}/\text{min}$ (e.g., 50 $\mu\text{l}/\text{min}$). In some

implementations, a microfluidic device, wherein the pump or syringe is configured to introduce human tubal fluid medium supplemented with 1% bovine serum albumin.

[0006] In some implementations, a microfluidic device, wherein, when a sample including human semen is introduced into the collection chamber, the collection chamber is configured to maintain motile sperm having positive rheotaxis when a flow of fluid is applied.

[0007] In some implementations, a microfluidic device, wherein a portion of less motile or non-functional sperm cannot maintain rheotaxis and are washed into the waste chamber.

[0008] In some implementations, a microfluidic device, further including sidewalls defining at least the main channel and the collection chamber, the sidewalls including polymethyl methacrylate (PMMA) and double-sided adhesive.

[0009] In another aspect, a method of testing for and selecting functional sperm from a sample, is disclosed, the method including: providing a microfluidic device having a buffer inlet, a main channel, a collection chamber, a secondary channel, and a waste chamber all in fluid communication along a flow direction; introducing a semen sample into the collection chamber; applying a flow of fluid to the buffer inlet and into the main channel along the flow direction; isolating motile sperm in the collection chamber based on their ability to maintain rheotaxis; and washing out less motile or non-functional sperm into the waste chamber.

[0010] In some implementations, a method, further including: collecting a sample of motile sperm from the collection chamber. In some implementations, a method, further including: analyzing the movement of sperm sample to evaluate velocity parameters. In some implementations, a method, further including: analyzing the sperm sample for DNA integrity by measuring fluorescence intensity of acridine orange (AO) bound to DNA.

[0011] In some implementations, a method, wherein applying a flow of fluid includes maintaining a flow of fluid for a predetermined period of time. In some implementations, a method, wherein the predetermined period of time is 45 minutes.

[0012] In some implementations, a method, wherein a cross-sectional shape of the main channel is rectangular. In some implementations, a method, wherein a height of the collection chamber is in a range of 0.5 mm to 50.0 mm (e.g., 1.5 to 10.0 mm).

[0013] In some implementations, a method, further including: coupling a pump or syringe to the buffer inlet; and applying, via the pump or syringe, a flow within the microfluidic device in

the flow direction. In some implementations, a method, wherein the applied flow rate through the microfluidic device is steady at 1 to 2000 $\mu\text{l}/\text{min}$ (e.g., 50 $\mu\text{l}/\text{min}$).

[0014] Additional advantages will be set forth in part in the description which follows or may be learned by practice. The advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1A shows a microfluidic device for sorting functional human sperm with a circular cross-section collection chamber, according to one implementation.

[0016] FIG. 1B shows a microfluidic device for sorting functional human sperm with a rectangular cross-section collection chamber, according to one implementation.

[0017] FIG. 1C shows an example diagram of velocity magnitude for the circular cross-section chamber, according to one implementation.

[0018] FIG. 1D shows an example diagram of velocity magnitude for the rectangular cross-section chamber, according to one implementation.

[0019] FIG. 2 shows a graph of sperm motility analysis, wherein the plot shows the percentage (%) motility of human sperm isolated using 50 $\mu\text{l}/\text{min}$ (*p value < 0.05, N = 3).

[0020] FIG. 3 shows a graph of sorting efficiency analysis displaying the isolation efficiency of sorted sperm using rheotaxis.

[0021] FIG. 4 shows a graph of sperm velocity analysis, wherein the sperm were analyzed for velocity parameters (i.e. VCL, VSL, VAP) and were collected from the collection chamber after 45 min. The sperm were sorted using 50 $\mu\text{l}/\text{min}$ flow rates and showed significantly improved velocity parameters compared to unprocessed semen samples (*p value < 0.05, N = 3).

[0022] FIGS. 5A-5C shows sperm morphology analysis. Specifically, FIG. 5A and FIG. 5B show representative images of sperm on a pre-stained morphology slide to assess sperm morphology for stock (in FIG. 5A) and 50 $\mu\text{l}/\text{min}$ flow (in FIG. 5B). FIG. 5C shows a plot showing normal morphology (%) for stock and sorted sperm. Sperm sorted with a 50 $\mu\text{l}/\text{min}$

flow rate showed a higher percentage of normal morphology compared to stock. (*p value <0.05 between raw and sorted semen samples).

[0023] FIGS. 6A-6B show sperm that were stained with acridine orange (AO) for quantification of DNA Fragmentation Index (DFI). The fluorescence images are shown after Acridine Orange (AO) staining for stock (in FIG. 6A), and 50 μ l/min flow (in FIG. 6B). The green fluorescence intensity of AO-stained nuclei is associated with native DNA stainability while the red fluorescence intensity represents fragmented DNA. FIG. 6C shows a graph of DNA fragmentation analysis wherein sperm sorted with the constant flow rate of 50 μ l/min showed significantly lesser DNA fragmentation compared to unsorted semen samples (*p < 0.05 between raw and sorted semen samples).

[0024] FIGS. 7A-7B show graphs of corner-swimming preference of sperm in rectangular cross-section, wherein the cross-sectional distribution of sperm in the rectangular chamber for 1.5 mm (in FIG. 7A), and 3mm (in FIG. 7B) height are shown.

DETAILED DESCRIPTION

[0025] Referring generally to the figures, systems, methods, and test results for improved sperm selection via a novel, microfluidic selection device is shown, according to various implementations.

Selection of Functional Sperm from Abnormal Semen Samples Using a Rheotaxis-based Microfluidic Device

[0026] Sperm selection plays a key role in successful fertilization in assisted reproduction. Current clinical sperm selection methods lack the efficiency to isolate high-quality sperm, especially from abnormal semen samples. Moreover, these selection methods circumvent the natural selection barriers. The currently used gradient and swim-up sperm sorting methods involve several centrifugal steps that further damage non-optimal samples, which is one of the main drawbacks.

[0027] Microfluidic-based selection methods have emerged as promising platforms for reproductive clinics. This disclosure describes a microfluidic device, inspired by the structure of the female reproductive tract, which helps to isolate high-quality sperm from unprocessed semen samples. The device exploits the rheotaxis (i.e., movement of motile sperm against the flow) behavior of the sperm in the selection process. It includes a collection and a waste chamber,

which are connected via microchannels. The collection chamber is designed such that highly motile and functional sperm can stay in the chamber while dead or less functional sperm are moved to a waste chamber. The device is tested with fresh clinically abnormal semen samples and cryopreserved samples. Results indicate that the device is capable of isolating highly functional motile sperm with normal morphology and a low level of DNA fragmentation.

[0028] Various objects, aspects, features, and advantages of the disclosure will become more apparent and better understood by referring to the detailed description taken in conjunction with the accompanying drawings, in which like reference characters identify corresponding elements throughout. In the drawings, like reference numbers generally indicate identical, functionally similar, and/or structurally similar elements. All dimensions displayed in the drawings or described herein are exemplary only and are not meant to limit the scope of the disclosure.

[0029] *Introduction*

[0030] Infertility, which is the inability of a couple to achieve a successful pregnancy, is on the rise worldwide and it affects almost 15% of couples (over 48 million couples). Male infertility is solely responsible for about 20% -30% of cases and contributes to 50% of infertility issues, such as low sperm count or poor sperm motility. Assisted reproductive technologies (ARTs) using In Vitro Fertilization (IVF) or Intra-Cytoplasmic Sperm Injection (ICSI) have revolutionized infertility management.

[0031] During an ART procedure, one of the key points for successful fertilization is the selection of high-quality healthy sperm. The existing ART procedures involve different techniques such as swim-up and density gradient centrifugation to select sperm. These techniques are either forcing sperm cells to swim through a density gradient using high centrifugation force or swim upward by enabling motile sperm to move away from a cohort of sedimented sperm into freshly layered media. Although these methods are developed to select good-quality sperm for ARTs, they ignore most of the natural selection barriers that sperm encounter in vivo which seems sub-optimal for the selection process. These centrifugal-based methods consist of multiple steps involving centrifugation that results in greater loss of sperm viability. One of the crucial factors for producing a good-quality embryo with a high implantation rate is sperm DNA integrity. Note that the level of DNA damage in sperm may be directly proportional to the spontaneous pregnancy loss. Conventional sperm selection methods involve a high centrifugal force that sediment healthy sperm along with dying sperm that can

generate excessive reactive oxygen species (ROS) and cause DNA fragmentation. Therefore, there is a need to develop a method that can select functional and healthy sperm in the sorting process while mimicking natural selection.

[0032] Microfluidic approaches are rapidly developing in the various areas of research and clinical applications. In the context of fertility, microfluidics can be a representative of the devices that can mimic in vivo sperm selection environment such that it can mimic the geometry of micro-confined regions within the female reproductive tract in a highly controlled fashion. Several microfluidic systems have been tested for sperm selection including a sorting chip that selected sperm based on their migration through the pores of a poly-carbonate membrane, or a rheotaxis-based microfluidic device.

[0033] In the journey of the sperm from the cervix to the site of fertilization in the ampulla, different guiding mechanisms are actively functioning; chemotaxis, thermotaxis, and rheotaxis. Among these guiding mechanisms rheotaxis, the ability of the sperm to reorient and align itself against the flow direction and swim upstream, is suggested as a long-distance biophysical guiding mechanism for human sperm. A preliminary study shows the effectiveness of the rheotaxis-based selection methods for selecting sperm with better sperm morphology and chromatin maturity. Previously reported rheotaxis-based microfluidic devices selected sperm based on sperm motility but were not very effective in selecting sperm from asthenospermic samples (low motility). To address these challenges, an innovative microfluidic device has been developed, where the device chamber has variable flow velocities that enable the collection of healthy motile sperm showing rheotaxis while swiping away sluggish and immotile sperm to the waste chamber.

[0034] Herein disclosed is a microfluidic device configured for filtering out non-functional sperm and isolating motile sperm. The microfluidic device exploits the rheotaxis effect where flow is maintained by a syringe pump. The syringe pump creates a flow along the micro-channel toward the collection chamber to guide and isolate sperm cells. To identify the effect of the shape and height of the collection chamber in the selection process, both rectangular and circular cross-sections with two different heights were tested. The quality of the isolated sperm cells was evaluated by measuring their motility, morphology, and DNA integrity. Additionally, the microfluidic device was tested using fresh abnormal patient semen samples and compared the results with the standard procedure for sperm selection; swim-up.

[0035] *Results*

[0036] The presented microfluidic device consists of two chambers (main/collection, and waste) separated by a micro-channel. FIG. 1A shows an example of the device 10 having a collection chamber 12 and a waste chamber 14 separated by a main channel 16 (e.g., a micro-channel). The main channel 16 has a flow direction from a first end towards a second end of the device 10. Additionally, secondary channel 18 (e.g., a micro-channel) is shown in fluid communication with the collection chamber 12 and a flow inlet 20. The flow direction of the device 10 is generally from the flow inlet 20 on the first end of the device 10 towards the waste chamber 14 on the second end of the device 10. Each of the collection chamber 12, the waste chamber 14, the main channel 16, and the secondary channel 18 are defined by an upper layer and a lower layer of the device 10. The flow inlet 20 extends through the upper layer to provide a flow path for the buffer fluid.

[0037] Similarly, FIG. 1B shows an example device 30 having a collection chamber 32 and a waste chamber 34 separated by a main channel 36 (e.g., a micro-channel). Additionally, a secondary channel 38 (e.g., a microchannel) is shown in fluid communication with the collection chamber 32 and a flow inlet 40. Each of the collection chamber 32, the waste chamber 34, and the main channel 36, and the secondary channel 38 are defined by an upper layer and a lower layer of the device 30. The flow inlet 40 extends through the upper layer to provide a flow path for the buffer fluid.

[0038] To evaluate the effect of the shape and height of the collection chamber in the sorting process, two different chamber shapes were considered (rectangular and elliptical) as well as two different chamber heights (1.5 mm and 3 mm) in the design of the device. For example, FIG. 1A shows a circular collection chamber 12, while the device 30 of FIG. 1B shows a rectangular collection chamber 32. However, in other implementations, a different height of the chamber may be used (e.g., a height in a range of 0.5 mm to 10.0 mm).

[0039] A flow of 50 $\mu\text{l}/\text{min}$ is generated inside the device by a syringe pump coupled to and in fluid communication with the flow inlet. However, in other implementations, a different flow rate of buffer fluid may be used (e.g., in the range of 1 to 2000 $\mu\text{l}/\text{min}$). FIGS. 1C and 1D show graphs of the velocity magnitude for each of the devices 10, 30, according to one experimental implementation. In the experiment, a sample of human semen is introduced to the main/collection chamber. It is expected that functional motile sperm swim against the flow

direction and remain in the main/collection chamber, i.e., positive rheotaxis. The dead and less motile sperm are washed out and moved to the waste chamber. After 45 min, the isolated samples are collected from the collection chamber.

[0040] In the experimental results shown in FIG. 1C, the velocity is higher in the center and it decreases by moving away from the center. In the experimental results shown in FIG. 1D, the velocity is higher in the center of the collection chamber and lower against the walls, and the velocity magnitude decreases moving toward the waste chamber.

[0041] While FIGS. 1A and 1B each show one embodiment of the device, other implementations are contemplated. For example, any chamber shapes may be used including circular, oval, square, rectangular, diamond, hexagonal, etc. The chamber height and dimension can also vary to allow large semen volumes to be processed using the example device. The buffer (or wash fluid) can be pumped using any type of pump including a syringe pump. The systems, methods, and devices of this disclosure include having another chamber filled with buffer (Buffer Chamber) where the buffer in this chamber will have more height compared to collection and waste chambers. This height difference allows the buffer to flow from this Buffer Chamber to other chambers. This setup may not need any pump to establish a flow in this device.

[0042] *The Effect of Shape and Height*

[0043] To determine an example optimal design, experiments were conducted with devices of two different shapes and heights. Elliptical and rectangular chamber shapes were used as well as 1.5 and 3 mm heights for the device chambers. Once the sperm was collected from the collection chamber, the motility of the sorted sperm was analyzed. FIG. 2 shows the experimental results which indicate higher motility for the rectangular-shaped chamber. The motility of sperm samples sorted with rectangular-1.5 mm, elliptical-1.5 mm, rectangular-3 mm, and elliptical-3 mm are determined to be $96.50\% \pm 0.087\%$, $45.85\% \pm 1.20\%$, $94.63\% \pm 2.16\%$, and $56.48\% \pm 7.15\%$ respectively.

[0044] Comparing the motility values of the rectangular cross-section with the motility of the stock sample of $38.65\% \pm 1.04\%$, it was found that the rectangular cross-section is an efficient design for isolating the sperm. The sperm isolation efficiency, i.e., the percentage of motile sperm that has made it to the collection chamber out of the total motile sperm of raw stock semen sample, was also calculated. It was observed that the rectangular shape with 3 mm height has the highest isolation efficiency value, as shown in FIG. 3. Results indicate that the rectangular cross-

section with 3 mm height might be an efficient design for isolating sperm using the presented device design and flow settings.

[0045] *Sperm Velocity Analysis for Rectangular Chamber Devices*

[0046] To quantify the motility of the sorted sperm more accurately, the sperm were tracked and their paths were generated by the Computer Aided Sperm Analysis (CASA) plugin (National Institute of Health, <http://rsbweb.nih.gov/ij/>). Then, three different velocity parameters were extracted: curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). Sorted sperm show a set of significantly higher velocity parameters than the unsorted sample, as shown in FIG. 4. The average sperm VCL values for sorted and stock semen samples are 64.22 ± 1.39 and 38.04 ± 1.39 $\mu\text{m/s}$ respectively (Table 1).

	VCL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)
Stock	38.04 ± 1.39	27.02 ± 1.35	24.47 ± 1.36
3 mm rectangular (18 μl volume)	64 ± 1.39	35.23 ± 1.97	28.12 ± 2.18
3 mm rectangular (1 ml volume)	69.17 ± 0.64	51.43 ± 4.83	42.08 ± 3.76

Table 1. Velocity parameters of sperm using the microfluidic device

[0047] *Sperm Morphology Analysis*

To determine the normal sperm population in sorted sperm, sperm morphology analysis was performed following the strict criteria defined by the WHO24. It was observed that sperm sorted by 50 $\mu\text{l/min}$ flow rate and rectangular-shaped device have a higher normal morphology compared to stock. It was found $40.78\% \pm 7.62\%$ of the sorted sperm were morphologically normal compared to stock which was $20.94\% \pm 5.84\%$. See FIGS. 5A-5C. Specifically, FIG. 5A and FIG. 5B show representative images of sperm on a pre-stained morphology slide to assess sperm morphology for stock (in FIG. 5A) and 50 $\mu\text{l/min}$ flow (in FIG. 5B). FIG. 5C shows a plot showing normal morphology (%) for stock and sorted sperm. Sperm sorted with 50 $\mu\text{l/min}$ flow rate showed a higher percentage of normal morphology compared to stock. (*p value <0.05 between raw and sorted semen samples).

[0048] *DNA Fragmentation Analysis*

[0049] To quantify the DNA integrity of each sample more accurately, the DNA Fragmentation Index (DFI) was calculated, which is obtained by using the measured

fluorescence intensity of acridine orange (AO) bound to DNA25. The DFI value was obtained as the ratio of red fluorescence intensity to the sum of red and green fluorescence intensities. The DFI (%) values were 26.63 ± 5.07 , and 12.89 ± 3.90 for stock and isolated samples respectively. FIGS. 6A-6B show images of sperm that were stained with acridine orange (AO) for quantification of DNA Fragmentation Index (DFI). The fluorescence images are shown after Acridine Orange (AO) staining for stock (in FIG. 6A), and 50 $\mu\text{l}/\text{min}$ flow (in FIG. 6B). The green fluorescence intensity of AO-stained nuclei is associated with native DNA stainability while the red fluorescence intensity represents fragmented DNA. FIG. 6C shows a graph of DNA fragmentation analysis wherein sperm sorted with the constant flow rate of 50 $\mu\text{l}/\text{min}$ showed significantly lesser DNA fragmentation compared to unsorted semen samples (* $p < 0.05$ between raw and sorted semen samples). Lower DNA fragmentation was observed for sperm that were isolated using the developed rheotaxis-based microfluidic device. Sperm cells with lower DFI values reflect a high-quality sperm sample.

[0050] *Processing Larger Volume of Sample*

[0051] The device(s) disclosed herein have the potential to handle a larger volume of semen samples by increasing the dimensions of the main/collection chamber to hold 1 ml of semen. The semen analysis was replaced with the higher volume. Higher velocity parameters were found for the device with the 1 ml chamber. The VCL and VAP were almost 1.5 times higher in 1 ml volume compared to the smaller volume device (Tables 1 and 2).

	Motility _{before} (%)	Motility _{after} (%)	Isolation Efficiency (%)	Normal Morphology _{before} (%)	Normal Morphology _{after} (%)
Device with 1ml volume	44 ± 3.16	99.15 ± 1.20	86.27 ± 5.18	16.33 ± 3.43	29.46 ± 4.03

Table 2. Analysis for the device with the higher sample volume. 150 sperm were analyzed 3 times for 3 different individuals.

[0052] *Abnormal Semen Fresh Sample and Rheotaxis-based Sorting*

[0053] For further evaluation of the efficiency and performance of our device, the device was tested with fresh de-identified clinical semen samples that have oligo- and astheno-zoospermic characteristics (low sperm concentration and low motility respectively). Sperm cells isolated using the developed device showed higher motility compared to the swim-up method. Viable

sperm cells were not recoverable from most of these abnormal clinical samples using the swim-up method; however, the presented microfluidic device successfully isolated functional sperm (Table 3).

	Concentration ($10^6/ml$)	Motility _{before} (%)	Motility _{after} (%)	Isolation Efficiency(%)
1	1.2 ± 0.15	24 ± 2.12	85 ± 2.83	55.56 ± 5.5
2	1 ± 0.12	20 ± 1.41	80 ± 2.12	20 ± 2.0
3	0.9 ± 0.14	4 ± 1.42	76 ± 1.41	53.33 ± 6.67
4	6.13 ± 0.90	44.04 ± 5.89	98.04 ± 3.39	75.71 ± 8.08
5	2 ± 0.17	35 ± 5.66	96.30 ± 6.41	70.83 ± 5.89
6	5.33 ± 0.58	23.07 ± 5.44	96.02 ± 3.51	63.33 ± 7.33
7	1.67 ± 0.57	30.95 ± 3.36	96.29 ± 6.41	71.33 ± 5.3
8	4.2 ± 0.57	18.52 ± 5.44	97.02 ± 3.60	66.33 ± 5.6
9	5.3 ± 0.63	22.31 ± 4.34	98.09 ± 3.88	65.18 ± 3.6
10	8.6 ± 0.69	19 ± 2.21	95.33 ± 2.56	68.45 ± 3.76
11	5.6 ± 0.43	16 ± 4.13	97.24 ± 2.83	64.92 ± 4.51

Table 3. Motility, isolation efficiency, and concentration analysis for the fresh semen samples with abnormalities using the device with 18 μ l volume.

[0054] Discussion and Conclusion

[0055] Several parameters can quantify the quality of the sperm samples including sperm concentration, motility, morphology, DNA integrity, and levels of ROS15. The conventional sperm sorting methods, such as swim-up, utilize centrifugation as a processing step, hence these methods are not inspired by natural sperm selection in the female genital tract where centrifugation is not involved. Multiple centrifugation steps can potentially damage sperm DNA and also be hazardous to the morphology of the sperm cells. Therefore, it is helpful to develop sperm processing and sorting techniques that are capable of mimicking natural selection and avoiding any damage to the sperm cells during sorting to achieve successful pregnancy outcomes.

[0056] In the presented microfluidic device, an example of height and chamber shapes that could isolate a higher population of functional and normal sperm was found experimentally. The highest isolation efficiency was found for the rectangular cross-section with 3 mm height, as it was shown previously that sperm cells have a preference for swimming near the corners. See FIGS. 7A and 7B showing graphs of corner-swimming preference of sperm in rectangular cross-

section, wherein the cross-sectional distribution of sperm in the rectangular chamber for 1.5 mm (in FIG. 7A), and 3mm (in FIG. 7B) height are shown.

[0057] Motility is one of the most important factors for sperm used in ART. The highest motility (~100%) was obtained using the presented microfluidic device for both sorted frozen and abnormal fresh semen samples. Results showed that the sperm selected using rheotaxis have higher motility than the swim-up technique for the fresh abnormal semen samples. Moreover, the sorted sperm showed higher velocity parameters (VCL, VSL, and VAP) compared to those of the unsorted samples. The higher velocity parameters are directly related to the higher fertilization rate. Only the cells with the highest velocity parameters can remain in the collection chamber and the dead and less motile cells are washed away. This observation validates that rheotaxis improves the selection of highly motile sperm with higher velocity parameters.

[0058] The higher percentage of mature and morphologically normal sperm may lead to a higher fertilization rate. It was observed that the sperm cells that showed positive rheotaxis (i.e. swim against the flow and remained in the collection chamber), had higher normal morphology compared to stock semen samples.

[0059] Sperm DNA integrity is a measurement of sperm chromatin organization/architecture, chromatin compaction, and DNA strand integrity. While high DNA integrity is linked to both improved fertilization rates and embryonic development in IVF and ICSI, poor integrity is correlated to the risk of pregnancy loss in both IVF and ICSI. DFI was used to quantify the DNA integrity of the isolated sperm cells and found that sperm populations selected by rheotaxis showed the lowest DFI, which is associated with low DNA strand breaks.

[0060] It was shown that the rheotaxis selection method is also capable of selecting healthy sperm in fresh abnormal semen samples. An example device was tested with clinical semen samples with abnormalities and observed that our device is efficient in isolating healthy sperm from samples having very low concentration and/or motility (abnormal semen samples). Using abnormal fresh samples, the swim-up method was performed for a few of the samples and it was not able to isolate any motile sperm cells due to low sperm quality and count.

[0061] The systems, methods, and devices described herein are highly scalable and can handle different volumes of samples. For example, in testing, the volume of the main/collection

chamber was increased to 1 ml. It was shown that the device with higher volume is also capable of isolating healthy sperm for both frozen and fresh abnormal samples (Table 2).

[0062] In conclusion, a novel microfluidic device that allows sperm cells to swim against a flow has been developed. It was observed that sperm isolated using the presented device had higher motility, velocity parameters, percentage of normal morphology, and lower DNA fragmentation. When the device's performance is compared to the swim-up method using abnormal semen samples (having very low concentration and/or motility), it was observed that the presented device was able to isolate healthy sperm with high isolation efficiency; however, the swim-up method was unable to isolate sperm from these samples.

Experimental Testing - Device Fabrication and Use

[0063] *Example Device Fabrication*

[0064] An example device was designed in AutoCAD 2015. The final design was uploaded to Universal Control Panel software for cutting. A combination of polymethyl methacrylate (PMMA) (McMaster-Carr, Atlanta, Georgia, and ePlastics, San Diego, California; 1.5 mm and 3 mm thick) and double-sided adhesive (DSA) (3M, St. Paul, Minnesota; 76-mm thick) was cut using a VLS (VersaLaser, Scottsdale, Arizona) 2.30 laser cutter to fabricate the device. The device includes three chambers: 1) flow inlet, 2) main/collection chamber, and 3) waste chamber. The collection chamber was cut to the area of 3.1 mm X 2.2 mm and the waste chamber was cut with a diameter of 18 mm. The device can easily be designed to handle different volumes of samples (up to 1ml). Two different shapes of collection chambers were tested; i) Circular, and ii) Rectangular chambers. Two different heights of the collection chamber were also tested; 1.5 mm and 3 mm heights (See FIG. 1A and FIG. 1B showing the example devices 10, 30). The chambers were attached to the glass slide using DSA. The syringe pump was connected to the flow inlet and generated a steady flow rate of 50 μ l/min.

[0065] Moreover, a computer simulation was performed to show the velocity profiles in the device (See FIGS. 1C and 1D showing velocity magnitude graphs). Water was used as the sample fluid for the velocity profile calculations. Navier Stokes equations for low-viscosity incompressible fluids, and no-slip boundary conditions were applied to the walls.

[0066] *Human Sperm Preparation*

[0067] Cryopreserved Semen Samples: Cryopreserved human semen samples were purchased from California Cryobank, Fairfax, VA, and Cryos International, Orlando, FL. The anonymous 1 mL semen vials were stored in liquid nitrogen. Human Tubal Fluid (HTF-HEPES) (InVitroCare, Fredrick, MD) was used and supplemented with 1% bovine serum albumin (BSA) (FisherSci, Fair Lawn, NJ) to fill the syringe pump. Sperm samples were thawed in the water bath at 37 °C for 15 min.

[0068] Clinical Abnormal Semen Samples: The abnormal fresh semen samples were collected by patients for clinical semen analyses. The samples used in this study were discarded and were provided to us for research purposes by Boca Fertility, Boca Raton, FL, and the University of Miami Health System, Urology Department. These samples were de-identified and could not be linked to the patients. The study was approved by the Institutional Biosafety Committee (IBC) of Florida Atlantic University. The approved study protocol no. is IBC # 2018-290.

[0069] *Sperm Sorting Using Microfluidic Device*

[0070] Thawed, raw semen sample (stock sperm) was introduced to the main/collection chamber of an example device (e.g., device 10 of FIG. 1A or device 30 of FIG. 1B). After introducing the raw semen sample, the main/collection chamber was covered with scotch tape. The total volume of the collection chamber is 18 μ l and 1ml for smaller and larger devices respectively. The steady flow rate of 50 μ l/min was maintained inside the microfluidic channel using a syringe pump. 1% BSA in HTF was loaded in the syringe to be pumped. The device was incubated at 37°C for the total time interval (45 min) of the experiment. The functional sperm were collected from the collection chamber after 45 minutes.

[0071] *Sperm Concentration and Motility Analysis*

[0072] A standard Makler counting chamber (Sef Medical, Israel) was used to manually analyze the concentration and motility of 1 μ l of sperm sample according to the manufacturer's instructions. Each sperm was labeled as motile or immotile based on its motility. Each count was taken three times and the average of those counts was used as the data point.

[0073] *Sperm Velocity Analysis*

[0074] The preparation of viable sperm samples for analysis was based on the methods described by the WHO laboratory manual. 10 μ l of semen sample was placed onto a glass slide

and covered with a 22×22 mm coverslip to give a depth of 20.67 μ l. A Nikon DS-Fi3 camera with NIS-Elements software (Nikon) was used to capture the movement of sperm samples for 1 min at 30 fps. To monitor sperm velocity parameters; VCL, VSL, and VAP; the captured videos were uploaded into ImageJ (National Institute of Health, <http://rsbweb.nih.gov/ij/>), and the CASA plugin was used to obtain these velocity parameters. 200 sperm were analyzed three times (N=3) for velocity parameters assessment. The experiments were repeated at least three times for different subjects.

[0075] *Sperm Morphology Assessment*

[0076] Recovered sperm cells with 50 μ l/min flow were collected after 45 min. 2–4 μ l of stock sample and each group were placed on a Prestained Morphology Slide (Fertility Stuff, Murphy, NC) and covered by 24×24 mm coverslip. The sperm were imaged using a 40X objective. Sperm morphology assessment was based on the WHO strict criteria (Head: spherical head; acrosome covering 40–70% of the head area; head length 3.7–4.7 μ m; head width 2.5–3.2 μ m; 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 beaks; thinner than the midpiece, the length of the principal piece should be approximately 10 times the head length. 200 sperm were analyzed three times for three different individuals for morphology assessment.

[0077] *DNA Fragmentation*

[0078] Sorted sperm cells were analyzed for DNA fragmentation. The DNA integrity was detected by measuring the fluorescence intensity of acridine orange (AO) bound to DNA. The DNA integrity was quantified in terms of the DNA Fragmentation Index (DFI). The slides for analysis were prepared by putting 4 μ l of sperm sample onto a microscope slide and covered by a 22×22 mm coverslip. Smears were air-dried and 3 μ l of freshly prepared AO stain was added to a dried sample. The AO stain was prepared as follows: 10 ml of 1% AO in distilled water was added to a mixture of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M Na₂HPO₄·7H₂O. Immediately each slide was imaged using a Nikon DS-Fi3 camera and 40X objective. The fluorescence images were captured (excitation of 450–490 nm). The exposure time was kept at 360 ms. The fluorescent images were loaded in ImageJ and intensities were measured. The

measured mean fluorescence intensity of the green and red sperm were used to find DFI. Note that 200 sperm cells were analyzed three times.

Configuration of Certain Implementations

[0079] The construction and arrangement of the systems and methods as shown in the various implementations are illustrative only. Although only a few implementations have been described in detail in this disclosure, many modifications are possible (e.g., variations in sizes, dimensions, structures, shapes, and proportions of the various elements, values of parameters, mounting arrangements, use of materials, colors, orientations, etc.). For example, the position of elements may be reversed or otherwise varied, and the nature or number of discrete elements or positions may be altered or varied. Accordingly, all such modifications are intended to be included within the scope of the present disclosure. The order or sequence of any process or method steps may be varied or re-sequenced according to alternative implementations. Other substitutions, modifications, changes, and omissions may be made in the design, operating conditions, and arrangement of the implementations without departing from the scope of the present disclosure.

[0080] Although the figures show a specific order of method steps, the order of the steps may differ from what is depicted. Also, two or more steps may be performed concurrently or with partial concurrence. It is to be understood that the methods and systems are not limited to specific synthetic methods, specific components, or to particular compositions. It is also to be understood that the terminology used herein is for the purpose of describing particular implementations only and is not intended to be limiting.

[0081] As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another implementation includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another implementation. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

[0082] “Optional” or “optionally” means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0083] Throughout the description and claims of this specification, the word “comprise” and variations of the word, such as “comprising” and “comprises,” means “including but not limited to,” and is not intended to exclude, for example, other additives, components, integers or steps. “Exemplary” means “an example of” and is not intended to convey an indication of a preferred or ideal implementation. “Such as” is not used in a restrictive sense, but for explanatory purposes.

[0084] Disclosed are components that can be used to perform the disclosed methods and systems. These and other components are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these components are disclosed that while specific reference of each various individual and collective combinations and permutation of these may not be explicitly disclosed, each is specifically contemplated and described herein, for all methods and systems. This applies to all aspects of this application including, but not limited to, steps in disclosed methods. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific implementation or combination of implementations of the disclosed methods.

WHAT IS CLAIMED IS:

1. A microfluidic device configured for filtering out non-functional sperm and isolating motile sperm, the microfluidic device comprising:
 - a main channel having a flow direction from a first end towards a second end of the main channel;
 - a buffer inlet in fluid communication with the first end of the main channel;
 - a collection chamber in fluid communication with the second end of the main channel;
 - a secondary channel in fluid communication with the collection chamber on a downstream side; and
 - a waste chamber in fluid communication with the secondary channel on a downstream side.
2. The microfluidic device of claim 1, wherein a cross-sectional shape of the main channel is circular.
3. The microfluidic device of any of claims 1-2, wherein a cross-sectional shape of the main channel is rectangular.
4. The microfluidic device of any of claims 1-3, wherein a height of the collection chamber is in a range of 0.5 mm to 50.0 mm.
5. The microfluidic device of any of claims 1-4, further comprising a pump or syringe coupled to the buffer inlet and configured to create a flow within the microfluidic device in the flow direction.
6. The microfluidic device of claim 5, wherein a steady flow rate through the microfluidic device is in a range of 1 to 2000 $\mu\text{l}/\text{min}$.
7. The microfluidic device of claim 5, wherein the pump or syringe is configured to introduce human tubal fluid medium supplemented with 1% bovine serum albumin.

8. The microfluidic device of any of claims 1-7, wherein, when a sample comprising human semen is introduced into the collection chamber, the collection chamber is configured to maintain motile sperm having positive rheotaxis when a flow of fluid is applied.
9. The microfluidic device of claim 8, wherein a portion of less motile or non-functional sperm cannot maintain rheotaxis and are washed into the waste chamber.
10. The microfluidic device of any of claims 1-9, further comprising sidewalls defining at least the main channel and the collection chamber, the sidewalls comprising polymethyl methacrylate (PMMA) and double-sided adhesive.
11. A method of testing for and selecting functional sperm from a sample, the method comprising:
 - providing a microfluidic device having a buffer inlet, a main channel, a collection chamber, a secondary channel, and a waste chamber all in fluid communication along a flow direction;
 - introducing a semen sample into the collection chamber;
 - applying a flow of fluid to the buffer inlet and into the main channel along the flow direction;
 - isolating motile sperm in the collection chamber based on their ability to maintain rheotaxis; and
 - washing out less motile or non-functional sperm into the waste chamber.
12. The method of claim 11, further comprising:
 - collecting a sample of motile sperm from the collection chamber.
13. The method of any of claims 11-12, further comprising:
 - analyzing the movement of sperm sample to evaluate velocity parameters.

14. The method of any of claims 11-13, further comprising:
analyzing the sperm sample for DNA integrity by measuring fluorescence intensity of acridine orange (AO) bound to DNA.
15. The method of any of claims 11-14, wherein applying a flow of fluid comprises maintaining a flow of fluid for a predetermined period of time.
16. The method of claim 15, wherein the predetermined period of time is 45 minutes.
17. The method of any of claims 11-16, wherein a cross-sectional shape of the main channel is rectangular.
18. The method of any of claims 11-17, wherein a height of the collection chamber is in a range of 0.5 mm to 50.0 mm.
19. The method of any of claims 11-18, further comprising:
coupling a pump or syringe to the buffer inlet; and
applying, via the pump or syringe, a flow within the microfluidic device in the flow direction.
20. The method of claim 19, wherein the applied flow rate through the microfluidic device is steady at a rate in the range of 5 - 2000 $\mu\text{l}/\text{min}$.