Individual sperm selection by microfluidics integrated with interferometric phase microscopy

Pinkie J. Eravuchira\textsuperscript{a,*}, Simcha K. Mirsky\textsuperscript{a}, Itay Barnea\textsuperscript{a}, Mattan Levi\textsuperscript{b}, Michal Balberg\textsuperscript{a,c}, Natan T. Shaked\textsuperscript{a,*}

\textsuperscript{a} Department of Biomedical Engineering, Faculty of Engineering, Tel Aviv University, Tel Aviv 69978, Israel
E-mail: pinkiee@mail.tau.ac.il, nshaked@tau.ac.il

\textsuperscript{b} Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel

\textsuperscript{c} Faculty of Engineering, Holon Institute of Technology, Holon 5810201, Israel

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Abstract

The selection of sperm cells possessing normal morphology and motility is crucial for many assisted reproductive technologies (ART), especially for intracytoplasmic sperm injection (ICSI), as sperm quality directly affects the probability of inducing healthy pregnancy. We present a novel platform for real-time quantitative analysis and selection of individual sperm cells without staining. Towards this end, we developed an integrated approach, combining interferometric phase microscopy (IPM), for stain-free sperm imaging and real-time automatic analysis based on the sperm cell 3D morphology and contents, with a disposable microfluidic device, for sperm selection and enrichment. On testing the capabilities of the microfluidic device, we obtained successful selection of sperm cells with a selectivity of 89.5±3.5%, with no negative-decision sperm cells being inadvertently selected. In addition, we demonstrate the accuracy of sperm cell analysis using IPM by comparing the quantitative analysis produced by our IPM-based algorithm to the qualitative visual analysis performed independently by an
experienced embryologist, which resulted in precision and specificity of 100%. We believe that the presented integrated approach has the potential to dramatically change the way sperm cells are selected for ICSI and other ART procedures, making the selection process more objective, quantitative and automatic, and thereby increasing success rates.

1. Introduction

Male infertility is defined as the incapability of sperm cells to fertilize a female oocyte [1]. As reported by the world health organization (WHO), roughly one half of the total cases of infertility can be attributed to male infertility, with factors such as low sperm count and motility being the primary causes [2,3]. Assisted reproduction technologies (ART), such as in-vitro fertilization (IVF) [4,5] and intracytoplasmic sperm injection (ICSI) [6,7], help scale down problems related to male infertility and improve fertilization success rate. Specifically, identification and isolation of sperm cells possessing high motility and normal morphological features, as defined by the WHO, are pivotal in achieving healthy pregnancies and increasing ART success rates.

In standard IVF, a female oocyte is incubated with motile sperms in order to achieve fertilization. Sperm cell enrichment for IVF is generally accomplished using traditional techniques for sperm cell sorting, such as swim-up or density-gradient centrifugation [8]. In contrast to standard IVF, in ICSI a single sperm cell possessing high motility and visibly normal morphology is selected from a sample of sperm cells, and is then injected into a female oocyte. The selection and injection of the sperm cell is typically executed under a light microscope using a micromanipulator connected to a micropipette for collecting and injecting the chosen sperm cell. Reports show that ICSI results in higher fertilization rates than its counterpart, standard IVF, especially in cases of severe male infertility [6,9].

However, due to the lack of quantitative stain-free imaging and selection tools for sperm cells, sperm selection for ICSI is done manually and subjectively by a trained clinical embryologist, whose decision is mostly based on the sperm cell motility and the two-
dimensional (2D) morphology of unstained cells. This introduces a significant margin of human error, making the selection of the best sperm cells for ICSI a challenging task.

Previous studies showed that highly motile sperm cells have higher fertilization potential. However, since the DNA is located in the sperm head, morphological characteristics such as head size and the relative sizes of the acrosome and nucleus are strongly connected to fertilization potential and the genetic normality of the offspring [10,11]. Unfortunately, the selection of sperm cells possessing normal morphological characteristics is challenging with the current imaging techniques, as the WHO [12] criteria for normal sperm morphology is based on the evaluation of fixed and stained cells, in which the sperm components are clearly visible. This is not the case in IVF and ICSI, where staining is not allowed.

The utilization of phase microscopy techniques, in which the delay of the light upon passing through the sample is recorded using optical interference, presents a stain-free alternative for sperm imaging. Differential interference contrast (DIC) [13] and Zernike’s phase contrast (PhC) [14] are two of the preeminent phase contrast microscopy techniques and provide improved contrast in comparison to bright-field microscopy (BFM). However, the images produced using these techniques do not provide quantitative contrast at all cell points, and significant imaging artifacts are apparent, especially around the cell and organelle edges. Contrary to DIC and PhC, interferometric phase microscopy (IPM) is a digital holographic microscopy technique that can quantitatively image sperm cells without staining and generate meaningful contrast at all cells points [15–18]. IPM records the quantitative phase map of the sample via interference between a beam passing through the sample and a reference beam. The resulting interferogram is translated into a 2D phase map image that provides the actual optical thickness, or optical path delay (OPD), of the sample at all points in the image [19,20]. This quantitative phase map provides meaningful, stain-free contrast that correlates well with that of stained sperm cells [15]. Additionally, new parameters that were not available to clinicians so far, such as cell dry mass, can be derived from these phase map
images [21]. When acquiring interferograms of sperm cells in a clinical setting, one cannot allow the meticulous stability and alignment conditions available in an optical laboratory. We therefore use a compact and external interferometric module that can be positioned at the exit of an existing imaging system, making IPM more accessible and affordable for sperm imaging in fertility clinics [15,16].

For the selection of individual sperm cells, we integrated this quantitative imaging technique with a specially designed disposable microfluidic slide, making this technique relevant for clinical use. Rapid, inexpensive fabrication as well as ease of handling make microfluidic devices a promising tool for biomedical and biotechnological applications [22–24]. Several works have been reported on the application of microfluidic models for the enrichment of sperm cells from raw semen based on cell motility [25,26] and DNA integrity [27–29]. With these tools, motile sperm cells are separated from cellular debris and immotile sperms, generally based on thermotaxis [30], chemoattractant driven [31], fluidic flow driven [32], or passively driven sorting [26]. Although, many studies have focused on sperm cell enrichment, only a few have attempted to select individual sperm cells. Moreover, in most of these models the sorting of sperm cells is almost entirely based on their motility, while little attention is given to morphological characteristics during the sorting process. As a result, most of these models are limited to IVF and not relevant for ICSI, mostly because they cannot be used to characterize the morphology of individual sperm cells selected in ICSI.

We therefore propose a new platform for individual sperm cell selection, integrating stain-free quantitative imaging and automatic real-time analysis using IPM with a disposable microfluidic device for the selection of individual sperm cells. After the selection process, the clinician is able to extract any single sperm cell from the selected sperm cell reservoir for injection into the female oocyte in an ICSI procedure. Thus, this new platform has great clinical potential for changing the subjective, manual, and non-quantitative sperm selection used in ICSI.
2. Materials and methods

2.1. Working principles of the integrated IPM-microfluidics platform

The proposed microfluidic device for sperm selection integrated with the IPM setup for stain-free quantitative imaging is shown in Fig. 1 inset. Being disposable and inexpensive is highly important for the sterile environment needed for ICSI. This device is comprised of three ports: one inlet port to introduce the sperm cells into the microchannel and two outlet ports, 1 and 2, which collect the abnormal and normal sperms, respectively. The flow of sperm cells along the microchannel to output port 1 and the routing of sperm cells into the selection port, outlet port 2, are achieved using two computer-controlled syringe pumps connected to the two outlets of the microfluidic device via vertically oriented Tygon tubes. As shown in Fig. 1, the microfluidic device is integrated into the sample stage of an inverted microscope. This microscope is illuminated by a low-coherence laser beam, which is cleaned by a spatial filter (lenses L1 and L2 and pinhole P1). The beam then passes through the microfluidic channel and is imaged by a microscope objective and a tube lens onto the output plane of the microscope, where a compact and external IPM module is connected. This module produces an off-axis image interferogram of the sample on the digital camera plane by splitting the beam into two identical beams using the beam splitter (BS), optically erasing the sample information from one of the beams by spatial filtering (lenses L3 and L4 and pinhole P2) in order to create a clean reference beam, and creating an off-axis angle between the sample and reference beams using a retro-reflector (RR) in the sample arm [33]. Each sperm cell is inspected by IPM, in a part of the predetermined field of view (FOV), which is positioned before the cell reaches the T junction of the channel, in order to enable a selection decision to be made prior to directing the sperm cell to the chosen sperm reservoir. As the height of the microchannel is 6 µm, cells are prevented from being out of focus. In any case, as IPM captures the complex wave front, numerical refocusing can be used to correct the focus for out-of-focus cells.
Fig. 1. Schematic of the experimental setup containing a disposable microfluidic device integrated with the IPM setup. The inset shows the top-view and cross-sectional view of the microfluidic device. L1-L4, achromatic lenses. MO, microscope objective, Sg1, Sg2, syringe pumps. P1, P2, pinholes. M, mirror. RR, retro-reflector mirror. BS, beam splitter.

Prior to the insertion of sperm cells into the microchannel, the microchannel is filled with medium and air bubbles are removed. Subsequently, to establish a smooth and consistent flow of medium through the microchannel, a withdrawal pressure of 2 µl/hr is applied using pump 1 and maintained throughout the experiment. After attaining a steady flow, sperm cells
are inserted into the inlet using a micropipette. Sperm cells along with the medium are then pulled by pump 1 towards the outlet port 1 using micropumps. Note that since the sperm cells are pulled along the microchannel at a slow flow rate, not all sperm cells start to flow along the microchannel right away. This limits the throughput of the current system. Upon detection of a normal sperm cell based on the quantitative IPM imaging, pump 2 is activated at a set flow rate of 100 µl/hr for 2 sec, and pulls one selected cell to outlet 2 during each activation.

2.2. Disposable microfluidic device fabrication

We have chosen polydimethylsiloxane (PDMS) (Silgards 184: Dow Corning, MI, USA), a silicon-based organic polymer, for lithographing the disposable microchannel for individual sperm cell selection. PDMS was chosen due to its inexpensive fabrication, optical transparency, mechanical, chemical and thermal stability, non-toxicity, and bio-compatibility.

Initially, a photomask was produced using direct laser writing and then the pattern on the photomask was transferred to SU-8 3050 (MicroChem, Westborough, MA, USA) coated silicon substrate using photolithography [34]. Micro-ridges of the required patterns were thus created on the silicon master mold. A standard soft lithography method was then adopted to produce the PDMS mold [35]. Soft lithography produced a negative pattern of the master on the PDMS, where the micro-ridges became microchannels. Subsequently, using a biopsy puncher, holes were punched to the inlet and outlets of the PDMS microchannels with the help of a stereomicroscope. PDMS was then bonded to the microscopic glass cover slip (500 µm thick) using oxygen plasma bonding (1 min at a power of 20 W) followed by thermal treatment in an oven (85°C for 20 min). The use of a microscope cover slip as the bottom of the microfluidic device is necessary in order to image the sperm at high resolution and magnification using an oil objective with a typically short working distance (see section 2.5.).
2.3. Disposable microfluidic device architecture

The proposed microfluidic device for sperm selection integrated with the IPM setup for stain-free quantitative imaging is shown in Fig. 1 inset. The height and width of the resulting microchannel were 6 µm and 10 µm respectively, as measured using a profilometer. The inlet and outlet ports had diameters of 2 mm. The dimensions of the microchannel were chosen based on the size of the sperm cells. The head of a sperm cell has a thickness of 3 µm and a length of 5 µm. Thus, the produced microchannel has a dimension that allows only one sperm to flow through the FOV at a time, preventing the flow of two or more cells in parallel. A width of 10 µm permits the flow of only a single sperm cell through the microchannel without leading to clogging or obstructions during flow. Furthermore, a height of 6 µm helps maintain a good focus on the flowing sperm cells without the need to focus while imaging. A taper with a length of 300 µm was designed at the inlet. The large and small ends of the taper had dimensions of 400 and 10 µm, respectively. The taper helped more sperm cells to enter into the microchannel amid possible blockages due to mucous in the sample or PDMS residues. We observed that during the flow, highly motile sperm cells moved along the microchannel at slightly higher speeds compared to less motile sperm cells. Therefore, optimization of microchannel length was necessary. Various lengths ranging from 0.5 to 4 mm were tested. From the measurements, we observed that when the channels were longer than 3 mm, the sperm cells became less motile as they swam along the microchannel. However, when the length was less than 3 mm, no considerable difference in the motility of sperm cells was observed. Nevertheless, when the length was less than 1 mm, the imaging FOV was blocked by the adjacent Tygon tube (outer diameter 2.2 mm) connected to the outlets. Therefore, the final optimal length of 2 mm was chosen, and it was observed that with this optimized length the sperm cells moved along the microchannel without any constraints.
2.4. Sperm sample preparation

Five healthy sperm donors participated in the study (ages 21-30). The study was approved by the institutional ethics committee of Tel Aviv University, Israel, and a signed consent from the sperm donors was obtained. First, the raw semen was allowed to liquefy at room temperature for 30 min. Then, standard density gradient-based centrifugation for sperm enrichment separated the cells based on their density. After liquefaction, the sperm cells were cleaned using the PureCeption Bi-layer density gradient kit (Origio, Knardrupvej, Denmark).

In short, a 15 ml sterile test tube was loaded with 2 ml of 40% PureCeption solution and 2 ml of 80% PureCeption solution. 1 ml of semen was placed on top of the gradient and was spun at a relative centrifugal force (RCF) of 400 g for 20 min at room temperature. After centrifugation the intact cells are concentrated in the pellet. Then the supernatant in the tube was removed and the pellet was washed with modified human tubal fluid (HFT) medium (Irvine Scientific, CA, USA) by centrifugation for 5 min at 400 RCF at room temperature. The gradient centrifugation cleaning method removes most of the seminal fluid, non-sperm cells, immature-sperm cells and non-viable sperm cells. The sorted sperm cells are then collected using a micropipette and mixed with modified HTF. Finally, a 1:1 v/v ratio of sperm cells to 7% polyvinylpyrrolidone (PVP) solution was prepared and introduced into the microchannel. PVP can increase the viscosity of the sperm cell solution, which helps slow down and regulate sperm cell movement through the microchannel. PVP is routinely used in ICSI, and it does not have to be removed while injecting the sperm cell into the oocyte.

2.5. IPM system and OPD map acquisition

The IPM system utilized light of partial temporal coherence (central wavelength: 650 nm, full width at half maximum: 7 nm), which originated from a supercontinuum laser (SC-400-4 Fianium) coupled to an acousto-optic tunable filter (SC-AOTF, Fianium), controlled by a computer. As shown in Fig. 1, the beam was spatially filtered using a beam-expander (two achromatic lenses, focal lengths 45 mm) and a 30 μm pinhole in order to produce a spatially
coherent Bessel beam. This beam subsequently illuminated the sample, which was imaged using 60× oil microscope objective (Olympus UPLSAPO60×O, 1.35 numerical aperture) and a tube lens (focal length 150 mm). The resultant sample beam then entered the \( \tau \) (tau) external interferometric module,[33] where it was divided into two beams of equal intensity using a beam splitter (BS). The first beam is focused onto a retroreflector mirror (RR) by the first module lens (focal length 100 mm), causing a small shift in the illumination angle on the camera. This was necessary in order to produce off-axis interference on the digital camera, enabling the acquisition of phase map images from a single interferogram. The second beam from the beam splitter was focused by the same lens onto a 15 \( \mu \)m mirror-mounted pinhole placed in the Fourier plane of the lens, thereby erasing all of the high spatial frequencies that encoded the sample information and creating a clean reference beam. The two beams then merged in the beam splitter and, after traversing another lens (focal length: 150 mm), an image interferogram was generated on the focal plane of the digital camera (DCC1545M, Thorlabs, monochromatic CMOS, 1280 \( \times \) 1024 square pixels of 5.2 \( \mu \)m each). This system possessed a total magnification of 76× with a resolution limit of 464 nm. The resulting image interferogram was then processed using digital spatial filtering to extract one of the cross-correlation terms [33] and the phase map is unwrapped using the Miguel 2D unwrapping algorithm in order to remove 2\( \pi \) ambiguities [36]. Finally, the resulting phase map was multiplied by the central illumination wavelength and divided by 2\( \pi \) to produce the OPD map.

2.6. Embryologist assessment

In the interest of determining the correlation between computer-based sperm morphology analysis using OPD map images and the current stain-free sperm morphology analysis done by a clinical-embryologist, phase map videos of 132 live sperm cells flowing through the microfluidic device were recorded. In order to provide the embryologist with familiar images that he would be capable of analyzing, the OPD map videos were displayed using a color map that closely approximated the colors seen by embryologists when examining sperm cells.
stained with Hemacolor while using the Sperm Class Analyzer software. We have shown previously [15] that these images, obtained without staining, enable the embryologist to reach the same decisions that would be reached when examining stained sperm using the WHO gold standard for the morphological analysis of stained sperm cells. In these videos, the sperm acrosome is indicated in yellow, while the nucleus is marked with blue. The OPD threshold used in order to produce a color map that would provide the acrosome and the nucleus in the phase map image with the correct colors was obtained from our previous research,[21] in which it was shown, by comparing the phase maps and stained images taken of the same cells, that the OPD threshold between acrosome and nucleus is approximately 105 nm for sperm in aqueous media.

The embryologist analyzed the phase map videos of each cell and determined whether it passed or failed each of the following five WHO sperm analysis criteria [12]:

1. Head shape: The head should be smooth, regularly contoured and generally oval in shape. The accepted normal ratio of head width to length is 3:5.

2. Acrosome size: There should be a well-defined acrosomal region comprising 40% - 70% of the head area.

3. Vacuoles: The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than 20% of the sperm head. The post-acrosomal region should not contain any vacuoles.

4. Midpiece: The midpiece should be slender, regular, and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head.

5. Residual cytoplasm: Residual cytoplasm is considered an anomaly only when in excess, i.e. when it exceeds one third of the sperm head size.
The manual analysis results performed by the clinical embryologist were compared to our computational decisions, as explained above.

3. Results and discussion

3.1. Sperm cell selection using microfluidic device integrated with IPM

To assess the device, 1 µl of the prepared sample was injected into the inlet. The small total volume required is advantageous for those patients with very poor sperm count and minimal motile sperms. The number of sperm cells in 1 µl of the prepared solutions was counted using a hemocytometer. The tests show that on average $3000 \pm 200$ sperm cells are present in each 1 µl solution for our donors. While this number is smaller than usually used for sperm cell selection in microfluidics, the number of sperm cells required to perform ICSI is only around 10 cells; thus our original sperm count is more than ample. Additionally, we have observed that an average of $8\pm2$ sperm cells flow along the microchannel per minute when the applied flow rate is 2 µl/hr. In order to establish and maintain a constant flow of sperm cells, inactive intervals of 5 sec were required after each cell selection. During this inactive interval, no cells were selected and constant flow was maintained using pump 1, directing all cells to outlet 1.

Figure 2a shows the microfluidic device with the sperm cells flowing along the microchannel as imaged using the IPM system. The IPM image is displayed only on the right side, located before the T junction. The rest of the channel displays the BFM intensity image. This allows the sperm cells to be imaged and analyzed by their OPD, and then directed towards outlet 1 or outlet 2 depending on their quality. Fig. 2b and c show video images of successful selection of abnormal and normal sperm cells into outlets 1 and 2, respectively. This selection process is repeated until the required number of normal sperm cells is selected. Finally, the Tygon tube from outlet 2 is removed and the normal sperm cells can be collected from the outlet 2 reservoir using an ICSI micropipette. Our system can analyze approximately 40 to 45 sperm cells in 6 min, and depending on the quality of the sperm cells the system can select one or more normal sperm cells in this time period. This sperm selection rate is
sufficient for performing ICSI. Further improvement of throughput is expected, as discussed below in Section 4.

Fig. 2. (a) The microfluidic device as imaged by the optical system, with a label-free OPD window on the right of the FOV, allowing quantitative imaging and analysis, and producing a selection decision before the sperm cell reaches the T junction. (b) If a cell should not be selected, it is directed to the outlet 1 on the left by the continuous pump. (c) If a cell should be selected, it is directed to outlet 2 on the bottom by the pulse pump. Video 1 (sperm cell selection using integrated microfluidic-IPM platform) demonstrates the entire process.

3.2. Selectivity of sperm selection

In order to calculate the selectivity of our microfluidic device, sperm selection based on arbitrary decisions was performed. We observed the sperm cells flowing in a microchannel using a 60× microscope objective and we arbitrarily designated each cell as either normal or abnormal and attempted to direct each cell to its designated outlet. Five trials with sperm samples from five different donors were carried out and in each trial the number of true positives (TP) was counted. TP is defined as the total number of sperm cells designated as
normal that were selected into outlet 2, and selectivity is defined as the total number of TP sperm cells divided by the total number of sperm cells designated as normal.

In order to calculate selectivity, we elected to arbitrarily designate 30 out of 100 sperm cells flowing through the microchannel as normal, and the remaining 70 as abnormal. During the tests, we were able to successfully select 26 to 28 normal cells, resulting in a selectivity of 89.5±3.5%. More importantly, during these tests no abnormal sperm cells were inadvertently directed into outlet 2 during the sperm cell selection process. This is due to the design of our model, since as long as syringe pump 2 is not activated all sperm cells will flow to outlet 1. This is of great importance as only one good sperm cell is needed for fertilizing the oocyte, and thus we cannot allow a single abnormal sperm to be directed into the normal-decision reservoir. On the other hand, missing some normal-decision sperm cells is not critical. Obtaining high selectivity with this selection protocol makes our approach a promising tool for sperm cell selection. Note that the calculation of the selectivity was performed by arbitrarily designating sperm cells as normal and abnormal. On the other hand, in the following section the sperm cells were known to actually be normal or abnormal based on the embryologist’s assessment, the current gold standard. The classification results of the computer algorithm were then compared to the results achieved by the embryologist.

3.3. Semi-automatic sperm analysis using IPM

A comparison study between the gold-standard method of sperm cell analysis for ICSI (visual inspection by an experienced embryologist) and a semi-automatic computer selection based on our IPM images was performed. In total, 132 living sperm cells were recorded using IPM as they flowed through the proposed microchannel and quantitative phase map videos of these flowing cells were generated, similar to the one shown in Fig. 2. The videos were recorded at a frame rate of 40 frames per second. The minimum frame rate required for proper imaging of the sperm cells is dependent on the size of the FOV as well as the flow rate through the device. The frame rate must be high enough and the exposure time must be short enough to avoid
motion blur. Practically, in our experiments, no significant blurring effects were observed by either the embryologist or the experimentalist. To verify this, we used a 5 µm polymer microsphere, flowing in the micro-channel under the same conditions of the sperm cells, where after image acquisition we measured the given diameter, and we found that the aspect ratio between the axis of flow and the orthogonal axis is 1. The analysis was performed both by our group (S.K.M. and I.B.), using a computer algorithm which analyzed a single image per cell, and independently by an experienced clinical embryologist (M.L.) who examined the dynamic OPD map, and classified the cells as normal and abnormal cells as explained above in the embryologist assessment section. In parallel to the clinical embryologist, a computer algorithm was used to determine the total sperm head area and acrosome head area, by isolating the sperm head from the single best OPD map image, in which the cell components are the most visible, and determining the nucleus and acrosome regions based on an OPD threshold of 105 nm [21]. It was found that only 67% of the measurements of the acrosome area relative to the total sperm head area (relative acrosome area, RAA) performed by our computer algorithm were in agreement with the embryologist’s visual assessment. This is illustrated in Fig. 3, in which it can be seen that a large number of the cells that the embryologist assessed as having RAA between 40% - 70% were found by our algorithm to possess RAA below 40%. In addition, in many of the cases where the embryologist determined that the RAA was below 40%, our algorithm was unable to distinguish such small acrosomes from the periphery of the cell and determined that these cells possessed no acrosome (corresponding to an RAA value of 0). In the cases where the algorithm was not in agreement with the embryologist, it was found that the embryologist overestimated the relative acrosome size by approximately 12% of the total head area, on average. This would imply that the algorithm-based analysis of RAA may be more accurate than the current method of visual analysis by embryologist.
Fig. 3. Distributions of RAA values of all 132 sperm cells, excluding those cells that the algorithm identified as not possessing an acrosome. Blue circles represent the cells that the embryologist determined as possessing RAAs between 40% - 70%. Red circles represent cells that the embryologist determined as possessing RAAs of less than 40% or greater than 70%. Dashed lines indicate mean values.

Furthermore, we classified sperm cells based on the dynamic OPD map. Taking into account four of the five WHO criteria (the midpiece criterion was excluded as only head characteristics were measured), 9 sperm possessed normal morphology as per the embryologist’s assessment, while the remaining 123 sperm failed in at least one of the four criteria. In order to classify the sperm cells, the maximum and minimum values of the head area, width to length ratio, and dry mass were calculated. Any cell with values falling within the ranges of these maximum and minimum values, as well as possessing a RAA between 30%-70%, was classified as a normal cell, while all other cells were classified as abnormal. The distributions of the parameters as well as the classification process are illustrated in Fig. 4. The range of 30%-70% was chosen in place of the typical 40%-70% in order to compensate for possible overestimation of the RAA on the part of the embryologist, as the embryologist tends to overestimate the area of small acrosomes by approximately 12%, as detailed above.
True positive (TP) in this case is defined as the number of correctly classified normal sperm, true negative (TN) is defined as the number of correctly classified abnormal sperm, false negative (FN) is defined as the number of incorrectly classified normal sperm, and false positive (FP) is defined as the number of incorrectly classified abnormal sperm.

This classification achieved precision \([\frac{TP}{TP + FP}]\) and specificity \([\frac{TN}{TN + FP}]\) of 100% with a corresponding sensitivity \([\frac{TP}{TP + FN}]\) of 67%.

Regarding the parameter of head width to length ratio, it is interesting to note that these values for the 9 normal sperm cells fall within the accepted normal range of approximately 3:5 or 0.6. In the case of the 9 sperm cells, this ratio has values between 0.55 – 0.64, while the abnormal sperms possess ratios as low as 0.40 and as high as 0.88. These results indicate that our algorithm is capable of accurately determining the head width to length ratio.

In order to determine the significance of the dry mass parameter, the classification process detailed above was repeated using all of the parameters except for the dry mass parameter. This classification maintained the same sensitivity of 67%, however the specificity dropped to 95% leading to a precision of only 50%. This result indicates that dry mass, a parameter only measurable through use of IPM, provides valuable information for the classification of sperm cell morphology.
Fig. 4. (a) Scatterplot of all 132 sperm cells based on 2 of the 4 classification parameters. Dashed lines indicate the maximum and minimum thresholds for each parameter, as used for classification. The cutoff values are equal to the maximum and minimum values achieved by cells that were determined by the embryologist to be normal in all of the four relevant WHO criteria. The exception to this is the relative acrosome area parameter, whose cutoffs of 30% - 70% are based on the WHO criteria for acrosome size. Blue circles represent sperm cells possessing normal morphology based on embryologist assessment of all four relevant WHO criteria. Red circles represent sperm cells possessing abnormal morphology in at least one out of the four WHO criteria, as per embryologist assessment. (b) Scatterplot of those 21 sperm cells from (a) whose data points fell within the yellow rectangle, based on the remaining two classification parameters. The rectangular cyan region illustrates the final classification; only those cells whose data points fell within this region as well as the yellow region in (a) were classified as normal cells. All others were classified as abnormal.

This paper presented a semi-automatic sperm selection. Further work will be carried out to construct a fully automatic sperm cell selection device that can perform real-time sperm cell analysis and selection. This device will integrate our machine-learning-based classification algorithm for automated analysis of sperm cell,[37] combined with motion
tracking algorithms in order to perform real-time analysis of the flowing sperm cells. The computer will then automatically activate the pump for sperm cell selection based on the algorithm decisions.

4. Conclusions
A disposable microfluidic platform for individual sperm cell selection integrating quantitative stain-free imaging was successfully developed. The results show that by controlling the flow rate, one-by-one selection of multiple sperm cells possessing normal morphological characteristics can be achieved in a single run with the proposed microfluidic device. We achieved a high selectivity of 89.5±3.5% with this selection protocol, and no abnormal sperm cells were selected. Furthermore, application of the computer-based sperm morphology analysis using OPD map images resulted in precision and specificity of 100% and sensitivity of 67%. While the typical embryologist requires an average of 10 seconds to analyze the quality of a single sperm cell, our system was capable of performing this analysis in less than a second. The possibilities inherent in the real-time imaging of flowing sperm cells are expected to motivate the integration of the real-time quantitative imaging setup with computerized selection tools, creating an automatic sperm cell imaging and selection system.

Additionally, the portability, ease of handling, and miniaturized size of the microfluidic device make it possible to integrate this proposed microfluidic platform with existing microscopy techniques that can be utilized for sperm cell selection, such as BFM, PhC, DIC, or IPM. Moreover, these microfluidic tools are disposable, and thus avoid the risk of cross-contamination of medium and cells between samples. Further work will be carried out to produce a lab-on-a-chip sperm cell selection device by integrating micro-pumps to the microchannel, enabling the induction of a higher throughput and more controlled flow, and making the wiring of electronics to the disposable device easier. This model demonstrates the possibility to analyze and select a single sperm cell using a single micro-channel device;
however, higher throughput can be achieved in the future by implementing a similar device containing multiple channels.

**Conflict of interest**

There are no conflicts to declare.

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