



Research article

Enrichment of bovine X-sperm using microfluidic dielectrophoretic chip: A proof-of- concept study



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ABSTRACT

The microfluidic dielectrophoretic (MF-DEP) chip is a new, economical and readily-available technology that might be used to enrich X-sperm for increasing female offspring in dairy farms. In this study, we sought to develop an MF-DEP chip to enrich X bovine sperm. The MF-DEP chip was composed of an electrode attached to a glass slide and a microchannel made from polydimethylsiloxane. Sex-sorted sperm from flow cytometry were used to identify optimal electric field conditions while unsorted sperm were later tested for sorting efficiency. The results show that during dielectrophoresis some sperm attached to the electrode (called positive DEP; pDEP) whereas other moved away from the electrode (called negative DEP; nDEP). X and Y-sperm responded to dielectrophoresis differently depending on various factors such as buffers, voltages, and frequencies. We found that the condition 4 V 1 MHz significantly reduced ($P < 0.05$) the percentage of Y-sperm to nearly 30 and therefore enriched X-sperm. The sorting efficiency was dependent on buffer, bull, sorting cycle, flow rate, electrical voltage, and frequency. Notably, the best sorting buffer found in this experiment was the conducting buffer, but this buffer significantly reduced sperm viability and motility. Other sperm-friendly buffers, TRIS and mHTF, were also used, but could not enrich X-sperm. In conclusion, this is a proof of concept that the MF-DEP chip can be effectively used to enrich bovine X-sperm. However, more research must be performed particularly to find the best sorting buffer to effectively sex-sort sperm while providing high motility and sperm viability.

1. Introduction

The development of sperm sexing technology (separation of X and Y-sperm) is important for the livestock industry, as it provides for economically flexible management practices, permits faster genetic progress, and facilitates higher productivity [1]. The most effective current method to separate mammalian X and Y-sperm is through the use of DNA staining (Hoechst 33342) and a flow cytometry machine [2]. However, this sorting technique is expensive and available only in some countries, thus limiting its availability for smaller, more local animal breeders. Moreover, the DNA staining may also cause abnormal development and genetic mutation [3, 4, 5]. Many alternative techniques such as antibody specific to sperm antigen, density gradient centrifugation, and albumin filtration have been demonstrated but inconsistent results,

and therefore unpopular [6, 7, 8]. Thus, a novel method for reliable and economical sperm sexing must be developed.

Microfluidics is a technology to control small volume fluid on the micro- and nanometre scale in channels with dimension less than thousand micrometres [9]. It can be used for the separation of many cell types including sperm [10]. Microfluidics can sort sperm for many applications such as separation of healthy sperm from unhealthy sperm [11], arrangement of sperm individually for single sperm analysis [12], and separation of sperm from other cells in forensic cases [13]. However, microfluidics alone has never been used to separate X and Y-sperm, except in combination with laser, fluorescence, and electromagnetic technologies, called cell sorting by flow cytometry [14].

Dielectrophoresis (DEP) is different from electrophoresis, it is a non-invasive technique for cell separation using non-uniform electric fields in

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a suitable solution to control the movement of cells [15]. Cells which possess different electrical surface properties are moved and separated by being drawn toward the point of highest electric field (termed positive DEP; pDEP), or repulsed from the highest field point (termed negative DEP; nDEP) [16]. DEP is commonly used together with a microfluidic chip technique to get high performance for sorting applications. The microfluidic dielectrophoresis (MF-DEP) technique for cell sorting has been reported in many types of mammalian cells. For example, it is used to separate tumour cells from normal cells [17], to sort different types of white blood cells [18] and for separation of mature sperm from immature cells [19].

Previous in situ studies showed that human X and Y-sperm move differently in regard to flagellum pattern and velocity, and that this difference is dependent on the dielectrophoretic field and media used [20, 21]. Our preliminary study also showed that bull sperm exhibited either pDEP or nDEP depending on electrical potentials and frequencies [18, 22]. The pDEP sperm are trapped within the high electric field region (attached to the electrode) whereas nDEP sperm float in solution, indicating the method might be used to separate X and Y-sperm [18]. Taken together, it could be possible to sort X and Y-sperm using the combined technology, the microfluidic dielectrophoretic system (MF-DEP).

The aim of this study was to investigate the effect of the frequencies, voltages, sorting buffers, bulls, flow rate, and round of sorting on sorting efficiency in order to assess and improve the efficiency of MF-DEP chip for sex-sorting bovine sperm. The outcome of this research may benefit the livestock industry worldwide.

2. Materials and methods

2.1. Bovine sperm

Frozen semen samples were obtained from 4 Holstein Friesian bulls, named A, B, C, and D. Samples were divided into two groups: sex-sorted and unsorted sperm. Samples from bull A were unsorted sperm, sorted X-sperm, and sorted Y-sperm. These sperm were purchased from Pornchai Intertrade Ltd. (Ratchaburi, Thailand). The separation of X and Y-sperm of bull A was done by flow cytometry (Sexing Technologies company, TX, USA). Sperm from bulls B, C, and D were unsorted sperm, and were donated from the Dairy Farming Promotion Organization of Thailand.

Sex-sorted sperm (X and Y) were obtained from the same bull, and they were used to identify optimal electrical condition (voltage and frequency) and validate sperm sex identification in the experiment. Unsorted sperm were obtained from 4 bulls and were used to study the efficiency of the MF-DEP chip by varying sorting conditions such as selected electrical stimulation, flow rates, number of cycles, and sorting buffers. Three inter-replications were performed in each experiment and for each bull.

Frozen semen was thawed in a 37 °C water bath for a minute, then semen was placed into a top layer of 4 mL 45–90% Percoll gradient solution (GE HealthCare Life Science, IL, USA) and centrifuged at $670 \times g$ for 10 min in order to remove cryomedia. Then the sperm pellets were re-suspended in sorting buffer (pre-warmed at 37 °C) before the experiment was performed.

2.2. Microchannel and electrode fabrication

The MF-DEP chip structure is shown in Figure 1. The system was assembled with a DEP electrode and microchannel. The microchannel was made from polydimethylsiloxane (PDMS) by moulding technique. The microchannel moulding was fabricated by photolithography and made from SU-8 film on glass. The height of the microchannel was about 100 μm , width 300 μm , and the length was 6 cm. The DEP electrode was made from conductive Indium Tin Oxide (ITO) film on a glass slide substrate (LT-G001ITO Glass 15 Ω , New Taipei City, Taiwan). The DEP pattern (dumbbell shape) layout was designed by using L-Edit Pro v8.03 and fabricated by a positive photolithography procedure.

2.3. Sorting process

The MF-DEP chip was connected to an arbitrary function generator (AFG3021B, Tektronix, OR, USA) by wire and carbon tape to provide different electric field gradients. A Minipuls-3 peristaltic pump (Gilson, WI, USA) was connected to the chip to manipulate the flow rate. The behaviour of sperm in the MF-DEP chip was observed under an inverted light microscope (Olympus CKX41, Tokyo, Japan).

Sperm (1.5×10^4 cells) were mixed into 60 μL of sorting buffer and were fully loaded into the MF-DEP chip via a silicone tube with an inner diameter of 0.5 mm (Cole-Parmer, Vernon Hills, IL, USA). The system

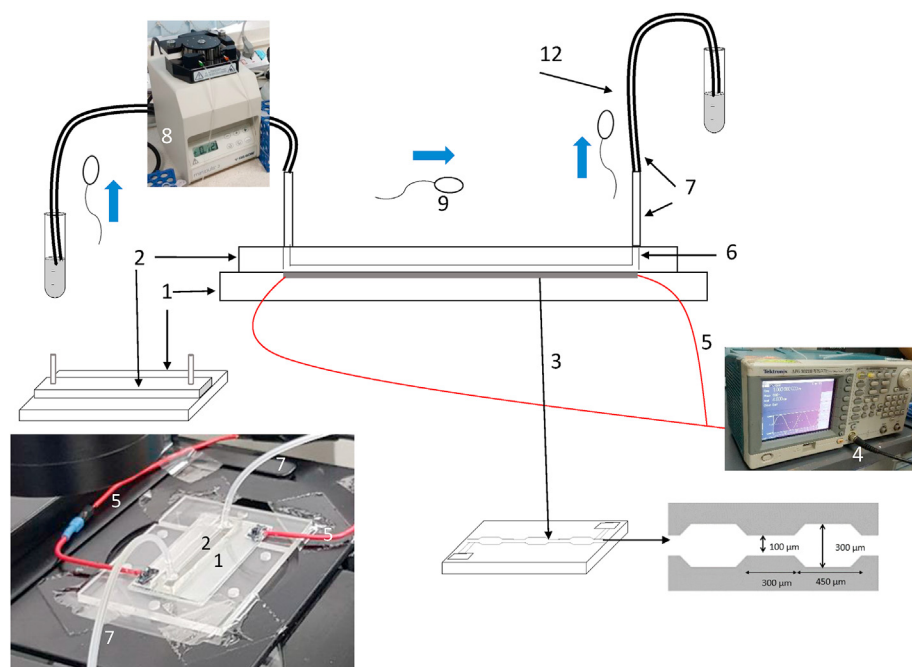


Figure 1. The illustrated image of the microfluidic dielectrophoretic (MF-DEP) system. The sorting chamber includes dielectrophoretic (1) and microchannel layers (2). The dielectrophoretic layer was made from glass and had an electrode pattern (3) which connected to the power generator (4) via electrical wire (5, red line). The microchannel layer was made from silicone and had a microchannel inside (6). The microchannel was connected to silicone tube (7), and the fluid was controlled by a peristaltic pump (8). Sperm (9) moves through the device following the direction represented by thick blue arrows.

Table 1. Primer sequence for qPCR.

Name		Sequence	Product size (bp)	Tm (°C)
Y chromosome	F	GATCACTATACATACACCACTCTCATCCTA	157	60
	R	GATCTTGTGATAAAAAGGCTATGCTACACA		
Satellite DNA	F	TGG AAGCAAAGAACC CCG CT	216	60
	R	TCGTGAGAAAACCGCACAC TG		

was operated for 15 min to sort sperm, unwanted sperm (pDEP) were trapped (attached) inside the chip while the wanted sperm (nDEP) passed through the chip. In the observational experiment, in order to count the number of sperm exhibiting pDEP/nDEP, the system was arrested after running for 5 min. After each experiment was finished, the chip was cleaned using detergent and rinsed with water. Unattached sperm moved through the chip channel and were collected at the end of the outlet tube while the attached sperm were washed by detergent after each experiment. The sorting process and mechanism of MF-DEP chip is demonstrated in the video file (Video 1).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.heliyon.2020.e05483>

2.4. Optimisation of voltage, frequency, and flow rate

AC sinusoidal wave using electrical potentials of 4, 6, and 8 V and electrical frequencies of 1, 4, 8, 12, 16, and 20 MHz were applied to the electrode in order to observe the behaviour of the sperm under these conditions. The numbers of sperm that exhibited pDEP or nDEP were counted under inverted microscope and then calculated as the percentage of pDEP ($\%pDEP = N_{pDEP}/N_{total}$). Most experiments used a flow rate of 0.1 $\mu\text{L/s}$, except for one experiment that specifically compared the effect of different flow rates (0.1, 0.2 and 0.3 $\mu\text{L/s}$).

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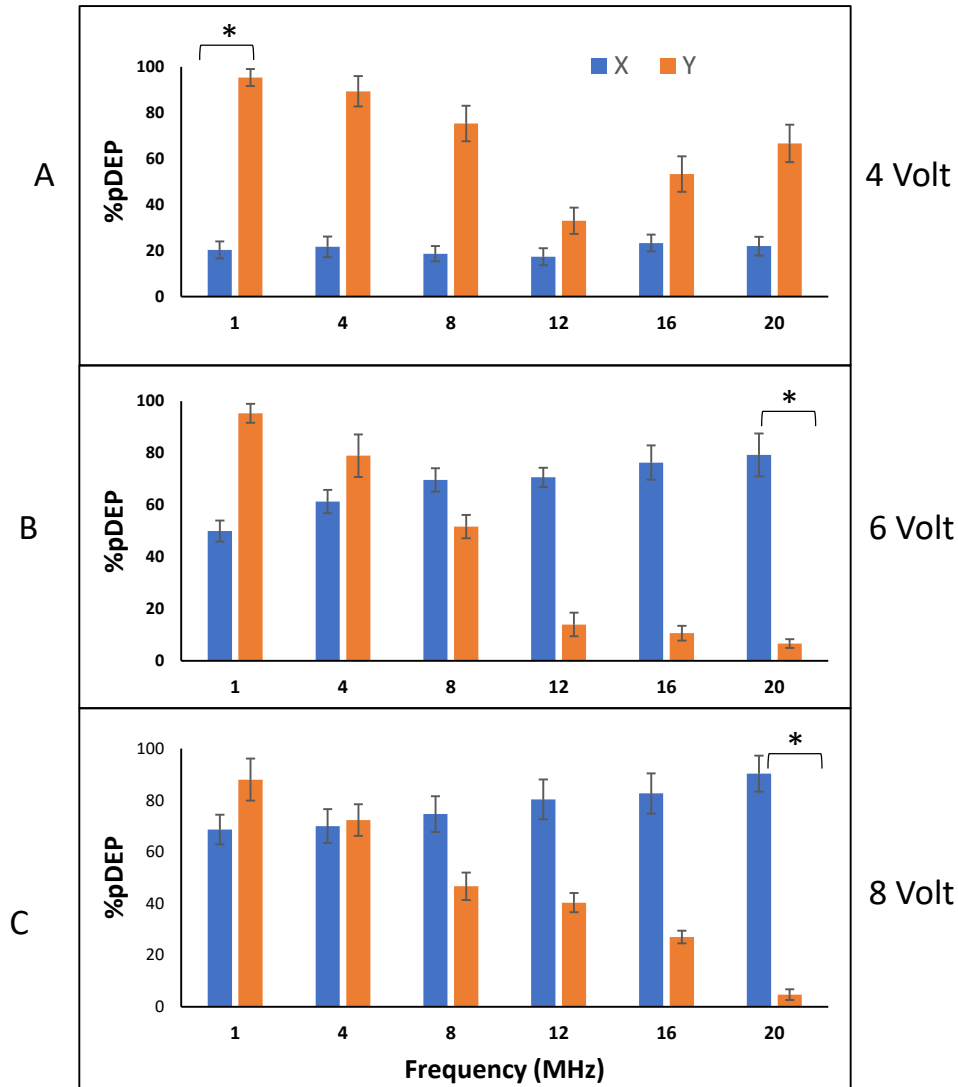


Figure 2. Effect of voltage and frequency on the dielectrophoretic (DEP) properties of X and Y-sperm. Sperm used in this experiment were commercially sex-sorted X- and Y-sperm. The percentage of positive DEP (pDEP) represents the ratio of sperm attached to the dielectrophoretic probe. Sperm of bull A was used in this experiment. *The most significant difference in percentage of pDEP sperm ($P < 0.01$).

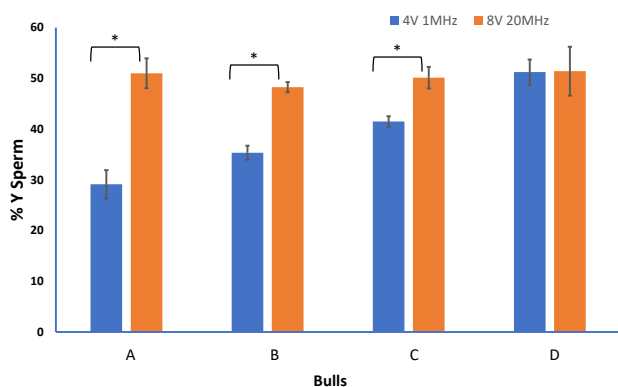


Figure 3. Sex-sorting efficiency of microfluidic dielectrophoretic (MF-DEP) chip for individual bulls. Samples were unsorted sperm from the bulls A, B, C, and D. *Significant difference ($P < 0.05$). Y-sperm of bull A, B, and C were significantly reduced.

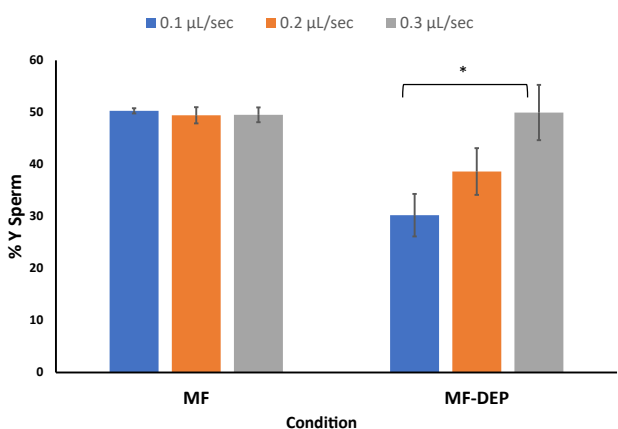


Figure 4. Effect of the flow rate (0.1, 0.2, and 0.3 µL/s) on sperm separation. The condition was 4 V 1 MHz. Sperm of bulls A and B were used in this experiment. *Statistical difference ($P < 0.01$). MF = microfluidic without dielectrophoresis. Y-sperm were significantly reduced at flow rate 0.1 and 0.2 µL/s.

2.5. Sorting buffer

Most experiments in this study used a conducting medium (CM) which contains 8.5% sucrose, 3% glucose, and 1M KCl, as a sorting buffer. This CM was used in our previous experiment with white blood cells and sperm [18], the conductivity of the CM was 10 mS/m.

Two other buffers were also used to compare with the CM buffer. They were the Tris buffer (containing 3.0% Tris aminomethane, 1.4% citric acid, 0.8% glucose, and 0.06% Na-benzylpenicillin) for which conductivity was 121.3 mS/m [23], and modified Human Tubal Fluid (mHTF) buffer (containing 0.29 M sucrose, 10 mM, HEPES, 30 g/L bovine serum albumin, and 0.5% HTF), for which conductivity was 40 mS/m [24].

2.6. Sperm sex identification

Sex identification of sperm was performed using Quantitative Real-time Polymerase Chain Reaction (qPCR). First, sperm DNA was extracted by Azupure DNA purification kit (BIOTEC, Bangkok, Thailand) and then DNA concentration was measured using NanoDrop machine (ThermoFisher Scientific, MA, USA). DNA was mixed with SYBR qPCR Master Mix buffer (KAPA Biosystem, MA, USA) and PCR primers, then the qPCR was performed using a Rotor Gene Q machine (Qiagen, Hilden, Germany). The qPCR reaction protocol was programmed according to a previous publication [25]. The percentage of Y-sperm of the treatment (sorted sperm) was calculated relative to the control (unsorted) by using the comparative quantification method of the Rotor Gene Q software (Qiagen, Hilden, Germany). The control (unsorted sperm) was set to 50% of Y-sperm. Quantification of bovine satellite DNA (found in both X and Y-sperm) was used for normalisation of chromosome Y quantification [26]. Melt curve analysis was used to confirm specific products of primers. Three internal PCR replicates were used for each sample.

The primer sequence used to identify bovine Y chromosome and bovine satellite DNA are shown in Table 1. These primers were used in previous studies [27, 28].

2.7. Sperm viability and motility

Sperm quality was evaluated by observing sperm motility and viability. Sperm motility was assessed under a light microscope at magnification 200X on a warm stage (37 °C), the motility was recorded as a percentage. Sperm viability was analysed by mixing (1:1) sperm

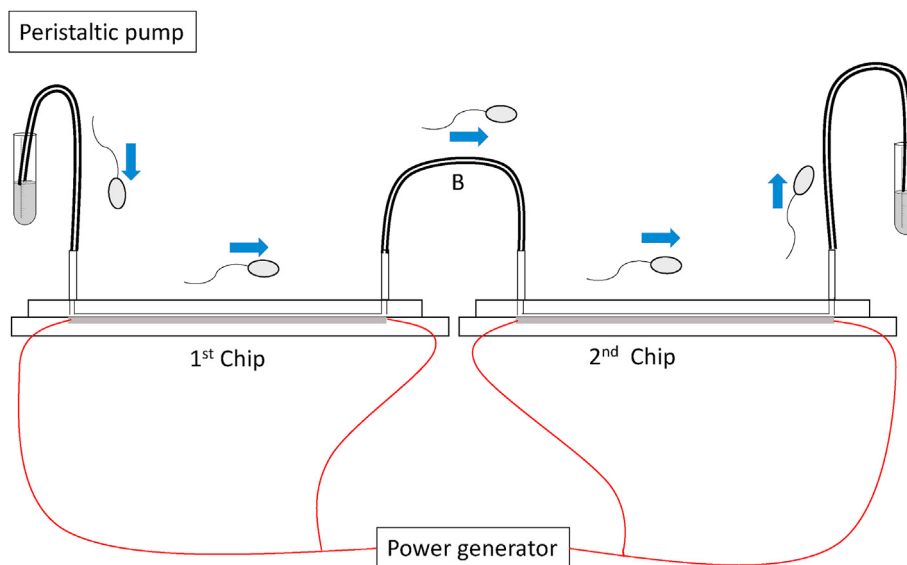


Figure 5. Illustrated image showing the setup of two cycle sorting by MF-DEP chip. Sperm move from the first chip to the second chip by bridge tube (B). Red line is electric wire, Grey bar is electrode, and blue arrows are the direction of the flow.

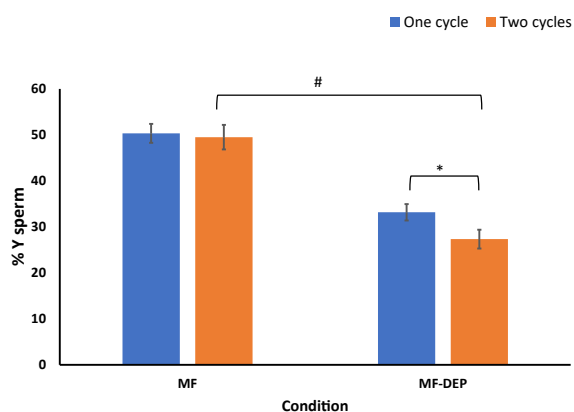


Figure 6. Comparison between one and two cycles (rounds) sorting by MF-DEP chip. The dielectrophoretic condition was 4 V 1 MHz, and the flow rate was 0.1 $\mu\text{L/s}$. MF = microfluidic without dielectrophoresis (control). Sperm of bulls A and B were used in this experiment. Significant difference was represented as * for $P < 0.05$ and # for $P < 0.01$. Two cycle sorting with MF-DEP chip significantly reduced Y-sperm.

solution with 0.4 % Trypan blue solution (ThermoFisher Scientific, MA, USA) and incubated for 2 min before counting dead/live sperm under light microscope at magnification 400X.

2.8. Statistics

Data are shown as Mean \pm Standard Deviation (SD), and tests of significant difference were calculated using one-way ANOVA and multiple comparison with LSD using SPSS 21 software (IBM, NY, USA).

3. Results

3.1. Effect of voltage and frequency on DEP properties of X and Y bovine sperm

This experiment used sex-sorted sperm from bull A. The effect of voltages and frequencies on the dielectrophoretic property of X and Y-

sperm is shown in Figure 2. In each condition, X and Y-sperm behaved differently. Most Y-sperm seemed more sensitive to a change in frequency than X-sperm. The Y-sperm exhibited the highest percentage of pDEP when the lowest frequency (1 MHz) was applied in all voltage conditions. Conversely, X-sperm exhibited the highest percentage of pDEP when the highest frequency (20 MHz) was used. For the voltage change, X-sperm seemed more sensitive than Y-sperm. When the voltage was increased, X-sperm clearly exhibited more pDEP while Y-sperm did not show a substantial difference. At 4 V condition, X-sperm exhibited a low percentage of pDEP (approximately 20%) in all conditions of frequencies. After increasing the voltage to 6 V, pDEP increased particularly at 20 MHz, but the highest percentage of pDEP of X-sperm was found at 8 V 20 MHz. For Y-sperm, the highest pDEP was found at 4 V 1 MHz, while the lowest pDEP was found at 8 V 20 MHz.

In order to separate X and Y-sperm with DEP, we chose the condition that showed the most substantial difference in percentage of pDEP between X and Y-sperm. The most difference was found at 4 V 1 MHz (Y > X, the difference = 75.00%) and 8 V 20MHz (X > Y, the difference = 85.66%).

3.2. Sex-sorting efficiency

To test the sorting efficiency of the MF-DEP chip, we chose two conditions: 4 V, 1 MHz (expected to reduce Y-sperm) and 8 V, 20 MHz (expected to reduce X-sperm). We initially tested with unsorted sperm from bull A. The results showed that the percentage of Y-sperm was significantly reduced ($P < 0.01$) after using the 4 V, 1 MHz condition, whereas the 8 V, 20 MHz condition could not reduce the percentage of X-sperm ($P > 0.05$) (Figure 3).

Then we applied these conditions to unsorted sperm from the other three bulls (named B, C and D). The results showed that sperm from each bull responded to the MF-DEP chip differently. At condition 4 V 1 MHz, X-sperm of bull B was more efficiently enriched than the other bulls. The percentage of Y-sperm of bulls B and C was significantly reduced ($P < 0.01$) to 35.34% and 41.5%, respectively. Unlike bulls B and C, the percentage of Y-sperm of bull D was not significantly altered ($P > 0.05$). For the condition 8 V 20 MHz, the percentage of Y-sperm for all bulls did not significantly change ($P > 0.05$) (Figure 3). We then investigated why Y-sperm of bull D and X-sperm of all bulls was not trapped in the chip,

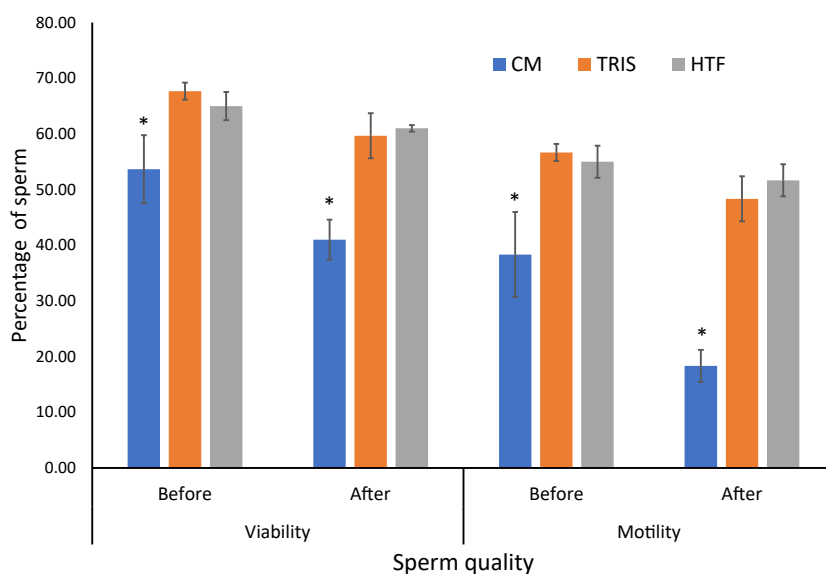


Figure 7. Sperm viability and motility before and after sorting with MF-DEP chip under different sorting buffers. The condition was 4 V 1 MHz, and the flow rate was 0.1 $\mu\text{L/s}$. Sperm from bulls A, B, C, and D were used in this experiment. *Statistical difference ($P < 0.01$). Motility and viability of sperm in the CM buffer was significantly lower than the other buffers.

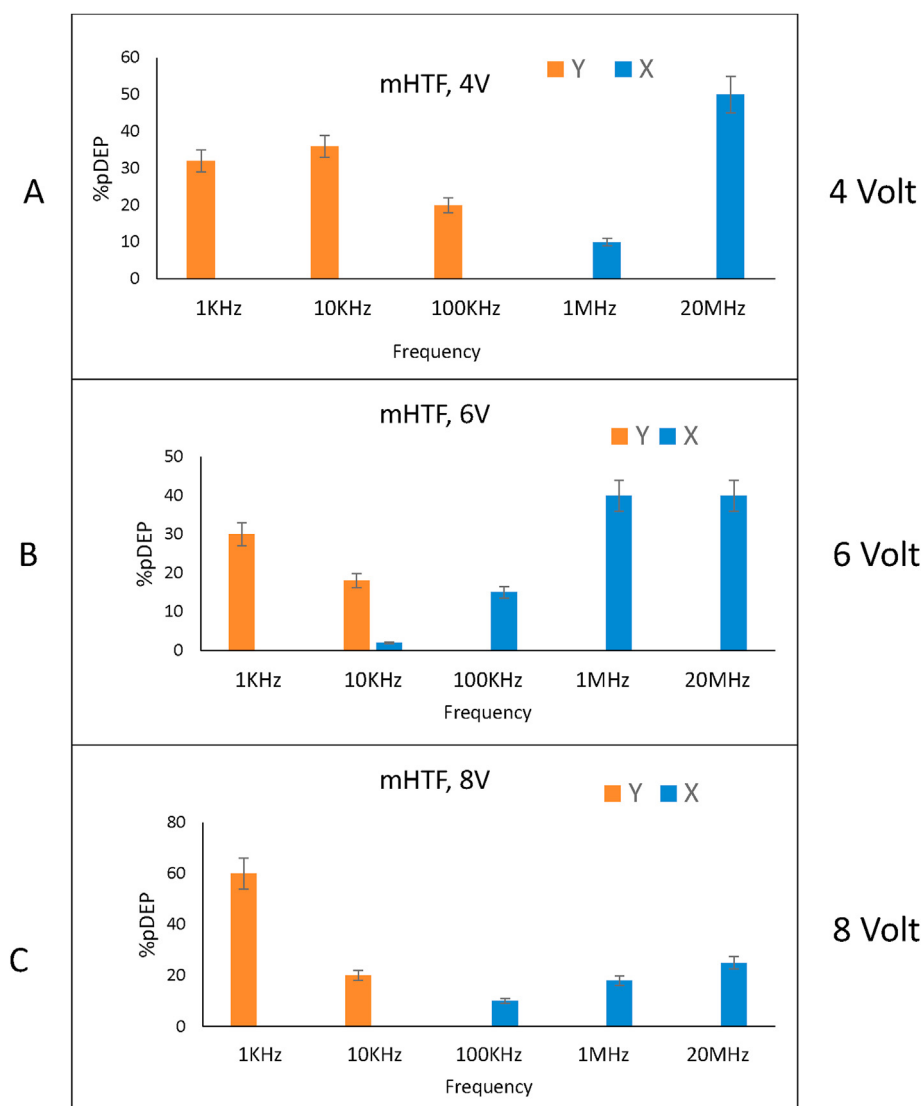


Figure 8. The percentage of positive DEP (pDEP) of Sorted X and Y-sperm in modified Human Tubal Fluid (mHTF) buffer, under different electrical conditions. Sperm from bull A was used in this experiment.

after carefully observing under microscopy we found that these sperm were only weakly bound to the electrode and thus were easily flushed away by the buffer.

3.3. Comparison of flow rates for sorting sperm with MF-DEP chip

To compare the effect of flow rates for sperm separation, we used sperm from bulls A and B, and applied the condition 4 V 1 MHz, but varied flow rate: 0.1, 0.2 and 0.3 $\mu\text{L/s}$. The control was sperm that passed through the chip without electricity (dielectrophoresis did not occur), the result showed (Figure 4) that the percentage of Y-sperm was not different in all flow rates for the control, while with dielectrophoresis, the flow rate 0.1 $\mu\text{L/s}$ seemed to enrich X-sperm more efficiently than the other flow rates. The difference was statistically significant ($P < 0.01$) between flow rate 0.1 $\mu\text{L/s}$ and 0.3 $\mu\text{L/s}$, but not between 0.1 $\mu\text{L/s}$ and 0.2 $\mu\text{L/s}$ or 0.2 $\mu\text{L/s}$ and 0.3 $\mu\text{L/s}$ ($P > 0.05$). The flow rates 0.1 and 0.2 $\mu\text{L/s}$ significantly reduced ($P < 0.05$) the percentage of Y-sperm when used together with dielectrophoresis compared to without dielectrophoresis.

3.4. Second round sorting (two cycles)

In this experiment, we used sperm from bulls A and B. We hypothesised that sorting two times might increase the sorting efficiency. In

order to sort two times, two MF-DEP chips were connected with a tube (Figure 5). Sperm passed from the first chip to the second chip and then to the collecting tube. The condition was 4 V 1 MHz and the flow rate was 0.1 $\mu\text{L/s}$. The result showed that two cycle sorting was significantly better ($P < 0.05$) than one cycle. It reduced Y-sperm from 50% to 27.33% compared to one cycle which reduced Y-sperm to 33.17%. Flowing sperm solution for two cycles without using dielectrophoresis (control group) did not change the percentage of Y-sperm (Figure 6).

3.5. Sorting buffers

In most experiments, we used the CM buffer as the sorting buffer; however, the CM buffer seemed unfavourable to sperm as it reduced sperm motility and viability (Figure 7). Therefore, in this experiment we tried to optimise dielectrophoretic condition for the Tris and mHTF buffers as sorting buffer. We used sperm from bulls A and B in this experiment. However, in the Tris buffer, pDEP was not observed in all electrical conditions we tested whereas in the mHTF buffer, pDEP was found (Figure 8), but at lower levels than in The CM buffer. With the similar frequencies used with the CM buffer (1–20 MHz) only X-sperm in the mHTF buffer exhibited pDEP. When we reduced the frequency down to 1 kHz, we found that Y sperm exhibited pDEP in 1–100 kHz while X-sperm rarely exhibited pDEP in this range of frequencies (Figure 8).

Based on this, we chose conditions 8 V 1 MHz (to reduce Y-sperm) and 4 V 20 MHz (to reduce X-sperm) to sort sperm with the mHTF buffer. However, after flowing the buffer, we did not observe trapped sperm in this buffer, suggesting that this buffer caused weak binding between sperm and the electrode. Expectedly, the percentage of Y-sperm in both electrical conditions was not different. (8 V 1 MHz = 49.67%), (4V 20 MHz = 51.33%) from unsorted sperm (50.00%).

3.6. Sperm motility and viability

Before and after going through the chip, the viability and motility of sperm in the CM buffer were substantially lower ($P < 0.01$) compared to the other buffers (Figure 7). The viability and motility of sperm in the TRIS and mHTS buffers were slightly reduced (<10%) after passing through the chip, but the viability and motility of sperm in the CM buffer were greatly reduced (>10%), and the difference was very significant ($P < 0.01$).

4. Discussion

The present study reveals that the MF-DEP chip can enrich X-sperm by trapping Y-sperm to the electric probes and allowing the X-sperm to pass through the chip. However, sorting efficiency varied widely and was dependent on donor bull, buffer, electrical voltage and frequency, flow rate, and number of sorting cycles. We suggest that the ability to sex-sort sperm by MF-DEP comes from the difference in dielectrophoretic properties between X and Y-sperm, which is related to different cell membrane proteins [29, 30] and the zeta potential of sperm [22, 31]. We believe that the sperm of any bull can be sorted with this technique, but it requires specific individual set up to achieve strong binding between the sperm and probes.

Although flow cytometry is most efficient for sorting X and Y-sperm, it still requires individual setup, because each male animal contains different amount of DNA content, and it is imperative to select the right bulls and to test the sorted samples on a routine basis [32, 33]. The inconsistent result of other alternative methods for sex-sorting by antibody, centrifugation, and albumin [6, 7, 8] may also result from difference in membrane potential, DNA content, and membrane proteins [22, 29, 30]. Taken together, this suggests that there is no universal condition that can be used to separate X-and Y-sperm for all male animals, and each bull requires a specific condition and setup to maximize efficiency of sperm sexing.

The best sorting efficiency in this experiment was achieved by using the CM buffer as a sorting buffer, but this buffer substantially reduced sperm motility and viability. So we tried to use the Tris buffer which is commonly used to dilute sperm [34], but the Tris buffer did not support dielectrophoretic separation (No pDEP was observed). This could be because the conductivity of the Tris buffer is too high. Another sperm-friendly buffer with low conductivity, the mHTF medium [24] was tested, and demonstrated a difference in dielectrophoretic properties between X and Y-sperm. Unfortunately, the binding between pDEP sperm and the probe in the mHTF buffer was too weak and sperm were easily washed away by the liquid flow. It is possible that albumin, a natural lubricant [35] and a main component in the mHTF medium weakened the bond between sperm and the electrode. So, a future experiment will be required to identify the ideal sorting buffer that will not affect sperm motility and viability, while allowing sperm to strongly bind to the electrode.

Electrophoresis was first used to separate X and Y-sperm around 40 years ago, this technique demonstrated the high efficiency of separation but was very toxic to sperm [36, 37]. Electrophoresis use a uniform electric field, generated using direct current (DC), this technique uses high energy to move the cell and can be more lethal to the cells than dielectrophoresis [38, 39]. Our study demonstrated that dielectrophoresis is safe for sperm in the proper buffer, the decrease in sperm quality was not affected by the electrical condition but the buffer choice.

Our study is the first study to demonstrate the possibility of separating X and Y- sperm using dielectrophoresis. However, more research is required to improve the efficiency of the protocol while maintaining the sperm quality. The essential step that need to be resolved before this protocol can be applied to the livestock industry include: identifying best sorting buffer, and the electrical condition specific to each breed and species, and developing multiple sets of chips for sorting a massive number of sperm for artificial insemination.

5. Conclusion

In conclusion, we propose that our MF-DEP chip is an affordable technology that, with further development, could be used to enrich X-bovine sperm for increasing female offspring in dairy farms without access to more complex and expensive techniques.

Declarations

Author contribution statement

Tuempong Wongtawan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Naruphorn Dararatana: Performed the experiments.

Chommanart Thongkittidilok: Analyzed and interpreted the data; Wrote the paper.

Sudsajjai Kornmatitsuk: Contributed reagents, materials, analysis tools or data.

Bovornlak Oonkhanond: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional Information

No additional information is available for this paper.

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