

# Clinically Translatable Passive Microfluidic Device for Preparation of Human Sperm with Variable Baseline Concentrations

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## Article

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# Abstract

The global decline in male sperm concentrations necessitates the development of effective protocols for the preparation of low-concentration samples for assisted reproductive technologies. Standard centrifugation protocols are characterized by limited cell yield and create unwanted mechanical stress. We developed a passive microfluidic system-based protocol combining a gentle, one-step preconcentration method with a PMMA device with 200 parallel channels. This platform allows the isolation of motile sperm by active migration over a wide range of initial concentrations. A clinical evaluation of 44 samples demonstrated the superiority of the microfluidic system over density gradient centrifugation (DGC) in enriching sperm showing higher progressive, overall motility, preserved DNA integrity, and normal morphology. The degree of improvement correlated with the initial concentration: the largest relative increase in quality was observed in samples with concentrations below  $40 \times 10^6$  sperm/mL. Analysis in three concentration ranges ( $> 115$ ,  $40\text{--}115$ , and  $< 40 \times 10^6$  sperm/ml) confirmed the consistent recovery of high-quality sperm, regardless of the initial parameters. Furthermore, the integration of a low-speed pre-concentration step simplified the processing of diluted samples, and proof-of-concept intracytoplasmic sperm injection (ICSI) experiments confirmed the full compatibility of the method with standard embryology procedures. Overall, this approach represents a reliable, safe alternative for working with clinical samples characterized by variable parameters.

## 1. Introduction

Approximately one in six couples worldwide experience infertility, with male-related factors accounting for nearly half of all cases. The quality of sperm chosen for fertilization has a direct impact on fertilization, embryo development, implantation, and pregnancy outcomes, making effective sperm preparation a crucial step in both conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).<sup>1,4–7</sup> Recent data show a persistent global decline in sperm concentration and total sperm count, along with rising rates of impaired sperm motility and DNA damage.<sup>1–5</sup>

Density gradient centrifugation (DGC) and swim-up remain the most popular sperm preparation methods in clinical practice because they efficiently enrich motile sperm while removing debris, leukocytes, and immotile cells.<sup>1,6–9</sup> However, both procedures rely on repeated centrifugation or prolonged incubation, which may increase reactive oxygen species (ROS) production, induce oxidative stress, and compromise sperm DNA integrity in susceptible samples.<sup>8–12</sup> Although DGC typically yields satisfactory recovery in increasingly motile semen samples, especially those with poor baseline quality.<sup>9–12</sup> Consequently, there is considerable interest in developing alternative sperm preparation technologies that minimize physical manipulation while maintaining or improving sperm quality.

To isolate highly motile sperm with superior DNA integrity, a variety of microfluidic platforms have been developed based on rheotaxis, boundary-following behavior, chemotaxis, thermotaxis, or passive migration through microchannels.<sup>13–18</sup> Several studies have shown that microfluidically selected sperm exhibit lower DNA fragmentation and improved functional quality compared with sperm prepared using

conventional DGC.<sup>14,15,17,19–22</sup> Meanwhile, advances in polymer microfabrication have made it easier to develop disposable and clinically translatable microfluidic devices.<sup>23–27</sup> Specifically, polymethyl methacrylate (PMMA) is a desirable substrate for routine clinical applications due to its exceptional optical transparency, mechanical robustness, low material cost, and compatibility with laser micromachining and large-scale manufacturing.<sup>25,26</sup>

Despite these developments, relatively little attention has been paid to a significant drawback of passive microfluidic sperm selection. The majority of passive microfluidic devices depend on sufficient motile sperm entering the sorting microchannels and operate with a fixed loading volume.<sup>12–17</sup> Therefore, passive microfluidic systems may not be applicable to one of the patient groups most likely to benefit from improved sperm selection because samples with lower baseline sperm concentration may contain an inadequate density of progressively motile sperm to achieve effective recovery. Few studies have so far thoroughly examined the effects of baseline sperm concentration on microfluidic sorting performance or proposed practical methods for extending passive microfluidic sperm selection to semen with lower concentrations.<sup>12,13,16</sup> Whether baseline sperm concentration influences the effectiveness of passive microfluidic sperm selection remains poorly understood.

In this work, we developed a pump-free, laser-fabricated PMMA microfluidic sperm-sorting platform for passive motility-based sperm selection. We combined the device with a straightforward single-step low-speed pre-concentration procedure prior to microfluidic sorting to process semen samples with lower baseline sperm concentrations. First, experimental characterization and computational fluid dynamics simulations were used to validate device performance. Subsequently, paired human semen samples were used to assess the microfluidic workflow and directly compare it with traditional DGC. Finally, we evaluated the viability of using microfluidically selected sperm for standard ICSI procedures and investigated whether baseline sperm concentration affects treatment response. By combining a clinically compatible preprocessing strategy with passive microfluidic selection, this work establishes a practical workflow for extending microfluidic sperm preparation to challenging clinical specimens while providing new insight into the influence of baseline sperm concentration on treatment efficacy.

## 2. Results

### 2.1. Device architecture and validation

The device consisted of a proximal loading chamber, a lateral waste chamber and a distal collection chamber connected by 200 parallel microchannels (20 mm long, 200  $\mu\text{m}$  wide and 130  $\mu\text{m}$  high). The PMMA device was fabricated by direct  $\text{CO}_2$  laser micromachining, followed by chloroform-vapour polishing and thermal bonding. The resulting device was optically clear and showed no visible delamination or leakage during assembly validation. A merged schematic and workflow overview is shown on Fig. 1.

In order to test the efficiency of the microchannel design and check if the selected migration distance of 20 mm is sufficient for passive selection of sperm, COMSOL simulation was performed. The particle tracing simulation showed that there was a lag in the arrival of motile sperm particles in the collection chamber, with the amount of particles increasing gradually with time (Fig. 2). A higher level of selection according to the swimming capacity was achieved in the 20 mm design because of its longer migration distance and the lower number of the sperm that entered the outlet during the simulation. This confirms the use of a longer migration distance in order to keep constant the passive transport conditions while selecting highly motile sperm. Computational details can be found in the Supplementary Material (S6).

## 2.2. Microfluidic enrichment of sperm motility, DNA integrity and morphology

Paired semen samples from 44 participants were processed by the microfluidic workflow and DGC. For the primary analysis, samples were stratified by baseline sperm concentration into two groups to assess its effect on microfluidic performance: Group A ( $< 40 \times 10^6$  sperm  $\text{mL}^{-1}$ ;  $n = 14$ ) and Group B ( $\geq 40 \times 10^6$  sperm  $\text{mL}^{-1}$ ;  $n = 30$ ). This study-specific threshold reflected the need for an additional low-speed concentration step before microfluidic processing in samples below  $40 \times 10^6$  sperm  $\text{mL}^{-1}$  and was not intended as a diagnostic threshold for oligozoospermia. Group A therefore included a heterogeneous range of semen quality, including oligozoospermic ( $n = 2$ ), asthenozoospermic ( $n = 5$ ), and oligo-teratozoospermic ( $n = 5$ ) samples. This stratification enabled assessment of device performance across clinically relevant baseline sperm concentrations.

**Table 1. Participant demographics and baseline semen characteristics. Concentrations expressed as  $\times 10^6/\text{mL}$ . Primary/secondary infertility refers to couple infertility diagnosis.**

| Characteristic                                    | Overall (n = 44) | Group A (n = 14) | Group B (n = 30)  |
|---------------------------------------------------|------------------|------------------|-------------------|
| Age, years (Mean $\pm$ SD)                        | 32.4 $\pm$ 5.8   | 32.9 $\pm$ 6.2   | 32.1 $\pm$ 5.6    |
| Primary infertility, n (%)                        | 31 (70.5%)       | 10 (71.4%)       | 21 (70.0%)        |
| Secondary infertility, n (%)                      | 13 (29.5%)       | 4 (28.6%)        | 9 (30.0%)         |
| Abstinence, days                                  | 3–10             | 3–10             | 3–10              |
| Ejaculate volume, mL (Mean $\pm$ SD)              | 3.2 $\pm$ 1.1    | 2.9 $\pm$ 1.2    | 3.4 $\pm$ 1.0     |
| Initial conc. ( $10^6/\text{mL}$ , Mean $\pm$ SD) | 79.6 $\pm$ 51.3  | 26.10 $\pm$ 2.22 | 104.61 $\pm$ 7.84 |
| Oligozoospermia, n                                | 2                | 2                | 0                 |
| Asthenozoospermia, n                              | 5                | 5                | 0                 |
| Oligo-teratozoospermia, n                         | 5                | 5                | 0                 |
| Normal spermiogram, n                             | 32               | 2                | 30                |

The most pronounced improvements were observed for sperm motility (Fig. 3a-3d). In the normal-concentration cohort, total motility increased from  $73.61 \pm 3.40\%$  in raw semen to  $95.49 \pm 0.76\%$  following microfluidic processing, compared with  $87.33 \pm 1.57\%$  after DGC (both  $p < 0.0001$  versus RAW). Progressive motility exhibited an even greater enhancement, increasing from  $29.57 \pm 2.07\%$  to  $81.37 \pm 1.15\%$  after chip processing, whereas DGC increased progressive motility to  $59.71 \pm 2.39\%$  ( $p < 0.0001$ ). Similar improvements were observed in the reduced-concentration cohort, where total motility increased from  $50.81 \pm 4.79\%$  to  $92.68 \pm 2.02\%$ , while progressive motility increased from  $24.24 \pm 4.27\%$  to  $77.25 \pm 3.24\%$  following microfluidic selection (both  $p < 0.0001$  versus RAW). In both cohorts, representative CASA images demonstrated a marked enrichment of progressively motile sperm following microfluidic processing (Fig. 3e-3j).

DNA integrity was also significantly improved following microfluidic selection (Fig. 4a-4b). In the normal-concentration cohort, the proportion of sperm with non-fragmented DNA increased from  $88.04 \pm 1.43\%$  in raw semen to  $98.04 \pm 0.40\%$  after chip processing, compared with  $95.81 \pm 0.80\%$  following DGC. Both processing methods significantly improved DNA integrity relative to RAW ( $p < 0.0001$ ), whereas no statistically significant difference was detected between the chip and DGC ( $p = 0.08892$ ). In the reduced-concentration cohort, DNA integrity increased from  $83.36 \pm 3.02\%$  in raw semen to  $96.64 \pm 0.86\%$  after chip processing, exceeding the improvement obtained with DGC ( $91.00 \pm 2.48\%$ ) (Fig. 4c-4e).

Representative CASA images (sperm chromatin dispersion (SCD) assay) of sperm DNA fragmentation in normal concentration specimens (c-e). (c) Raw semen: mixed population, frequent fragmented nuclei (red boxes). (d) DGC fraction: mostly intact nuclei with residual fragmented cells. (e) Chip fraction: near-uniform enrichment of intact-DNA spermatozoa. Red = fragmented DNA; green = intact DNA.

In the normal-concentration cohort, normal morphology increased from  $3.20 \pm 0.49\%$  in raw semen to  $6.87 \pm 0.68\%$  following chip processing, compared with  $5.32 \pm 0.64\%$  after DGC. Improvement following chip processing was significant relative to RAW ( $p < 0.0001$ ), while the difference between the chip and DGC approached statistical significance ( $p = 0.05254$ ). In the reduced-concentration cohort, morphology increased from  $2.14 \pm 0.91\%$  to  $3.36 \pm 0.94\%$  after chip processing, whereas DGC produced no measurable improvement ( $1.50 \pm 0.50\%$ ). However, neither treatment reached statistical significance relative to RAW within this subgroup (Fig. 5).

Following Holm–Bonferroni correction, DGC significantly increased total motility, progressive motility, and DNA integrity compared with raw semen. Passive microfluidic preparation produced significantly greater improvements in all three parameters than both DGC and raw semen in both concentration cohorts. However, post-processing sperm concentration remained significantly lower than the DGC and raw baseline in both groups ( $p < 0.0001$ ), reflecting sperm selection rather than recovery of the entire sperm population. Complete within-group comparisons are summarized in Table 2.

Table 2

Semen parameters after raw, DGC, and microfluidic chip processing. Mean  $\pm$  SD. P-values from One-way Anova followed by Dunnett's and Tukey multiple comparison testing in OriginPro2026. For DNA integrity in the reduced concentration group, three p-values indicate DGC vs. RAW, CHIP vs. RAW, DGC vs. CHIP.

| Group                                                                           | Parameter                                                   | RAW                  | DGC                 | CHIP                | p-value<br>DGC-<br>RAW | p-value<br>CHIP-<br>RAW | p-value<br>CHIP-<br>DGC |
|---------------------------------------------------------------------------------|-------------------------------------------------------------|----------------------|---------------------|---------------------|------------------------|-------------------------|-------------------------|
| Normal<br>concentration<br>( $> 40 \times 10^6$<br>$\text{mL}^{-1}$ , n = 30)   | Concentration<br>( $\times 10^6 \text{ mL}^{-1}$ )          | 104.61<br>$\pm 7.84$ | 27.96<br>$\pm 3.65$ | 3.50<br>$\pm 0.65$  | $< 0.0001$             | $< 0.0001$              | 2.66379E-4              |
|                                                                                 | Total motility<br>(%)                                       | 73.61<br>$\pm 3.40$  | 87.33<br>$\pm 1.57$ | 95.49<br>$\pm 0.76$ | $< 0.0001$             | $< 0.0001$              | 0.00602                 |
|                                                                                 | Progressive<br>motility (%)                                 | 29.57<br>$\pm 2.07$  | 59.71<br>$\pm 2.39$ | 81.37<br>$\pm 1.15$ | $< 0.0001$             | $< 0.0001$              | $< 0.0001$              |
|                                                                                 | Normal<br>morphology<br>(%)                                 | 3.20 $\pm$<br>0.49   | 5.32<br>$\pm 0.64$  | 6.87<br>$\pm 0.68$  | 0.00623                | $< 0.0001$              | 0.05254                 |
|                                                                                 | DNA integrity<br>(%)                                        | 88.04<br>$\pm 1.43$  | 95.81<br>$\pm 0.80$ | 98.04<br>$\pm 0.40$ | $< 0.0001$             | $< 0.0001$              | 0.08892                 |
|                                                                                 | Total motile<br>sperm ( $\times 10^6$<br>$\text{mL}^{-1}$ ) | 81.43<br>$\pm 8.43$  | 25.29<br>$\pm 3.52$ | 3.37<br>$\pm 0.65$  | $< 0.0001$             | $< 0.0001$              | 0.00183                 |
| Reduced-<br>concentration<br>( $< 40 \times 10^6$<br>$\text{mL}^{-1}$ , n = 14) | Concentration<br>( $\times 10^6 \text{ mL}^{-1}$ )          | 26.10<br>$\pm 2.22$  | 8.15<br>$\pm 1.48$  | 9.47<br>$\pm 2.46$  | $< 0.0001$             | $< 0.0001$              | 0.85041                 |
|                                                                                 | Total motility<br>(%)                                       | 50.81<br>$\pm 4.79$  | 60.69<br>$\pm 5.00$ | 92.68<br>$\pm 2.02$ | 0.0135                 | $< 0.0001$              | $< 0.0001$              |
|                                                                                 | Progressive<br>motility (%)                                 | 24.24<br>$\pm 4.27$  | 36.16<br>$\pm 5.13$ | 77.25<br>$\pm 3.24$ | 0.00201                | $< 0.0001$              | $< 0.0001$              |
|                                                                                 | Normal<br>morphology<br>(%)                                 | 2.14 $\pm$<br>0.91   | 1.5 $\pm$<br>0.5    | 3.36<br>$\pm 0.94$  | 0.53221                | 0.13938                 | 0.01808                 |
|                                                                                 | DNA integrity<br>(%)                                        | 83.36<br>$\pm 3.02$  | 91 $\pm$<br>2.48    | 96.64<br>$\pm 0.86$ | 0.00379                | $< 0.0001$              | 0.03004                 |
|                                                                                 | Total motile<br>sperm ( $\times 10^6$<br>$\text{mL}^{-1}$ ) | 13.62<br>$\pm 1.81$  | 5.64<br>$\pm 1.24$  | 8.97<br>$\pm 2.42$  | 0.00229                | 0.07896                 | 0.23919                 |

## 2.3. Baseline sperm concentration modifies treatment response

To test whether baseline sperm concentration altered the magnitude of microfluidic benefit, treatment response was expressed as  $\Delta = \text{CHIP} - \text{RAW}$ . A significant Group  $\times$  Method interaction was observed for total motility ( $F(1,42) = 11.84, p = 0.001$ ), indicating that the effect of processing differed between concentration groups. The reduced-concentration group showed a larger increase in total motility than the higher-concentration group (median  $\Delta = 41.87\%$  versus  $21.88\%$ ; Mann–Whitney  $U = 108.0, p = 0.002$ , Holm–Bonferroni-adjusted  $p = 0.008$ ). Significant concentration-dependent interactions were also observed for progressive motility and DNA integrity (both  $p < 0.01$ ), whereas normal morphology showed no significant interaction ( $p = 0.312$ ).

The magnitude of treatment response for each outcome measure is illustrated in Fig. 6. Progressive motility increased substantially following passive microfluidic sperm selection in both cohorts, with mean improvements of  $53.01\%$  in the reduced-concentration cohort and  $51.80\%$  in the higher-concentration cohort (Fig. 6a). The largest concentration-dependent effect was observed for total motility (Fig. 6b), which increased by  $41.87\%$  in the reduced-concentration cohort compared with  $21.88\%$  in the higher-concentration cohort, representing an approximately two-fold greater improvement. DNA integrity also exhibited a concentration-dependent response (Fig. 6c), with the proportion of sperm possessing intact DNA increasing by  $13.28\%$  and  $10.00\%$  in the reduced- and higher-concentration cohorts, respectively. In contrast, improvements in normal morphology were comparable between groups ( $1.22\%$  versus  $3.67\%$ ; Fig. 6d), consistent with the absence of a significant Group  $\times$  Method interaction.

All these results show that baseline sperm concentration plays a significant role in determining the effectiveness of passive microfluidic sperm preparation. The workflow greatly enhanced sperm quality in both concentration cohorts, but in samples with lower baseline sperm concentration, it was most beneficial for motility and DNA integrity. On the other hand, improvements in sperm morphology appeared largely independent of the initial sperm concentration, suggesting that in reduced-concentration semen, passive microfluidic sperm selection preferentially improves functional rather than structural sperm characteristics.

## 2.4. Secondary analysis across three baseline concentration groups

An exploratory secondary analysis divided the cohort into three approximately equal-sized groups:  $>115 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 15$ ),  $40\text{--}115 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 15$ ), and  $< 40 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 14$ ). The  $115 \times 10^6$  sperm  $\text{mL}^{-1}$  threshold was selected to balance subgroup sizes and was not intended as a biological or clinical cutoff. Across all three groups, microfluidic processing produced high post-processing DNA integrity, progressive motility and total motility compared to DGC. DNA integrity reached  $98.40 \pm 1.72\%$ ,  $97.67 \pm 2.60\%$  and  $96.65 \pm 2.64\%$  in the three groups, respectively, while progressive motility reached  $82.48 \pm 7.03\%$ ,  $80.27 \pm 5.48\%$  and  $77.25 \pm 12.14\%$ . Total motility reached  $96.23 \pm 2.56\%$ ,  $94.74 \pm 5.26\%$  and  $92.67 \pm 7.56\%$ .

The reduced-concentration subgroup ( $< 40 \times 10^6$  sperm  $\text{mL}^{-1}$ ) showed the greatest absolute improvements after microfluidic processing, particularly in progressive and total motility, despite having

substantially poorer initial semen quality. Notably, the final sperm quality in this group approached that observed in the higher-concentration groups, indicating that the workflow could effectively enrich a highly functional sperm population across a broad range of starting concentrations. The recovered sperm concentration was also higher in the  $< 40 \times 10^6/\text{mL}$  group ( $9.46 \pm 9.21 \times 10^6/\text{mL}$ ) than in the  $> 115 \times 10^6/\text{mL}$  and  $40\text{--}115 \times 10^6/\text{mL}$  groups ( $4.69 \pm 4.59$  and  $2.32 \pm 1.59 \times 10^6/\text{mL}$ , respectively); however, this difference primarily reflects the additional low-speed pre-concentration step used for reduced-concentration samples before chip loading and should not be interpreted as an intrinsic concentration-enrichment effect of the microfluidic device. Full three-group data are provided in Supplementary Table S1.

## 2.5. Translational feasibility of the passive microfluidic workflow for ICSI

In this proof-of-concept assessment, sperm selected using the passive microfluidic chip were successfully used for conventional ICSI without modification of the standard laboratory procedure. Embryological outcomes were comparable with those obtained using conventional DGC. Among 19 embryos generated using microfluidically selected sperm and 18 embryos generated using DGC-prepared sperm, the mean pronuclear score was similar between groups (1.79 vs. 1.83, respectively;  $p = 0.84$ ), as was the mean iDAScore (7.41 vs. 7.91;  $p = 0.5243$ ). Ten embryos in the microfluidic group and six in the DGC group were cryopreserved; three embryos from the DGC group underwent fresh embryo transfer. The three fresh transfers reported here involved embryos generated using DGC-prepared sperm and were performed in human recipients as part of routine ART care. We obtained written informed consent for embryo transfer from each recipient before the procedure.

## 3. Discussion

This study presents a clinically compatible passive microfluidic sperm preparation workflow combining a rapidly fabricated laser-fabricated PMMA device with a single-step pre-concentration protocol. The workflow performed better than traditional density gradient centrifugation (DGC) and improved progressive motility, total motility, DNA integrity, and normal morphology relative to raw semen. The magnitude of improvement differed across the concentration groups, with the largest gains in motility and DNA integrity observed in samples with lower initial concentrations. Sperm recovered from the device was also successfully used for routine ICSI, providing an initial indication of compatibility with established embryology procedures. Pronuclear (PN) scores were comparable to those obtained after DGC, while a greater proportion of embryos met cryopreservation criteria (52.6% vs 33.3%). These embryological findings remain preliminary because only three proof-of-concept cases were evaluated. Conventional sperm preparation methods such as density gradient centrifugation and swim-up are widely used in assisted reproduction, but centrifugation and repeated handling can contribute to mechanical and oxidative stress and may affect sperm DNA integrity.<sup>6–9</sup> Microfluidic approaches have therefore been investigated as alternatives for recovering motile sperm with less centrifugation-related handling.<sup>12–17</sup> A practical limitation of passive microfluidic sperm selection is that reduced-concentration samples may

not provide sufficient sperm for direct loading and recovery. We addressed this limitation by introducing a single low-speed pre-concentration step before chip loading, without increasing device complexity. The purpose of this step was to increase sperm density within the fixed loading volume and ensure adequate loading of the device, while sperm selection within the microchannels remained driven by progressive motility. This approach enabled reduced-concentration samples in the present cohort to be processed using the same passive, pump-free device architecture.

Baseline sperm concentration was associated with differences in the magnitude of response to microfluidic selection. Improvements in total motility, progressive motility, and DNA integrity were greater in samples with lower initial concentrations, although sperm quality improved across the cohort. The secondary analysis using three concentration groups ( $> 115$ ,  $40-115$ , and  $< 40 \times 10^6/\text{mL}$ ) further showed high post-processing sperm quality across the concentration range examined. Adding a single pre-concentration step enabled the workflow to accommodate lower-concentration samples and may have contributed to the greater response observed in this group. The step consisted of low-speed centrifugation at 1000 rpm (approximately  $180 \times g$ ) for 10 minutes to increase sperm concentration per unit volume before loading. Its purpose was to standardize sperm availability within the fixed 200  $\mu\text{L}$  loading volume rather than to perform density-based sperm selection. The greater improvement observed in reduced-concentration samples may reflect the larger proportion of functionally heterogeneous sperm available for motility-based enrichment. Thus, the pre-concentration step primarily enabled efficient device loading, while the subsequent microfluidic process provided the motility-based selection responsible for sperm enrichment.

The enrichment in DNA-intact sperm provides an additional indication of improvement in sperm quality beyond motility. Sperm DNA damage has been investigated as a complementary measure of sperm function in male infertility and assisted reproduction.<sup>9,31</sup> In contrast, the response in normal morphology did not differ significantly between groups, indicating that the concentration-dependent effect was primarily associated with functional sperm characteristics rather than morphology.

Previous studies have established passive microfluidic sperm selection as a biomimetic approach for recovering highly motile sperm while reducing handling associated with conventional centrifugation-based preparation.<sup>12-17</sup> The improvements in motility and DNA integrity observed here are consistent with this previous work. Studies by Shirota, Quinn and others have also reported improved sperm quality or reduced DNA damage using passive microfluidic platforms.<sup>14,15</sup> Most previous studies have focused on device-level parameters, including channel geometry, flow configuration, fabrication, and recovery efficiency.<sup>12-17</sup> In comparison, the practical adaptation of passive devices to lower-concentration semen has received less attention. Our findings suggest that this limitation can be addressed through relatively simple workflow modification rather than by increasing device complexity. A single pre-concentration step enabled lower-concentration samples to be processed while retaining passive, motility-based selection within the same device architecture. Crucially, the PMMA device can be made directly using laser machining, eschewing the cleanroom processing, photomasks, photoresists, and specialized lithography equipment that are usually involved in traditional photolithographic microfabrication. Rapid

prototyping and repeatable production are made possible by this fabrication method, which may also reduce the infrastructure needed for implementation in labs without specialized microfabrication facilities. Another significant translational benefit is the combination of a straightforward fabrication route and a standardized sample-preparation workflow. The standardized workflow may also reduce operator-dependent variability by limiting the number of manual processing steps. The suggested method allows for variation in initial sperm concentration through a specified preprocessing step, as opposed to necessitating modifications to the underlying microchannel architecture for various semen characteristics. When adapting passive microfluidic sperm selection to heterogeneous clinical samples, this separation of device function from specimen-specific preparation may help standardize the workflow and lessen the need for customized device designs. The results also suggest that treatment response is influenced by the characteristics of the starting sample. This is relevant to translational microfluidics because device performance is closely linked to the biological material being processed. Future development of microfluidic sperm-selection systems may therefore benefit from considering specimen-specific processing strategies alongside device design.

The proposed workflow provides a way to extend passive biomimetic sperm preparation to lower-concentration samples without requiring active sorting, external pumping, or additional device complexity. The pre-concentration step uses conventional laboratory procedures and could potentially be incorporated into existing IVF workflows. The combination of a disposable PMMA device, pump-free operation, and simple sample preparation provides a basis for further evaluation in routine ART settings. The use of microfluidically selected sperm for ICSI provides an additional proof-of-concept for translational compatibility. Previous studies have evaluated microfluidic sperm selection in relation to ICSI and embryological outcomes.<sup>19–22</sup> In the present study, microfluidically selected sperm were used without modification of standard embryology procedures. PN scores were comparable to those obtained after DGC, and embryos were subsequently evaluated during routine preimplantation development. The higher proportion of embryos meeting cryopreservation criteria in the microfluidic group is encouraging, but the number of procedures was too small to draw conclusions regarding embryological or clinical outcomes. In addition to its biological performance, the workflow has a number of useful benefits for application in reproductive medicine. By limiting the number of manual processing steps and avoiding complicated fluidic control or external pumps during sperm selection, the standard operating procedure may lessen operator-dependent variability and make implementation in clinical laboratories easier. Pump-free operation, and standardized sample preparation may lessen the technical burden associated with traditional sperm-selection workflows, even though processing time and operator workload were not formally quantified in this study. Several limitations should be acknowledged. This was a single-center study involving 44 semen samples. The explanation for the greater response in lower-concentration samples remains inferential because sperm trajectories, channel occupancy, and hydrodynamic interactions were not directly measured. The ICSI evaluation was also limited to three proof-of-concept cases and was not designed to assess fertilization rates, embryo quality in a larger population, pregnancy, or live-birth outcomes. The present findings therefore demonstrate technical and biological feasibility but do not establish clinical superiority over existing sperm preparation methods. Larger

prospective multicenter studies will be needed to determine whether the observed differences in treatment response are reproducible across broader patient populations and whether improvements in sperm quality translate into differences in reproductive outcomes. Mechanistic studies using particle tracking, high-speed imaging, and computational modelling could further clarify how sperm concentration influences transport and selection within the microchannels. Overall, the present findings indicate that baseline sperm concentration is associated with the magnitude of response to passive microfluidic sperm selection. A simple pre-concentration step enabled lower-concentration samples in this cohort to be processed using the same passive workflow, while the largest relative improvements were observed in samples with lower initial concentrations. This combination of simple device architecture and adaptable sample preparation provides a basis for further translational evaluation of passive microfluidic sperm-selection technology.

## **4. Materials and Methods**

### **4.1. Microfluidic Chip Design and Fabrication**

The passive microfluidic device was designed in SOLIDWORKS 2022 (Dassault Systèmes SOLIDWORKS Corporation, Waltham, MA, USA) and fabricated from 2-mm-thick polymethyl methacrylate (PMMA) sheets (SIGNTRADE LLC, Dubai, UAE) using a CO<sub>2</sub> laser system (VLS3.50, Universal Laser Systems, Scottsdale, AZ, USA). The device comprised 200 parallel microchannels connecting a loading chamber to a collection chamber. Each microchannel was 200  $\mu\text{m}$  wide, 130  $\mu\text{m}$  deep, and 20 mm long. PMMA surfaces were treated with chloroform vapour before thermal bonding to improve optical transparency and reduce surface roughness. Three PMMA layers were aligned and thermally bonded under light uniaxial compression to form the enclosed microfluidic structure. The fabricated devices were visually inspected for channel integrity, leakage, and delamination before use and sterilized by electron-beam irradiation. Direct laser fabrication was used to enable rapid and reproducible production without photomasks, photoresists, or conventional photolithographic processing.

The channel length was chosen to allow enough migration distance for passive motility-based selection while maintaining a stable fluidic interface during sample loading and sperm recovery. Shorter channels were more vulnerable to liquid instability during manual loading and collection during initial device tuning, which led to less consistent device performance. The array's 200 parallel channels reflect the highest channel density that could be consistently produced while preserving dimensional accuracy throughout the available device area. The center-to-center spacing between adjacent channels was set to approximately 250  $\mu\text{m}$ , corresponding to the effective laser spot size and fabrication resolution of the CO<sub>2</sub> laser system. This spacing minimized thermal overlap between adjacent laser passes, preserved channel integrity, and enabled high-throughput sperm processing within a compact device footprint. Detailed laser parameters, layer dimensions, surface treatment, bonding conditions, and fabrication procedures are provided in the Supplementary Methods (S1).

Biocompatibility of the fabricated PMMA microfluidic devices was evaluated before their use for sperm preparation. Devices were subjected to HSSA according to a protocol. Briefly, comparative test between evaluated plastic used in the laboratory and tested sample consumables for sperm survive in 24 hours. The resulting biological response was assessed by measuring cell viability. Devices were considered biocompatible when no difference is observed from a control.

## 4.2. Computational modelling

Fluid flow and idealized sperm transport were simulated using COMSOL Multiphysics (COMSOL AB, Sweden) to compute the hydrodynamic conditions and motility-driven migration within the microfluidic sperm-sorting device. The model computed velocity distribution, pressure profile, and flow behavior throughout the loading chamber, microchannels, and collection chamber under the experimental operating conditions.

The modelling results showed uniform distribution of the fluid in the sorting device and absence of convective flow which proves that the movement of sperm cells in the channel occurs mainly due to their motility. These findings support the passive operating principle of the device and complement the experimental validation.

Detailed descriptions of the computational model, governing equations, boundary conditions, mesh independence analysis, and simulation results are provided in the Supplementary Materials (S6).

## 4.3. Patient cohort and stratification

The study included 44 men (mean age,  $32.4 \pm 5.8$  years) presenting for routine semen analysis. Participants were 18–50 years of age and provided semen samples following 3–10 days of sexual abstinence. Samples were allowed to liquefy at  $37^{\circ}\text{C}$  for 30–60 min in accordance with the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen, 6th edition (2021). An ejaculate volume of  $\geq 1.0$  mL was required for inclusion. Samples with azoospermia, leukocytospermia, incomplete liquefaction, excessive viscosity, or known genetic causes of impaired spermatogenesis were excluded. Of 49 recruited participants, five samples were excluded, including three with leukocytospermia and two with excessive viscosity. Baseline demographic and semen characteristics are summarized in Table 1. The study was conducted under institutional ethical approval, and written informed consent was obtained from all participants before sample collection.

For the primary analysis, samples were divided according to baseline sperm concentration into a reduced-concentration group ( $< 40 \times 10^6$  sperm  $\text{mL}^{-1}$ ;  $n = 14$ ) and a higher-concentration group ( $\geq 40 \times 10^6$  sperm  $\text{mL}^{-1}$ ;  $n = 30$ ). This study-specific threshold was selected because samples below  $40 \times 10^6$  sperm  $\text{mL}^{-1}$  required pre-concentration before microfluidic processing and was not intended to represent a diagnostic threshold for oligozoospermia.

An exploratory secondary analysis divided the cohort into three approximately equal groups:  $>115 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 15$ ),  $40\text{--}115 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 15$ ), and  $< 40 \times 10^6$  sperm  $\text{mL}^{-1}$  ( $n = 14$ ). The  $115 \times$

$10^6$  sperm  $\text{mL}^{-1}$  threshold was selected to obtain approximately balanced subgroup sizes and was not intended as a biological cutoff.

## 4.4. Experimental workflow

Following routine semen analysis, each sample was processed using both DGC and the passive microfluidic workflow. Samples with a baseline sperm concentration  $\geq 40 \times 10^6$  sperm  $\text{mL}^{-1}$  were loaded directly into the device. Samples below  $40 \times 10^6$  sperm  $\text{mL}^{-1}$  underwent a single low-speed pre-concentration step before loading.

For pre-concentration, 1.0–1.5 mL of liquefied semen was diluted in 5 mL of pre-warmed HEPES-buffered sperm wash medium (Vitromed GmbH, Heidelberg, Germany) and centrifuged once at 1000 rpm (approximately  $180 \times g$ ) for 10 min. The supernatant was removed, and the sperm pellet was gently resuspended in 200–300  $\mu\text{L}$  of fresh medium. A 200  $\mu\text{L}$  aliquot was then loaded into the microfluidic device. This procedure was performed solely to increase sperm density within the fixed loading volume and did not constitute a sperm-selection step. Biological enrichment in the microfluidic workflow occurred exclusively through passive sperm migration across the microchannel array.

For DGC, 1.0 mL of liquefied semen was layered onto a discontinuous 40%/80% density gradient and centrifuged at  $300\text{--}400 \times g$  for 15–20 min. The sperm pellet was recovered, washed at  $200 \times g$  for 10 min, and resuspended in 0.3–0.5 mL of fresh sperm washing medium. The detailed DGC protocol is provided in the Supplementary Methods.

Following each preparation method, sperm concentration, progressive motility, total motility, normal morphology, and DNA integrity were assessed using the same analytical procedures.

## 4.5. Microfluidic chip operation

Before sample loading, the device was primed with 1.0 mL of pre-warmed HEPES-buffered sperm washing medium to fill the microchannels and collection chamber and remove trapped air. A 200  $\mu\text{L}$  semen sample was then introduced into the loading chamber, and the device was incubated at  $25^\circ\text{C}$  for 1.5 h. During incubation, progressively motile sperm migrated through the 20-mm microchannels into the collection chamber by active swimming, whereas poorly motile and immotile sperm remained predominantly in the loading chamber.

Following incubation, the enriched sperm fraction was collected from the collection chamber and immediately analyzed for concentration, total and progressive motility, morphology, and DNA integrity. Detailed protocols for standardized assessment of sperm quality parameters are provided in the Supplementary Materials (S5).

## 4.6. Statistical analysis

Statistical analyses were performed using OriginPro 2026 (OriginLab Corporation, Northampton, MA, USA). Processing effects were evaluated using mixed-design ANOVA, with processing method (RAW, DGC, and CHIP) as the within-subject factor and baseline concentration group as the between-subject

factor. The Group  $\times$  Method interaction was used to determine whether the response to processing differed between concentration groups. Normality of model residuals was assessed using the Shapiro–Wilk test and sphericity using Mauchly's test; the Greenhouse–Geisser correction was applied when required.

Treatment response was additionally expressed as  $\Delta = \text{CHIP} - \text{RAW}$  and compared between concentration groups using the Mann–Whitney U test. Holm–Bonferroni correction was applied across the four outcome measures, with adjusted  $P < 0.05$  considered statistically significant. One-way ANOVA with Tukey's post hoc test was used for normally distributed data, and the corresponding non-parametric analysis was used where distributional assumptions were not met.

## **4.7. Translational proof-of-concept using ICSI**

A separate translational proof-of-concept experiment evaluated whether sperm recovered using the passive microfluidic workflow could be used in routine ICSI. Microfluidically selected sperm from three independent semen samples were used by experienced embryologists for conventional ICSI under standard clinical laboratory conditions.

Fertilization was assessed by the presence of two pronuclei (2PN) 16–18 h after injection. Embryo development was subsequently monitored using routine clinical procedures. PN score, embryo morphokinetic development, and suitability for cryopreservation or transfer were recorded. Embryological outcomes were compared descriptively with those obtained during the same study period using DGC-prepared sperm. Because only three microfluidic ICSI cases were evaluated, this analysis was considered exploratory and was not designed to assess clinical superiority or reproductive outcomes.

## **4.8. Ethics approval and informed consent**

The National Center for Biotechnology Institutional Research Ethics Committee reviewed and approved the study protocol (reference number 7 on 21 October 2024). All methods involving human participants and human gametes were performed in accordance with the relevant institutional and national guidelines and regulations and with the principles of the Declaration of Helsinki. Written informed consent was obtained from all semen-sample participants before sample collection and use of their samples and associated data for research. Embryo recipients provided written consent before fresh embryo transfers.

## **5. Conclusion**

To enable passive sperm selection across a wide range of baseline sperm concentrations, this study presents a clinically compatible passive microfluidic sperm preparation workflow that combines a disposable pump-free PMMA microfluidic device with a standardized single-step pre-concentration protocol. Compared with raw semen, the workflow greatly enhanced sperm motility, DNA integrity, and morphology while achieving performance on par with or better than traditional density gradient centrifugation. Significantly, baseline sperm concentration was a major predictor of treatment response, with lower concentration samples showing the greatest gains in DNA integrity and motility. These results

show that the application of biomimetic sperm preparation can be expanded without increasing device complexity by combining a straightforward preprocessing strategy with passive microfluidic sperm selection. This workflow provides a practical framework for broader clinical implementation of passive microfluidic sperm selection and establishes a foundation for future multicenter studies evaluating reproductive outcomes.

## **Declarations**

## **Competing of interest**

The authors declare no competing interests or conflicts of interest

### **Author contributions**

Damir Mukhamedyarov and Almas Rakhymzhanov contributed equally to this work.

Damir Mukhamedyarov: Conceptualization (lead); Formal analysis (lead); Investigation (supporting); Writing – review & editing (lead).

Almas Rakhymzhanov: Methodology (lead); Device fabrication (lead); Formal analysis (supporting); Writing – review & editing (lead).

Shyray Ashim: Investigation (lead); Formal analysis (lead); Data curation (supporting); Writing – review & editing (supporting).

Alina Abdykadyr: Software (COMSOL simulations) (lead); Validation and interpretation of simulation results (lead); Writing – review & editing (supporting).

Sergey Yegorov: Conceptualization (Supporting); Writing – review & editing (supporting).

Aidana Irgebayeva: Interpretation of research findings (supporting); Interpretation of clinical results (supporting); Writing – review & editing (supporting).

Dinara Makhadiyeva: Interpretation of research findings (supporting); Manuscript organization and structure (supporting); Writing – review & editing (supporting).

Gulsim Kulsharova: conceptualization (supporting), project administration (lead), supervision (lead), writing – review and editing (equal)

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## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Figures

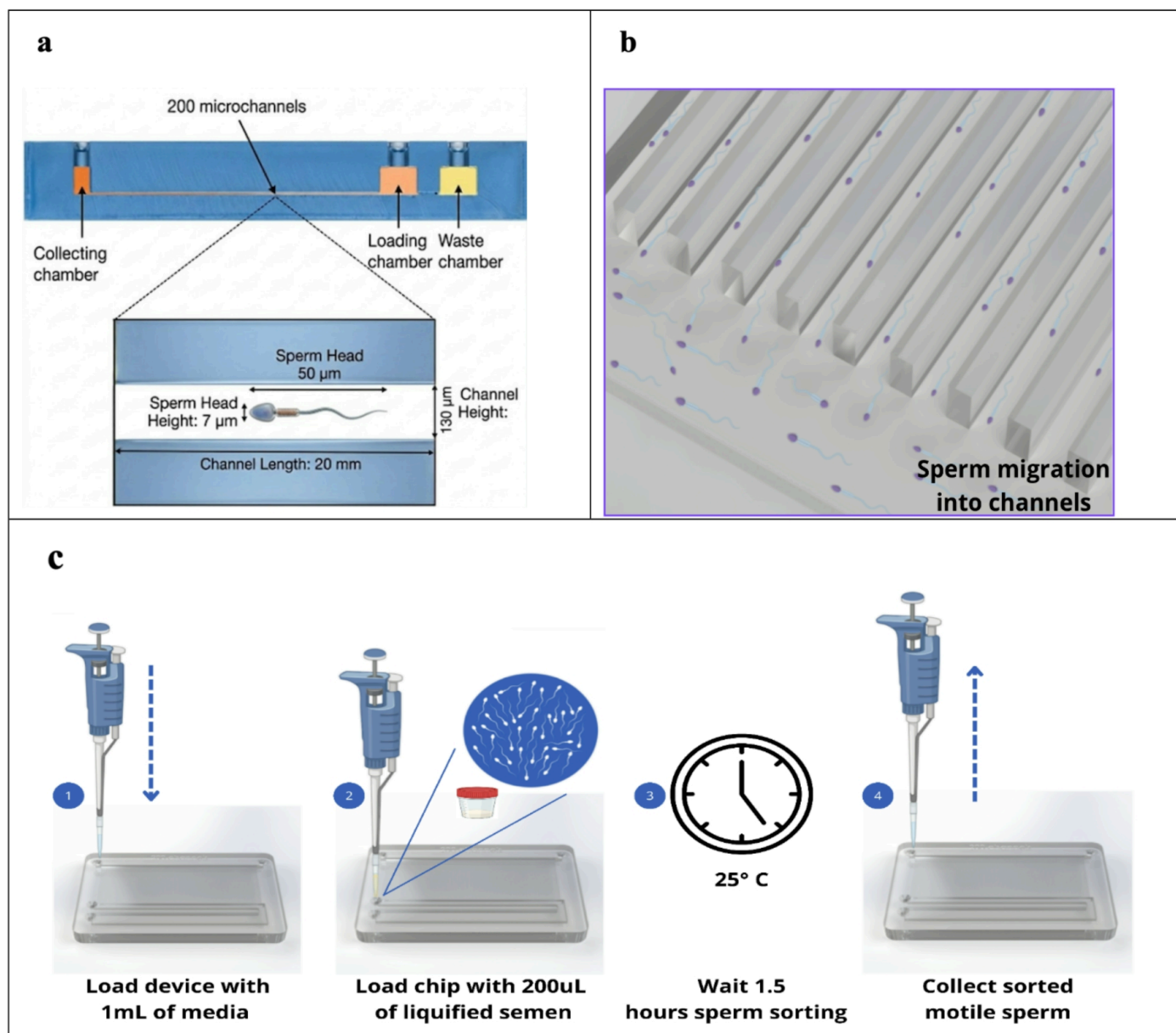


**Figure 1**

Concept figure. **a)** Laser fabricated PMMA microfluidic chip. Top: assembled device with collecting (orange), loading (peach), and waste (yellow) chambers connected by 200 parallel microchannels, and Bottom: channel cross-section schematic showing sperm head dimensions (7  $\mu\text{m}$  height) relative to channel height (130  $\mu\text{m}$ ) and length (20 mm). **b)** Illustration of sperm migration into channels. **c)** Workflow for sample loading, passive migration and recovery of the selected sperm fraction. The figure combines the original device concept and workflow illustrations.

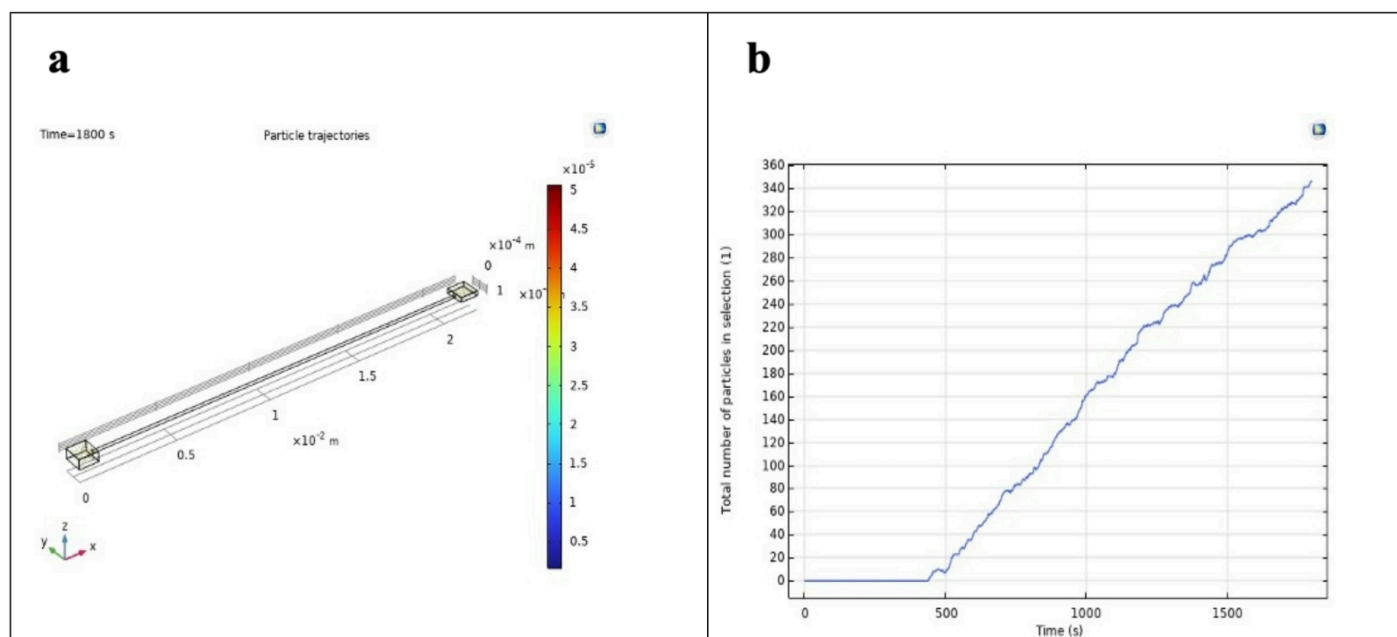


**Figure 2**

Simulated sperm trajectories and sperm accumulation in the outlet chamber for microchannels of different lengths. **a)** Simulated sperm trajectories at  $t = 1800$  s for a 20 mm. channel. **b)** Cumulative number of sperm in the outlet chamber (Domain 3) as a function of time for a 20 mm. channel.

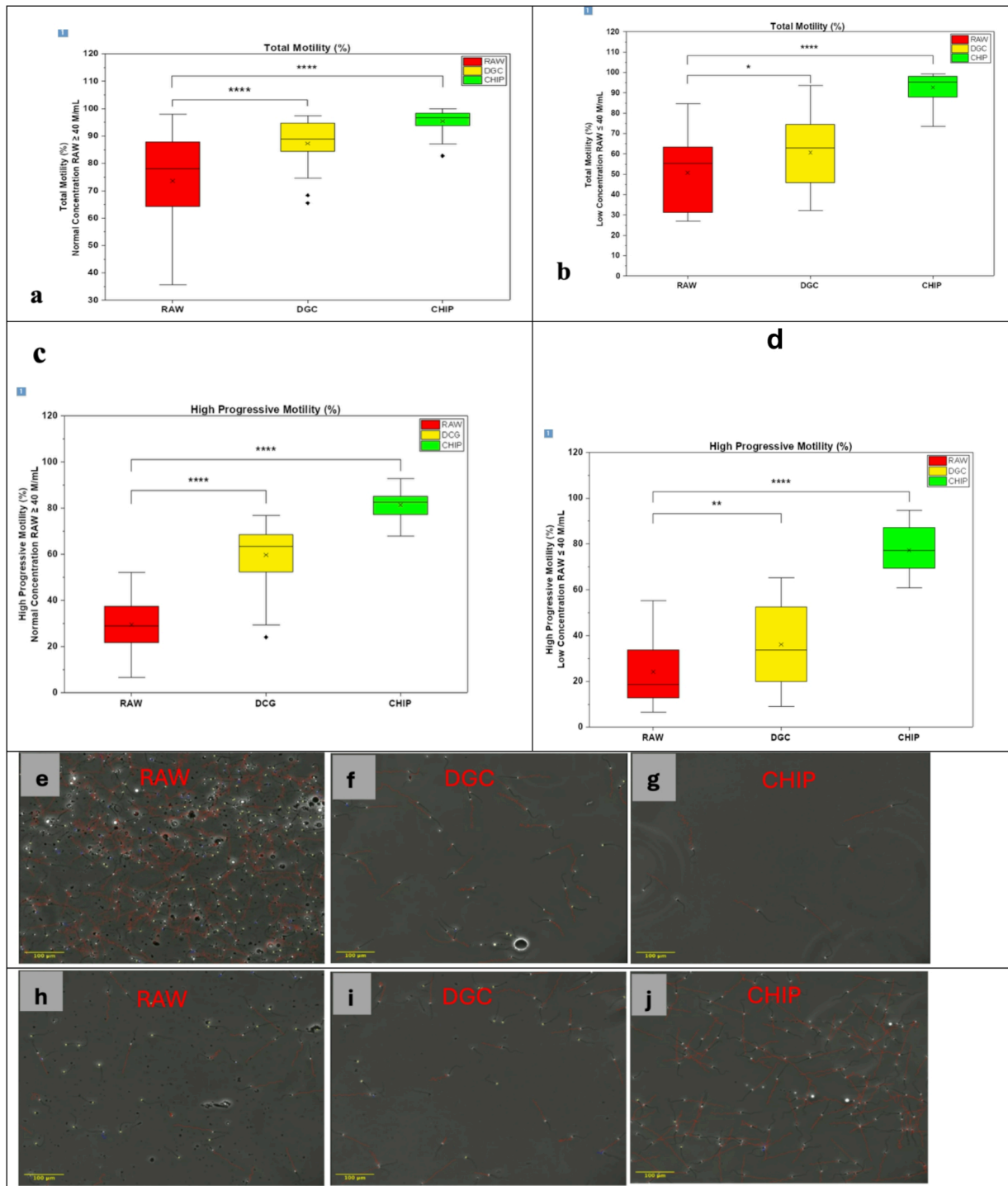


**Figure 3**

Sperm motility (%) following RAW, DGC, and CHIP processing stratified by sperm concentration, with representative CASA visualizations. **(a)** Total motility of normal-concentration cohort ( $\text{RAW} \geq 40 \times 10^6 \text{ mL}^{-1}$ ), both density gradient centrifugation (DGC) and microfluidic CHIP processing significantly increased total motility compared with RAW semen in normal concentration samples (\*\*\*\* $p < 0.0001$ ). **(b)** Total Motility of Low-concentration cohort ( $\text{RAW} \leq 40 \times 10^6 \text{ mL}^{-1}$ ), in low-concentration samples motile

sperm statistically higher in CHIP processed group compared RAW (\*\*\*\*p < 0.0001), DGC revealed compared RAW (\*p < 0.05, p=0.0135). (c) High progressive motility of normal-concentration cohort (RAW  $\geq 40 \times 10^6 \text{ mL}^{-1}$ ), both density gradient centrifugation (DGC) and microfluidic CHIP processing significantly increased high progressive motility compared with RAW semen in normal concentration samples (\*\*\*\*p < 0.0001). (d) In high concentration motility low-concentration cohort (RAW  $\leq 40 \times 10^6 \text{ mL}^{-1}$ ) the CHIP group showed a highly significant improvement compared to RAW (\*\*\*\*p < 0.0001), whereas the DGC group demonstrated a significant improvement (\*\*p < 0.00201). (E) Representative CASA visualization of RAW semen (normal concentration group), (F) DGC-processed sample (normal concentration group), (g) CHIP-processed sample (normal concentration group), (h) RAW semen (low-concentration group), (i) DGC-processed sample (low-concentration group), (j) CHIP-processed sample (low-concentration group). Red trajectories indicate sperm motility tracks captured during CASA analysis, illustrating progressive motility enrichment following processing, most pronounced in CHIP-prepared samples. Box plots represent median, interquartile range (IQR), and full data range. Statistical comparisons were performed using One-way ANOVA followed by Dunnett's multiple comparisons test in OriginPro2026.

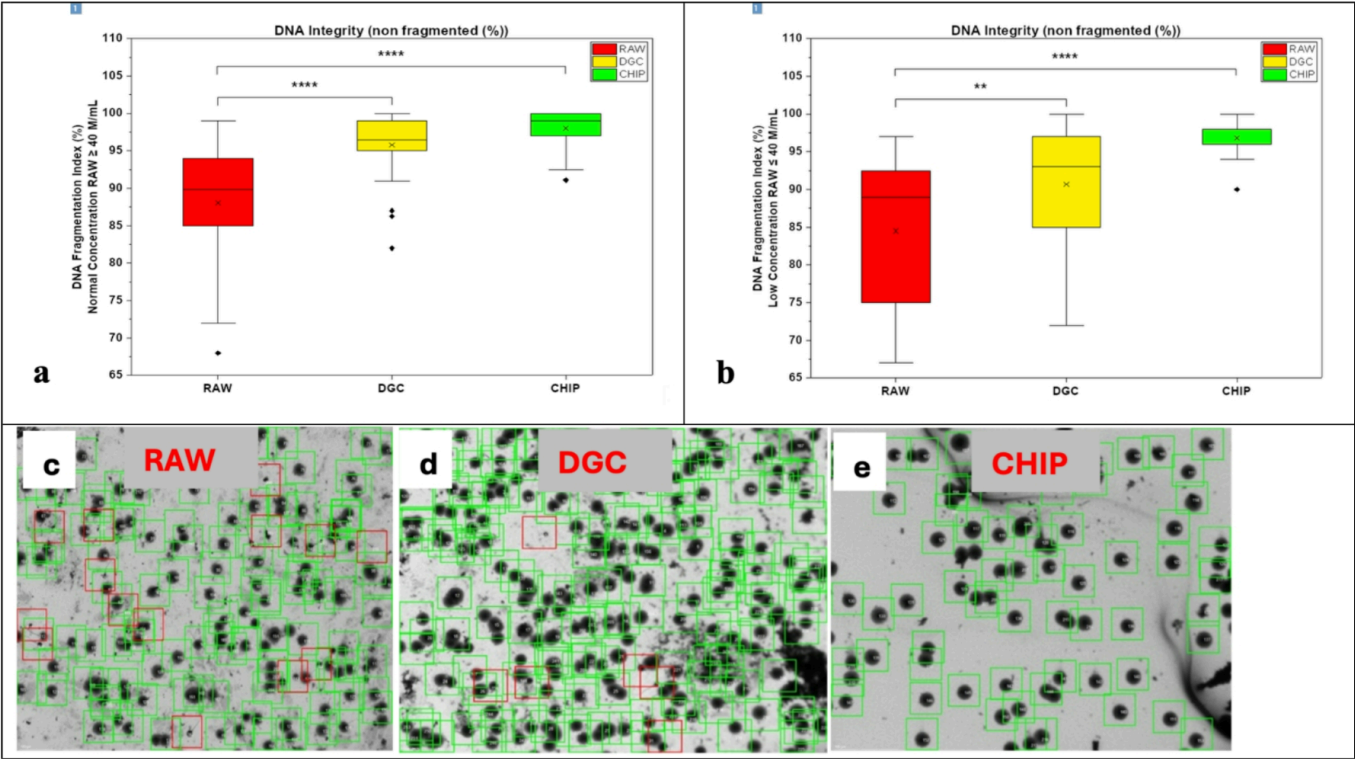


**Figure 4**

DNA fragmentation integrity (%) following RAW, DGC, and CHIP processing stratified by sperm concentration. (a)Both density gradient centrifugation (DGC) and microfluidic CHIP processing significantly increased non-fragmented DNA compared with RAW semen in normal concentration

samples (\*\*\*\* $p < 0.0001$ ). (b) Reduced concentration cohort ( $\text{RAW} \leq 40 \times 10^6 \text{ mL}^{-1}$ ) revealed significantly higher non fragmented result DGC compared RAW (\*\* $p < 0.01$ ,  $p=0.00379$ ), CHIP group compared RAW (\*\*\*\* $p < 0.0001$ ). Box plots display median, interquartile range, and full data range. Statistical significance was assessed using One-way ANOVA followed by Dunnett's multiple comparisons test in OriginPro2026.

Representative CASA images (sperm chromatin dispersion (SCD) assay) of sperm DNA fragmentation in normal concentration specimens (c-e). (c) Raw semen: mixed population, frequent fragmented nuclei (red boxes). (d) DGC fraction: mostly intact nuclei with residual fragmented cells. (e) Chip fraction: near-uniform enrichment of intact-DNA spermatozoa. Red = fragmented DNA; green = intact DNA.

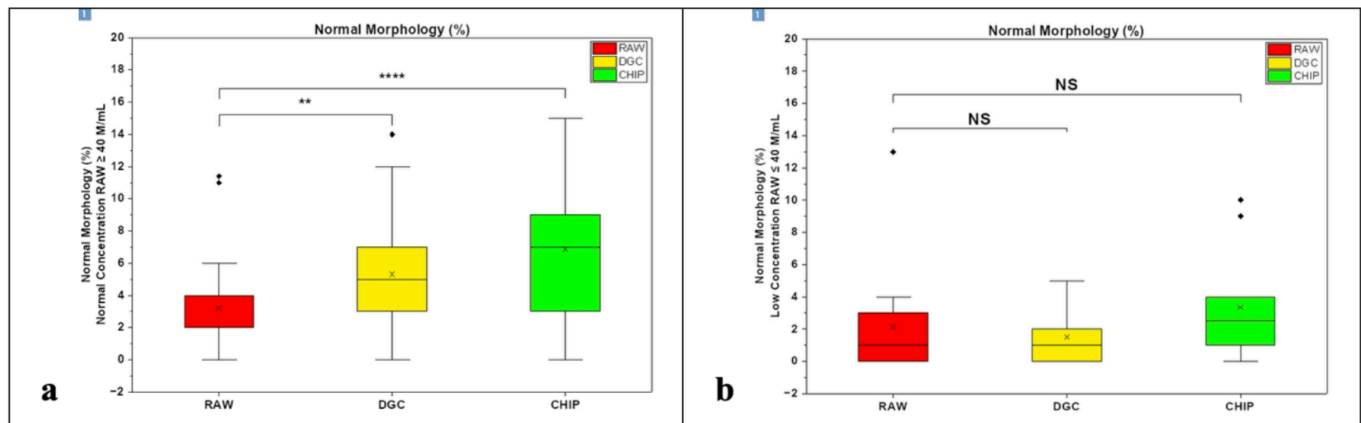


**Figure 5**

Normal sperm morphology (%) following RAW, DGC, and CHIP processing stratified by sperm concentration. **(a)** normal-concentration cohort ( $\text{RAW} \geq 40 \times 10^6 \text{ mL}^{-1}$ ); **(b)** Low-concentration cohort ( $\text{RAW} \leq 40 \times 10^6 \text{ mL}^{-1}$ ). Box plots display median, interquartile range, and full data range. Statistical significance was assessed using One-way ANOVA followed by Dunnett's multiple comparisons test in OriginPro2026. In the normal-concentration cohort, DGC compared RAW revealed (\*\* $p < 0.01$ ,  $p=0.00623$ ), CHIP numerical enriched morphologically normal spermatozoa compared to RAW (\*\*\*\* $p < 0.0001$ ), with CHIP yielding the highest median morphology and widest upper range. In the low-concentration cohort **(b)** both groups (DGC, CHIP) revealed non-significant result (ns).

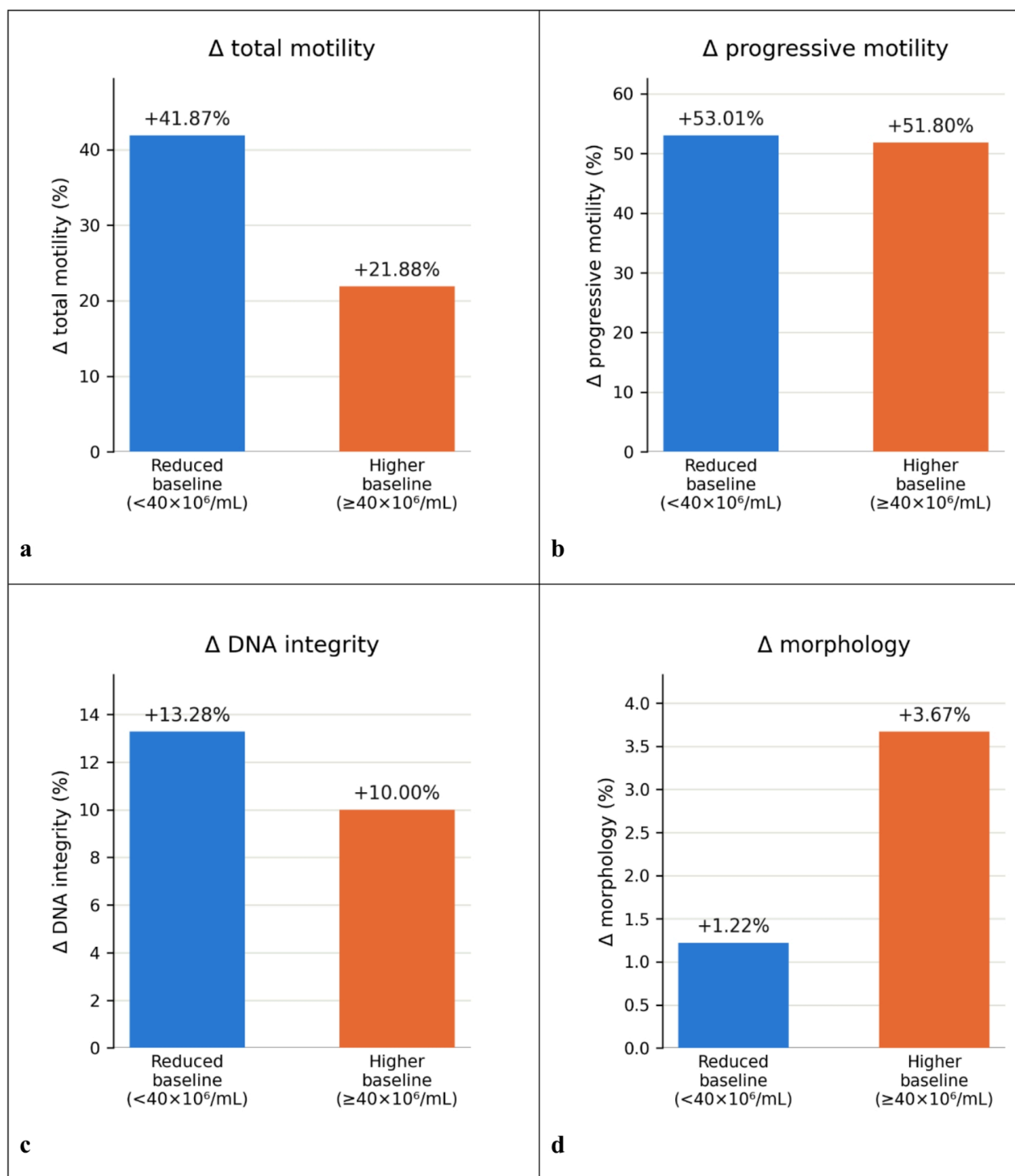


**Figure 6**

(a) Progressive motility: larger improvement in Group A. (b) Total motility: much larger improvement in Group A. (c) DNA integrity: slightly larger improvement in Group A (d) Morphology: larger improvement in Group B.

## Supplementary Files

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